J. Plant Biotechnology (2006) Vol. 8 (1). pp. 9~14

Journal of Plant Biotechnology

Expression of a Functional Anti-Cucumber Mosaic Virus Single-Chain Variable Fragment Antibody in Tobacco Plants (*Nacotiana tabacum*)

Chua Kek Heng[1*], Norzulaani Khalid[2], Rofina Yasmin Othman[2]

[1]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Malaysia; [2]Institute of Biological Sciences, Faculty of Science University of Malaya, Malaysia

Abstract

As an alternative method to produce low cost reagents for immunodiagnosis and protect the plants from viral disease, a gene encoding a single chain variable fragment (scFv) recombinant antibody targeted to the coat protein of cucumber mosaic virus (CMV) was expressed in *Nacotiana tabacum*. The source of the scFv recombinant antibody gene was from spleen tissue of an immunized mouse. The gene was initially cloned into the pCANTAB5E phagemid and expressed in *E. coli*. In the following study, the antibody gene was subcloned into the plant expression vector, pCAMBIA-1301 and introduced into tobacco leaf tissue via *Agrobacterium tumefacients* mediated transformation. After transformation, 56 out of 58 plants were shown to carry the desired anti-CMV scFv gene by PCR analysis. Overall, only 12.5% of the 56 putative transgenic plants were found to express the antibody to a detectable level.

Key words: scFv, CMV, *E. coli*, Tobacco, Expression

Introduction

Cucumber mosaic virus (CMV) is the type member of cucumoviruses. It was first found in *Cucumis sativas* in USA (Doolittle 1916; Jagger 1916).

CMV is a single-stranded, positive-sense RNA, isometric, non-enveloped virus and exists as many strains. In nature, it is easily transmitted by a number of vectors commonly aphid species. However, CMV can also be transmissible by mechanical inoculation. It infects forages, cereals, woody and herbaceous ornamentals, vegetables, fruit crops and other important agriculture crops covering over 1000 plant species (Palukaitis et al. 1992). Systemic mosaic is the typical symptom exhibited in most of the infected plants (Kaper and Waterworth 1981). It has been reported that the coat protein of CMV is important in aphid transmission (Gera et al. 1979; Chen and Francki 1990), virus encapsidation (Harrison 1984) and may regulate the RNA genome synthesis either directly or as a consequence of encapsidation (Houwing and Jaspars 1986). The expression of the viral coat protein in plant has also become an approach to control CMV infection (Tricoli et al. 1995; Yie et al. 1995; Fuchs et al. 1996; Fuchs et al. 1997). Coat protein also provides the best target for virus detection in infected tissue (Gonda and Symons 1979; Lin et al. 1996). The virus can be detected using standard techniques such as ELISA and western blot, where monoclonal antibodies are the main diagnostic agent.

A new cucumber mosaic virus (CMV) isolate was isolated from chilli plants in Malaysia. In this study, we have cloned the viral coat protein gene into an expression vector (pRSET, Invitrogen) to form construct CP.pRSET (Figure 1). Sequence of the cloned fragment is identical to the published CMV stain C7-2 sequence (GenBank accession no. D42079). The CMV coat protein expressed from the CP.pRSET construct was used as an antigen source for mouse immunisation to generate soluble Flag-tag fusion scFv antibody.

The approach of antibody production in animal cells can now be bypassed through the engineering of the antibody genes using recombinant DNA technology. Antibodies are generally expressed in bacterial system for rapid and large-scale production (Skerra and Pluckthun 1988).

* Corresponding author, E-mail: khchua@um.edu.my
Received Nov. 15, 2005; Accepted Mar. 20, 2006

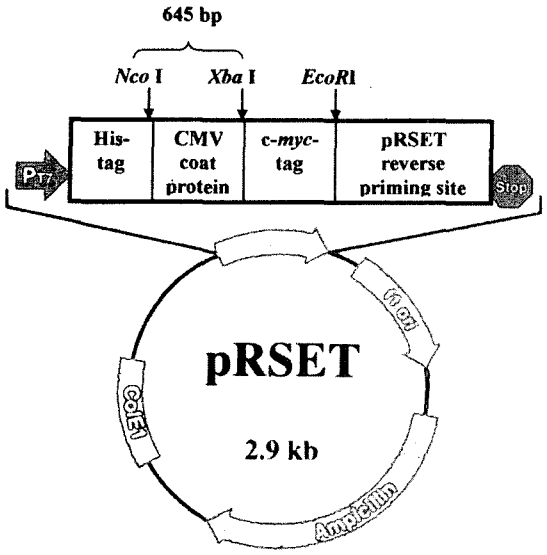

Figure 1. CP.pRSET Construct. This construct was obtained from MARDI, Malaysia. Schematic diagram shows the CMV coat protein gene construct. The coat protein gene of the local isolate CMV was cloned into pRSET vector (Invitrogen) forming the CP.pRSET construct. His-tag and c-*myc*-tag markers were also incorporated into the vector for purification and detection purposes respectively. Several important restriction sites, T7 promoter and sequencing priming site are also shown in the diagram. The expected size of CMV coat protein is ≈645 bp.

Generation of the single-chain variable fragment (scFv) is an efficient technique for production of soluble antibodies in bacterial system. An artificial peptide linker is used to join the variable heavy (V_H) and variable light (V_L) of an antibody molecule to form the scFv (Bird et al. 1988; Hutson et al. 1988), which has antigen recognition and binding affinity. ScFvs are mainly constructed using either hybridoma (Hutson et al. 1988) or spleen tissue of immunized mice (Clackson et al. 1991). The scFv can then be easily displayed as a fusion protein with the minor coat protein (PIII) of fd phage (McCafferty et al. 1990) using M13 phage display. An anti-CMV scFv gene was isolated using immunized mouse spleen cells, M13 phage display of the resulting scFv library and 3 rounds of biopanning in a bacteriophage system using the purified expressed CMV coat protein as the target antigen (Chua et al. 2003). In this study, we have successfully expressed the anti-CMV specific scFv antibody in tobacco plant (*Nacotiana tabacum*).

Materials and Methods

Single-chain variable fragment (scFv) construction

Expressed CMV coat protein was preparation for mouse immunization. After that, the mouse was sacrificed for its spleen. Total RNA was then extracted from the spleen cell followed by antibody variable fragment amplification and single-chain variable fragment (scFv) construction. The desired anti-CMV scFv was further selected through phage display and biopanning and confirmed with ELISA and western blot. The whole procedures and results were described in previous study (Chua et al. 2003).

Expression of single-chain variable fragment (scFv) recombinant antibody in *E. coli*

Following scFv construction and biopanning, the desired scFv clone was introduced into *E. coli*. The transformed *E. coli* was then induced after overnight culture with 1 mM IPTG in super broth medium (0.035% w/v bacto-tryptone, 0.02% w/v bacto-yeast extract, 0.085 M sodium chloride). After that, the scFv recombinant antibody was obtained by lysing the induced bacterial cells through osmotic shock in 1X TES (0.2 M tris hydrochloride, 0.5 mM ethylenediaminetetra-acetate acid, 0.5 M sucrose) followed by 1/5X TES buffer treatment. The detail procedures were described in Chua et al. (2003).

Western blot and ELISA assay

The scFv antibody was electrophoresed on 12% SDS-PAGE in denaturing buffer. Fractionated protein on the gel was transferred to a nitrocellulose membrane using a trans-blotter (Bio-Rad). Blocking, incubating and washing steps of the membrane were performed as described in Sambrook et al. (1989). The presence of the soluble scFv antibody protein was detected using anti-Flag M2 monoclonal antibody (Sigma) and Fc specific goat anti-mouse Ig G conjugated with Horseradish peroxidase (HRP) (Pierce). On the other hand, the ELISA assay detection method was descried by Micheal et al. (1988).

Cloning of anti-CMV ScFv into modified pCAMBIA-1301 vector

The two primers, SCFVF (5'-CATGCCATGGATGCATC-ACCATCACCATCACATGGTGCAGCTGCAGGAG-3') and SCFVR (5'-CATGCACGTGTCAGTCGACCTTGTCATCGTC-GTCCTT-3') were used in scFv DNA fragment amplification and sub-cloning as described in Chua et al. (2005).

Electroporation of *Agrobacterium tumefaciens* with plasmid

Agrobacterium tumefaciens LBA4404 (Clontech) was

grown overnight in 200 ml LB broth at 28℃ on a 2-inch stroke shaker (New Brunswick Scientific) to an OD_{600} of 0.8 Cells were cooled on ice and harvested by centrifugation at 4,000 x g for 15 min at 4℃. The cells were washed in cold distilled water twice and pelleted as before. The collected cells were resuspended in cold 10% glycerol.

Agrobacterium cell suspension with a volume of 30 µl was transferred to a pre-cooled 0.2 cm electroporation cuvette (Bio-Rad). Ten ng of the appropriate plasmid DNA was mixed with the cell suspension on ice and subjected to a 2.5 kV charge dissipated at 25 µF and 250 Ω using a Gene Pulser (Bio-Rad). Charge dissipation required approximately 4 sec for successful transformation. The *Agrobacterium* cells were then immediately transferred to 1 ml YM medium (0.04% yeast extract, 1.0% mannitol, 1.7 mM NaCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 2.2 mM $K_2HPO_4 \cdot 3H_2O$) and shaken at 30℃, 225 rpm for 3 hours. Aliquots of 100 µl were plated on YM agar containing streptomycin and kanamycin at 100 µg ml^{-1} each and incubated for 3 days at 28℃.

Co-cultivation of tobacco leaf with *Agrobacterium*

A lawn of *Agrobacterium* LBA4404 carrying the desired construct was grown on a YM agar plate containing 100 µg ml^{-1} of streptomycin and kanamycin. *Agrobacterium* suspensions were prepared by resuspending the *Agrobacterium* in 10 ml of minimal A media (4% glucose, 0.05% sodium citrate, 60mM K_2HPO_4, 31.25 mM KH_2PO_4, 7.57 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4 \cdot 7H_2O$) until the OD_{600nm} of the culture reached 0.8. Two expanded leaves from young tobacco plants were sterilized by dipping into 70% ethanol and then immersing in 10% Clorox for 5 min. The leaves were rinsed with sterile distilled water and processed into small disks (≈ 32 mm^2). Ten leaf disks were transferred into the recombinant *Agrobacterium* suspension for 15 sec. The leaf disks were then blotted on a filter paper and placed upside down on RMOP agar (0.6% Bacto-agar, 0.005% Fe-EDTA, 0.0001% thiamine, 0.00001% 1-Naphthaleneacetic acid, 0.001% 6-benzylaminopurine, 0.01% inositol, 3% sucrose, 18.81 mM KNO3, 1.50 mM $MgSO_4 \cdot 7H_2O$, 20.63 mM $(NH_4)NO_3$, 3.01 mM $CaCl_2 \cdot 2H_2O$, 1.25 mM KH_2PO_4, 0.09 mM $MnSO_4 \cdot 2H_2O$, 0.1 mM H_3BO_3, 0.07 mM $ZnSO_4 \cdot 7H_2O$, 5 µM KI, 1 µM $Na_2MoO_4 \cdot 2H_2O$, 0.1 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $CoCl_2 \cdot 6H_2O$, pH 5.8) at 26℃. After 2 days, the leaf disks were transferred to RMOP medium containing 200 µg ml^{-1} hygromycin and 500 µg ml^{-1} carbinicillin and incubated at 26℃ with 16 hours illumination (2,000 lux) per day for 2-3 weeks until the calli or shoots were formed. The calli or shoots were dissected and transferred to MS medium (0.6% Bacto-agar, 0.005% Fe-EDTA, 3% sucrose, 18.81 mM KNO_3, 1.50 mM $MgSO_4 \cdot 7H_2O$, 20.63 mM $(NH_4)NO_3$, 3.01 mM $CaCl_2 \cdot 2H_2O$, 1.25 mM KH_2PO_4, 0.09 mM $MnSO_4 \cdot 2H_2O$, 0.1 mM H_3BO_3, 0.07 mM $ZnSO_4 \cdot 7H_2O$, 5 µM KI, 1 µM $Na_2MoO_4 \cdot 2H_2O$, 0.1 µM $CuSO_4 \cdot 5H2O$, 0.1 µM $CoCl_2 \cdot 6H_2O$, pH 5.8) containing 300 µg ml^{-1} hygromycin, incubated for 7-14 days under the same conditions until roots formed. Finally, the rooted shoots were transferred to the soil for growing.

Genomic DNA extraction from tobacco plants

Leaves to be analysed (0.1 g) were ground to fine powder in liquid nitrogen. The powder was then transferred to a 1.5 ml microfuge tube and 800 µl of the extraction buffer (100 mM Tris, 500 mM EDTA, 2% SDS, pH 8.0) with 2 µl β-mercaptoethanol was added. Incubation at 65℃ for 10 min was carried out and 200 µl of 3M sodium acetate was added. The mixture was centrifuged at 10,000 x g for 10 min, the supernatant was transferred to a new microfuge tube and 500 µl of isopropanol was added, mixed and incubated at -20℃ for 30 min. The pellet was then collected by centrifugation at 10,000 x g for 10 min and dissolved in 200 µl of TE buffer. Two µl of 10 mg ml^{-1} ribonuclease A was added to the solution and incubated at 65℃ for 10 min. The process was continued by adding 2 µl of 100 mg ml^{-1} proteinase K into the solution and incubated under the same conditions. After that, phenol extraction followed by chloroform extraction was carried out and the aqueous phase was mixed with 20% PEG 8000/1.2 M NaCl solution. Incubation on ice for 20 min followed by centrifugation was done to collect the pellet. The DNA pellet was then washed with 70% ethanol and dissolved in 20 µl of TE. The DNA was stored at -20℃ for future analysis.

Total Protein Extraction from Tobacco Plants

Two leaves from each of the 2-month old putative transgenic tobacco plants were cut and ground with a pestle in a 1.5 ml microfuge tube on ice. The sample was centrifuged at 10,000 x g for 10 min at 4℃ and the sap (supernatant) was collected and used for protein analysis.

Results and Discussion

Generally, a new isolate of CMV isolated from chilli plants in Malaysia and known to cause serious economical loss of this major agriculture crop has coat protein sequence with close identity to the published CMV coat protein sequences. We report on the construction and expression of an anti-

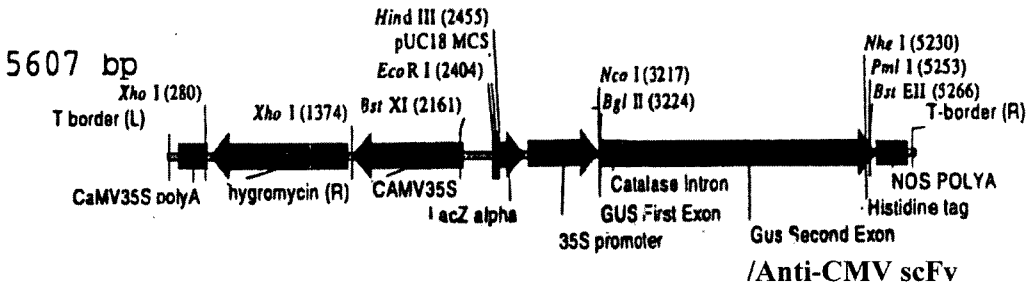

Figure 2. pUMSCFV-CMV1 construct. T-DNA sector of pCAMBIA-1301. After double digestion using *Nco* I and *Pml* I endonucleases, the Gus second exon cassette was removed from the vector and replaced with an anti-CMV scFv DNA fragment to form pUMSCFV-CMV1.

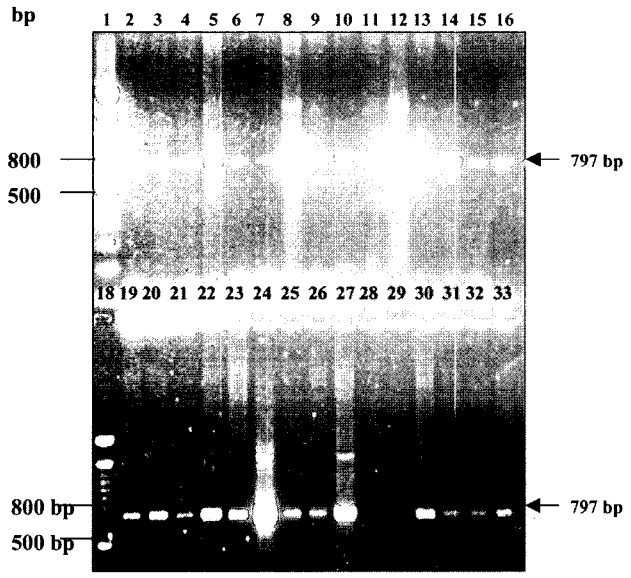

Figure 3. PCR screening of putative transgenic plants. Lane 1 and 18 : One hundred bp ladder (Gibco BRL); Lane 2-16 and 19-33: PCR samples amplified using SCFVF and SCFVR primers on individual putative transgenic plant total genomic DNA. The samples were separated on a 1% agarose gel followed by ethidium bromide staining. The arrows show that the expected 797 bp DNA fragment.

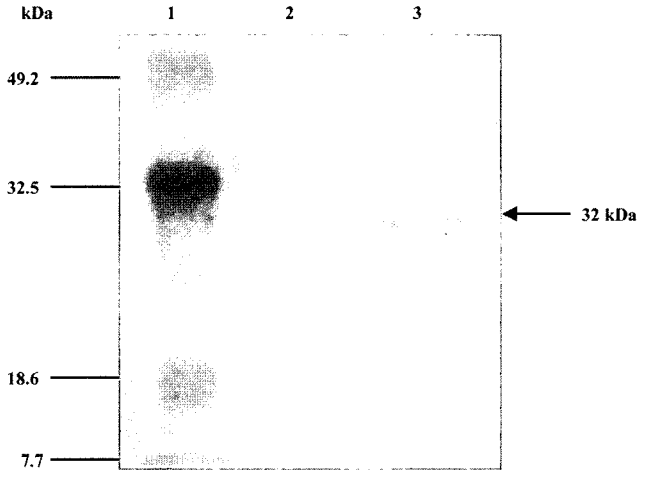

Figure 4. Western blot analysis of anti-CMV scFv recombinant protein. Lane 1: kaleidoscope prestained protein marker (Bio-Rad); Lane 2: total protein isolated from wild type tobacco leaves as a negative control; Lane 3: Total protein isolated from transgenic tobacco leaves. The 32 kDa scFv antibody as shown by an arrow was detected using anti-FLAG as the primary antibody, followed by goat anti-mouse IgG-HRP conjugated antibody.

CMV scFv for cost effective production of antibodies in bacterial system to be used for CMV diagnostics in previous study (Chua et al. 2003).

In this study, the same antibody which produced in bacterial system was also successfully expressed in transgenic tobacco *Nacotiana tabacum* as an alternative system of production. Initially, the selected anti-CMV scFv was subcloned into pCAMBIA-1301 to form pUMSCFV-CMV1 construct as shown in Figure 2. Following transformation of tobacco with pUMSCFV-CMV1, 58 plants, or 35.8% of 162 shoots and calli selected from 4 events, survived after the second selection on MS medium with 300 μg ml^{-1} of hygromycin. Fifty six out of 58 plants were shown to carry the desired anti-CMV scFv gene by PCR analysis. Part of the results is shown in Figure 3. For detection of recombinant antibody expression in plants, total plant protein was extracted from the tobacco leaves and separated on a 12% SDS-PAGE gel and western dot-blot was carried out using anti-Flag M2 monoclonal antibody. Only 12.5% of the 56 putative transgenic plants were found to express the antibody to a detectable level. A representative sample together with total protein from a wild type plant was used for western blot analysis. A band of the expected anti-CMV scFv antibody of 32 kDa was visualized as shown in Figure 4. Functional analysis of the plant-derived antibody was then carried out using competition western blot. The presence of a 35 kDa

confirmed that the plant-derived antibody is functional for

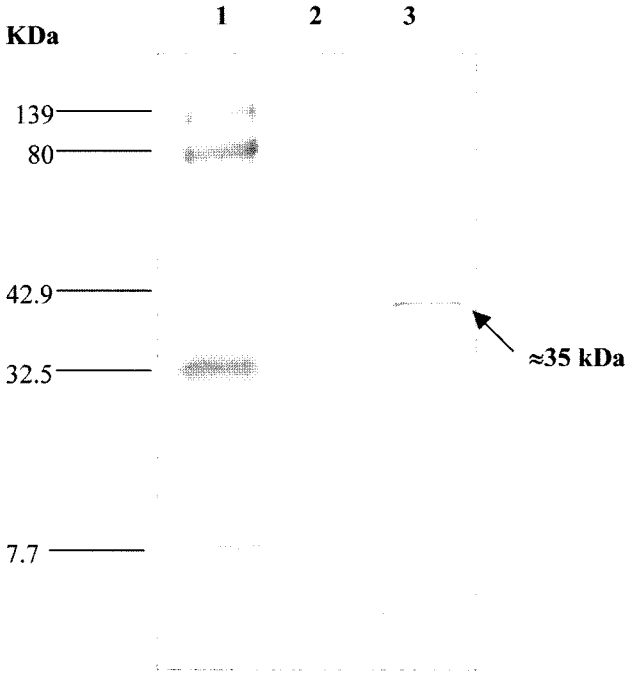

Figure 5. Functional analysis of recombinant plant-produced antibody by competition western blot. Lane 1: kaleidoscope prestained protein marker (Bio-Rad); Lane 2: total protein of wild type *E. coli* as a negative control; Lane 3: total protein extracted from induced *E. coli* carrying CP.pRSET construct. The 35 kDa band as shown by an arrow above was detected by plant-derived anti-CMV scFv as the primary antibody, followed by anti-Flag and goat anti-mouse IgG-HRP conjugated antibody detection.

detection of CMV coat protein (Figure 5). The application of the transgene for efficient and large scale production of antibodies will require several further steps and modifications. This would include the addition of tag sequences in the construct to promote specific targeting leading to higher scFv stability and accumulation and less degradation (Schouten et al. 1996; Schouten et al. 1997; Fiedler et al. 1997).

There has been a great interest in the possibility of using plants for the heterologous production of antibodies and antibody fragment. Apart from safety issues, the cost of antibody production in plants can be lower than production costs using bacteria, such as *E. coli* especially when the production reaches field scales (Larrick et al. 1998). This study also shows that the scFv expressed in tobacco in this study were structurally functional.

In addition to antibody production in plants for diagnostic reagent use, plant expression of antibodies that interfere with plant viral infection can provide an alternate strategy for plant disease control (Tavladoraki et al. 1993; Zimmermann et al. 1998). The antibodies might interfere with viral replication or perturb translocation of viral glycoprotein, however, the actual mechanism remains unclear (Tavladoraki et al. 1993; Tavladoraki et al. 1999; Franconi et al. 1999). Antibody produced against the virus coat protein could have high neutralizing properties to protect the host from virus infection.

In conclusion, anti-CMV scFv antibody production was successfully demonstrated in transgenic plants in this study. The competition western blot results showed that the plant-produced antibody has the same antigen-binding functionality as those of bacterially produced scFv antibody.

Acknowledgements

This study was funded by IRPA R&D grant ID: 09-02-03-0521, and Vote F of University of Malaya. Special thanks to Dr. Tan Chong Seng for the viral construct, Dr Jennifer, Professor Sazaly, Hui Yee and all the members of the Microbiology lab, Medical Faculty, UM, members from AMCAL, UM for their help and advice.

References

Bird RE, Hardman KD, Jacobson JW, Johnson S, Kaufman BM, Lee T, Pope HS, Riordan GS, Whitlow M (1988) Single-chain antibody binding proteins. Science 242: 423-426

Chen B, Francki RIB (1990) Cucumovirus transmission by the aphid Myzus-persicae is determined solely by the viral coat protein. J Gen Virol 71: 939-944

Chua KH, Noorzulaani K, Tan CS, JA Harikhrisna, Rofina YO (2003) Synthesis of a soluble flag-tagged single chain variable fragment (scFv) antibody targeting cucumber mosaic virus (CMV) coat protein. Asia Pacific J Mol Biol Biotechnol 11: 93-100

Chua KH, JA Harikrishna, Norzulaani K, Rofina YO (2005) Direct cloning approach for expression of an anti-cucumber mosaic virus single chain variable fragment in plants. Biotechnol 4: 206-210

Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1991) Making antibody fragments using phage display libraries. Nature 352: 624-628

Doolittle SP (1916). A new infectious mosaic disease of cucumber. Phytopathology 6: 145

Fiedler U, Philips J, Artsaenko O, Conrad U (1997) Optimisation of scFv antibody production in transgenic plants. Immunotechnol 3: 205-216

Franconi R, Roggero P, Pirazzi P, Arias FJ, Desiderio A, Bitti O, Pashkoulov D, Mattei B, Bracci L, Masenga V, Milne RG, Benvenuto E (1999) Functional expression in bacteria and plants of an scFv antibody fragment against tospoviruses. Immunotechnol 4: 189-201

Fuchs M, Provvidenti R, Slightom JL, Gonsalves D (1996) Evaluation of transgenic tomato plants expressing the coat protein gene of cucumber mosaic-virus strain WL under field conditions. Plant Dis 80: 270-275

Fuchs M, McFerson JR, Tricoli DM, McMaster JR, Deng RZ, Boeshore ML, Reynolds JF, Russell PF, Quemada HD (1997) Cantaloupe line CZW-30 containing coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus-2 is resistant to these three viruses in the field. Mol Breeding 3: 279-290

Gera A, Leobenstein G, Raccah B (1979) Protein coats of two strains of cucumber mosaic virus affect transmission by Aphis gossypii. Phytopathology 69: 369-399

Gonda TJ, Symons RH (1979) Cucumber mosaic virus replication in cowpea protoplasts: time course of virus, coat protein and RNA synthesis. J Gen Virol 45: 723-736

Harrison SC (1984) ultiple-modes of subunit association in the structures of simple spherical viruses. Trends Biochem Sci 9: 345-351

Houwing CJ, Jaspars EMJ (1986) Coat protein blocks the in vitro transcription of virion RNAs of Alfafa mosaiv-virus. FEBS Lett 209: 284-288

Hutson JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, Crea R, Oppermann H (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. Proc Natl Acad Sci USA 85: 5879-5883

Jagger IC (1916) Experiments with the cucumber mosaic disease. Phytopathology 6: 148

Kaper JM, Waterworth HE (1981) Cucumoviruses. In: Kurstak E (ed), Handbook of Plant Virus Infectious and Comparative Diagnosis. Elsevier/North-Holland Biomedical Press, The Netherlands, pp 257-332

Lin NS, Hsieh CE, Hsu YH (1996) Capsid protein of cucumber mosaic virus accumulates in the nuclei and at the periphery of the nucleoli in infected cells. Arch Virol 141: 727-732

Larrick JW, Yu L, Chen J, Jaiswal S, Wycoff K (1998) Production of antibodies in transgenic plants. Res Immunol 149: 603-608

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348: 552-554

Michael, F. C., Richard M.L. and Moshe B. J. (1988) Methods for plant molecular biology: ELISA Techniques (Arthur W. and W. Herbert (ed.). Academic Press, London, pp 507-530

Palukaitis P, Roossinck MJ, Dietzgen RG, Francki RIB (1992) Cucumber mosaic virus Avd Virus Res 41: 281-384

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual (2nd eds) Cold Spring Harbor: Cold Spring Harbor laboratory Press

Schouten A, Roosien J, De Boer JM, Wilmink A, Rosso MN, Bosch D, Stiekema WJ, Gommers FJ, Bakker J, Schots A (1997) Improving scFv antibody expression level in the plant cytosol. FEBS Lett 415: 234-241

Schouten A, Roosien J, Van Engelen FA, De Jong GAM, Borst-Vrenssen AWM, Zilverentant JF, Bosch D, Stiekema WJ, Gommers FJ, Schots A, Bakker J (1996) The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both cytosol and the secretory pathway in transgenic tobacco. Plant Mol Biol 30: 781-793

Skerra, A, Pluckthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. Science 240: 1038-1040

Tavladoraki P, Benvenuto E, Trinca S, De Martinis D, Cattaneo A, Galeffi P (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature 366: 469-472

Tavladoraki P, Girotti A, Donini M, Arias FJ, Mancini C, Morea K, Chiaraluce R, Consalvi V, Benvenuto E (1999) A single-chain antibody fragment is functionally expressed in the cytoplasm of both *Escherichia coli* and transgenic plants. Eur J Biochem 262: 617-624

Tricoli DM, Carney KJ, Russell PF, McMaster R, Groffl DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD (1995) Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to cucumber mosaic virus, watermelon mosaic virus 2, and zucchini yellow mosaic virus. Biotechnol 13: 1458-1465

Yie Y, Wu ZX, Wang SY, Zhoa SZ, Zhang TQ, Yao GY, Tien P (1995) Rapid production and field testing of homozygous transgenic tobacco lines with virus-resistance conferred by expression of satellite RNA and coat protein of cucumber mosaic-virus. Transgen Res 4: 256-263

Zimmermann S, Schillberg S, Liao YC, Fischer R (1998) Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in *Nicotiana tabacum*. Mol Breed 4: 369-379