Ph.D. Thesis

Expression of dsRNA-specific monoclonal antibodies in transgenic plants

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This modest work is
dedicated to my Father for his
faith, love and support along
all my studies.

Bogdan Morgun

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1. Acknowledgement

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2. List of Abbreviations

AN-ELISA	Anti-nucleic acid ELISA
BCIP	5-Bromo-4-chloro-3-indolyl phosphate disodium salt
BSA	Bovine serum albumin
CDR	Complementarity Determining Regions
DEPC	Diethyl pyrocarbonate
dNTP	Deoxynucleoside triphosphate
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Monovalent antibody fragment composed of variable (VH and VL) and constant (CL and CH1) domains
IgG	Immunoglobulin of class G
IPTG	Isopropyl-beta-D-thiogalactopyranoside
mAb	(Intact) monoclonal antibody
MOPS	3-(N-Morpholino)propanesulfonic acid
MW	Molecular weight
NBT	p-Nitro-blue tetrazolium chloride
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PAA	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonyl fluoride
RPAS	Recombinant Phage Antibody System
rpm	Revolutions per minute
RT	Room temperature
scFv	Single-chain variable fragment
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA buffer for agarose gel electrophoresis of DNA
TBE	Tris- borate-EDTA buffer for agarose gel electrophoresis of DNA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tm	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
X-Gal	5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside

3. Introduction

3.1. Antibodies: structures and functions

Antibodies are complex glycoproteins, which bind to target antigens with great specificity and play a major role in the specific immune response of vertebrates. Vertebrates produce them in response to antigens, such as infectious agents (e.g. bacteria or viruses) and other non-self substances (e.g. proteins or polysaccharides in pollens). The presence of an antigen induces the production of different antibodies, each of which may bind to a different region (epitope) of the antigen. Antibodies act as specific sensors for antigens, forming antibody-antigen complexes that initiate a cascade of protective reactions in cells of the immune system.

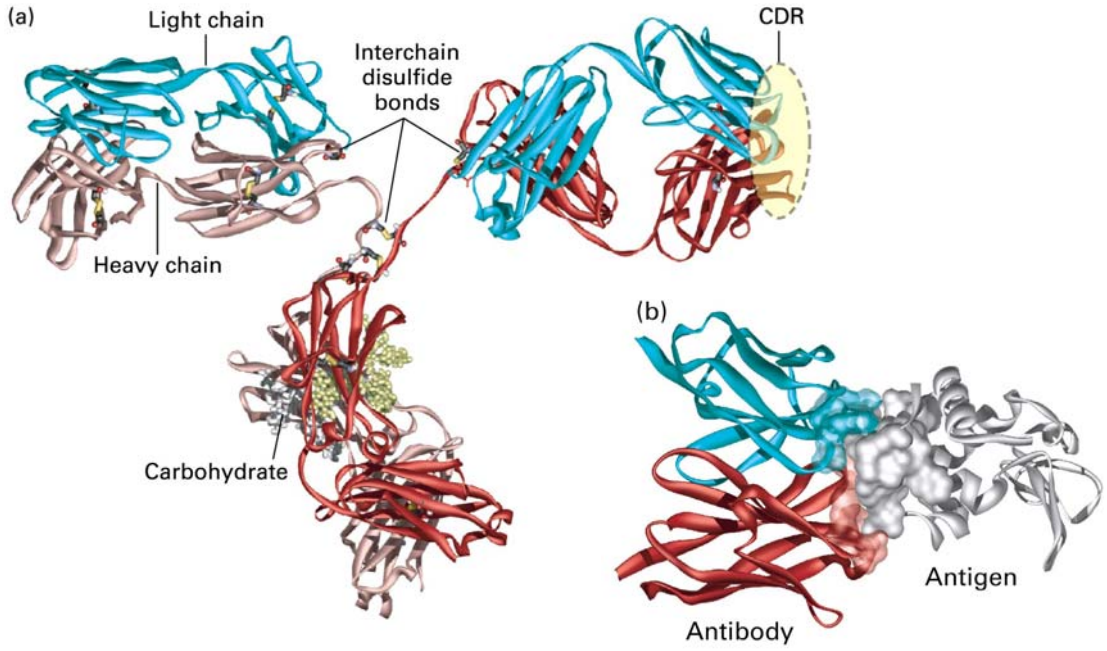

Figure 3-1: Antibody structure and antibody-antigen interaction. (a) Ribbon model of an antibody. Every antibody molecule consists of two identical heavy chains (red) and two identical light chains (blue) covalently linked by disulfide bonds. (b) The hand-in-glove fit between an antibody and an epitope on its antigen - in this case, chicken egg-white lysozyme. Regions where the two molecules make contact are shown as surfaces. The antibody contacts the antigen with residues from all its complementarity-determining regions (CDRs). In this view, the complementarity of the antigen and antibody is especially apparent where "fingers" extending from the antigen surface are opposed to "clefts" in the antibody surface (Lodish et al., 2003)

Antibody molecules carry out dual tasks - binding on the one hand to a wide variety of antigens, and on the other hand to a limited number of effector molecules and cells. Each of these tasks is carried out by separable parts of the molecule: The N-terminal variable (V) domains are involved in antigen binding, whereas the far less variable constant (C) domains interact with effector cells and molecules (Figure 3-2).

All antibodies are constructed in the same way from paired heavy and light polypeptide chains, and the generic term immunoglobulin is used for all such proteins. Within this general category, however, five different classes of immunoglobulins IgM, IgD, IgG, IgA, and IgE can be distinguished by their C regions. More subtle differences confined to the V region account for the specificity of antigen binding.

IgG antibodies are formed from two identical heavy chains and two identical light chains (Figure 3-1a, Figure 3-2). Each chain consists of a series of discrete, compactly

folded domains, each about 110 amino acids long. The light chain is made up of two such immunoglobulin domains, whereas the heavy chain of the IgG antibody contains four. The amino-terminal variable or V domains of the heavy and light chains (VH and VL, respectively) together make up the V region of the antibody and confer the ability to bind specific antigen, while the constant domains (C domains) of the heavy and light chains (CH and CL, respectively) make up the C region (see Figure 3-2). Each arm of an antibody molecule contains a single light chain linked to a heavy chain by a disulfide bond and by non-covalent interactions. Near the N-termini of the chains are 3-3 highly variable loops, called complementarity determining regions (CDRs), which form the antigen-binding sites. High affinity between the antigen binding site and the antigen epitope recognized is achieved by high surface complementarity (Figure 3-1b). The intimate contact between these two surfaces, stabilized by numerous noncovalent bonds, is responsible for the exquisite binding specificity exhibited by an antibody.

3.2. Antibody engineering

Natural antibodies can be elicited by immunizing animals or by the hybridoma technology. Although hybridoma technology is expensive and time consuming, hybridoma cell lines still provide a permanent source of monoclonal antibodies and rearranged antibody sequences. Hybridoma lines are propagated as individual clones, each of which produces a single type of monoclonal antibody. This antibody recognizes a single type of antigenic site (epitope), for example, a particular cluster of five to six amino acids on the surface of a protein. Their uniform specificity makes monoclonal antibodies much more useful for most purposes than conventional antisera, which generally contain a mixture of antibodies that recognize a variety of different antigenic sites on macromolecules.

The most important advantage of the hybridoma technique is that monoclonal antibodies can be made against molecules that constitute only a minor component of a complex mixture. In an ordinary antiserum made against such a mixture, the proportion of antibody molecules that recognize the minor component would be too small to be useful. But if the B lymphocytes that produce the various components of this antiserum are made into hybridoma cell lines, it becomes possible to select lines producing the desired type of monoclonal antibody and to propagate the selected hybridoma indefinitely to produce that antibody in unlimited quantities. In principle, therefore, a monoclonal antibody can be made against any protein in a biological sample. Once an antibody has been made, it can be used as a specific probe both to track down and localize its Protein Antigen and to purify that protein in order to study its structure and function.

A few years later after the development of monoclonal antibodies by Kohler and Milstein in 1975, gene technology revolutionized the potential to obtain a single species of antibody with a desired specificity to an antigen (Conrad et al., 1994). Immunoglobulin genes could now be modified in order to obtain different recombinant antibody fragments or fusion proteins for almost any applications (Winter et al., 1991). There are many advantages of producing small antibody fragments by genetic engineering technology: high level of expression (Fiedler et al., 1995), stability, possibility to influence biological processes (Artsaenko et al., 1994; Richardson et al., 1995), incorporation of 'tag' sequences, construction of libraries, design of bifunctional and bispecific molecules, etc. A number of the applications could be found, among them the introduction of pathogen resistance, diagnostic and therapeutic usage, inexpensive high level production of antibodies and their fragments, development of powerful tools for cellular and structural studies and so on (Tavladoraki et al., 1993; Voss et al., 1994;

Fecker et al., 1996). Recently established expression systems for genetically engineered antibodies utilize the now trusted vectors in bacteria, yeast, plant and mammalian cell (Hiatt, 1990; Benvenuto et al., 1991; Swain, 1991).

An alternative to hybridoma approach is to use phage display libraries based on the human immune repertoires for the production of scFvs (Figure 3-3 and Figure 3-4). In 1990, McCafferty and colleagues demonstrated in a model experiment that it was possible to use the filamentous bacteriophage fd as a tool to select an antibody fragment from a large population of non-binding proteins (McCafferty et al., 1990). This work, based on earlier fundamental findings concerning peptide phage display by Smith's group (Scott et al., 1990), was the basis of the development of the technique that has revolutionized the field of antibody engineering.

Phage display is advantageous, because high-affinity antibodies can be rapidly identified, novel combinations of heavy and light chains can be tested and the DNA sequence encoding the antibody is packed in the same particle where the antibody itself is localized (Griffiths et al., 1998; Sidhu, 2000). This avoids the laborious isolation of cDNA or genomic immunoglobulin sequences from hybridoma cell lines. Phage display allows the isolation of antigen-binding scFv-encoding sequences directly from established libraries or from the spleen of immunized mice. The technique makes use of generally applied recombinant DNA techniques and avoids animal cell cultures.

The basic principle is to express a protein, in this case an antibody fragment, on the surface of a phage particle as a fusion protein of a phage protein (e.g. g3). The resulting particle can be affinity-purified on immobilized antigen. After washings and elution, the selected phages are amplified by infection of *E. coli*. This cycle can be repeated several times. On average, a binder is enriched 10^3-10^5-fold over non-binding clones. This technique can be used to select antibodies from large repertoires. Two types of repertoires have been built. Immunized repertoires where the V genes used to assemble the antibody fragments are taken from a mouse immunized by antigen, and naive "single pot repertoires" where V genes are taken from peripheral blood lymphocytes of non-immunized individuals. The main advantage of the first method is that antibodies selected from immune repertoires are expected to have high affinities on the basis that immunization will have induced a large bias toward B-cells specific for the immunogen and having undergone affinity maturation. Obviously the main drawback of such repertoires is that a new repertoire is required for each new antigen.

In this respect, naive libraries are very convenient since once they are constructed, they can then be used almost indefinitely against a diverse range of antigens. Most importantly, these libraries can be built using human B-cells, thereby allowing the direct isolation of human antibodies. A major drawback of naive repertoires is that they need to be very large to yield reasonable affinities. Typically a naive library of 10^{10} clones is expected to yield affinities in the 10^{-8} M range. If higher affinities are desired, for example for *in vivo* applications, affinity maturation techniques can be used. Alternatively, "synthetic" libraries have been made using PCR techniques and degenerate primers to introduce diversity at precise locations (typically CDR3s). The possibility to control the selection process (by depletion, competition) and to obtain fully human high-affinity antibodies against any antigen including toxic, conserved or non-immunogenic targets, possibly in an automated fashion, should ultimately set phage display technology to replace hybridoma technology. Antibody engineering also provides the possibility to tailor these binding fragments for diagnostic or targeting purposes as well as for use as "intrabodies", a rapidly growing field of application of antibody fragments (Cesaro-Tadic et al., 2003; Bai et al., 2004; Pini et al., 2004).

From the bacterial clones, antibody fragment-encoding sequences are available for further cloning in plant expression vectors. Using this approach, it is feasible to isolate antibody fragment-encoding sequences from immunized mice in about 4 months starting from immunization. However, even further improvements in antibody gene isolation are possible by exploiting recent developments in recombinant antibody engineering, which promise to bypass immunization. These advances have been achieved by constructing huge naïve Fab and scFv phage display libraries (Griffiths et al., 1994; Vaughan et al., 1996) from which high-affinity antibody fragment-encoding sequences can be isolated in only a few weeks against any antigen. Although applications of such libraries have been limited largely to medical research, library construction strategies are being continuously optimized (Sheets et al., 1998; de Haard et al., 1999; Sblattero et al., 2000) and the construction of phage display libraries for use in plant biology seems feasible in the near future (Eeckhout et al., 2004).

Worth mentioning is also the *in vitro* production of antibody fragments by ribosome display (Hanes et al., 1997), which should allow even library construction in *E. coli* to be bypassed. The unit, selected in this system, consists of the recombinant protein connected to the encoding RNA via the ribosome. Library construction is carried out by PCR, *in vitro* transcription, and *in vitro* translation. No transformation is necessary and very large libraries could become accessible in a single step. Moreover, ribosome display avoids the tedious alternation between *in vitro* and *in vivo* steps. Furthermore, after each selection round, additional genetic diversity can be generated by error-prone PCR, expanding the size of the sequence space that can be screened and thus increasing the chance to find a specific antibody with the desired specificity and high affinity.

3.3. Expression of antibodies in plants

Production of biomolecules in plants began in 1989 with the remarkable demonstration that functional recombinant antibodies could be expressed in tobacco (Hiatt et al., 1989). Before this result was published, there was little support for the idea that plants could be used to produce therapeutic proteins. Since then, it has been shown that transgenic plants are extremely versatile and they have been used to produce a wide range of pharmaceutical proteins. These include blood substitutes (Magnuson et al., 1998), growth hormones (Barta et al., 1986; Staub et al., 2000; Leite et al., 2000), vaccines (Haq et al., 1995; Arakawa et al., 1998; Kapusta et al., 1999; Walmsley et al., 2000; Mason et al., 2002), and mammalian antibodies (Ma et al., 1995; Conrad et al., 1998; Zeitlin et al., 1998; Fischer et al., 2004).

Different classes of intact immunoglobulins (Ig) have been produced successfully in plants, including IgG (various subclasses), secretory IgA and a chimeric IgA/G (Figure 3-2) (Ma et al., 2003). These have ranged in sequence from completely murine to fully humanized. Secretory IgAs are dimers of the typical serum-type immunoglobulins and include two extra components: the secretory component and the joining chain. Four separate transgenes are required to produce such molecules (Larrick et al., 2001). The simplest natural antibody, camelid heavy-chain antibody lacks a light chain and advantageously can be expressed as a single transgene. Smaller engineered antibody derivatives have also been expressed in plants (Stoger et al., 2002a; Schillberg et al., 2003).

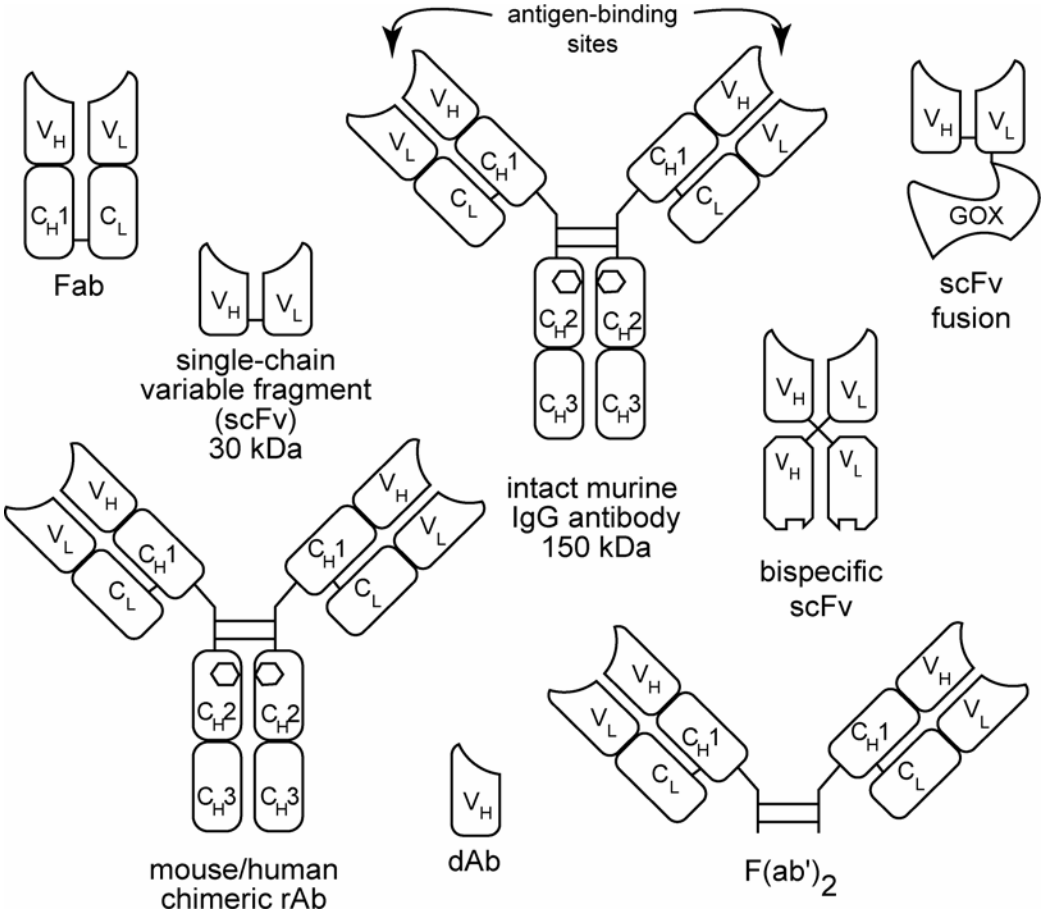

Figure 3-2: Full-length IgG and artificially constructed antibody fragments that were expressed in plants

Antibody genes could be obtained from monoclonal hybridoma cell lines, from immunized animals or phage display libraries.

In phage display libraries the coding sequences of variable domains are amplified from isolated mRNAs and assembled into one DNA fragment by PCR (Figure 3-3). After ligation to a vector, recombinant molecules are transformed and studied in *E. coli*.

Currently the most popular antibody fragment for plant expression is the single-chain Fv-fragment (scFv). In scFv the variable regions of the heavy and the light immunoglobulin chains are joined covalently by a short flexible hydrophilic peptide linker (Figure 3-4). It permits formation of the antigen-binding site. Frequently the coding sequence of scFv is flanked with unique restriction endonuclease sites to facilitate its transfer from vector to vector and fusion of necessary regulatory signals. When expressed some plantibody derivatives form spontaneous dimers. For some specialized applications antibody fragments could be made in a form of minibodies, diabodies, large single chains, bispecific scFvs that contain the variable regions from two parent immunoglobulins and recognize two unrelated antigens; scFv fusion proteins in which the scFv is genetically fused to a toxin, cytokine or enzyme (Ma et al., 2003).

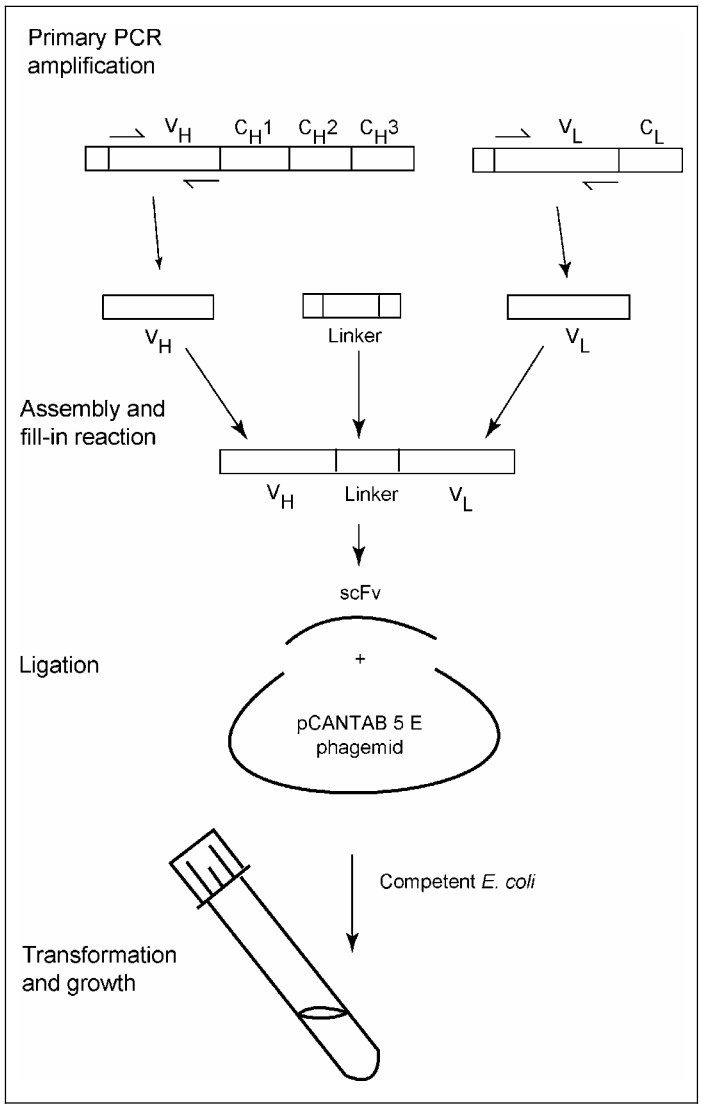

Figure 3-3: Construction and cloning scFv using poly A$^+$ mRNA as source of VH- and VL-sequences

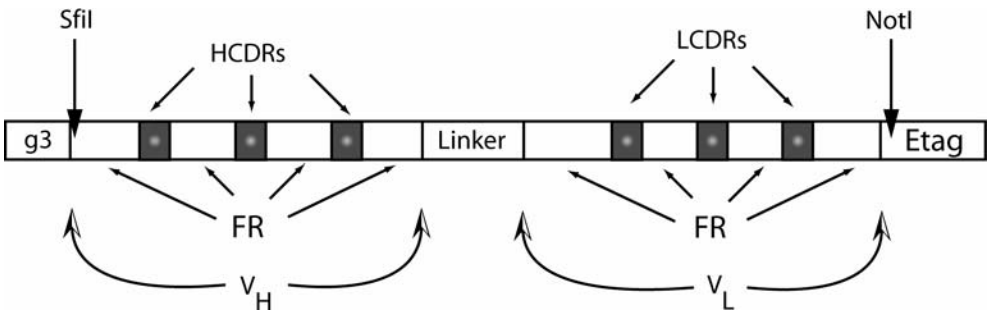

Figure 3-4: General map of scFv constructed in pCANTAB 5E phagemid and expressed in E. coli*. Complementarity determining regions (CDR) and framework regions (FR) are shown*

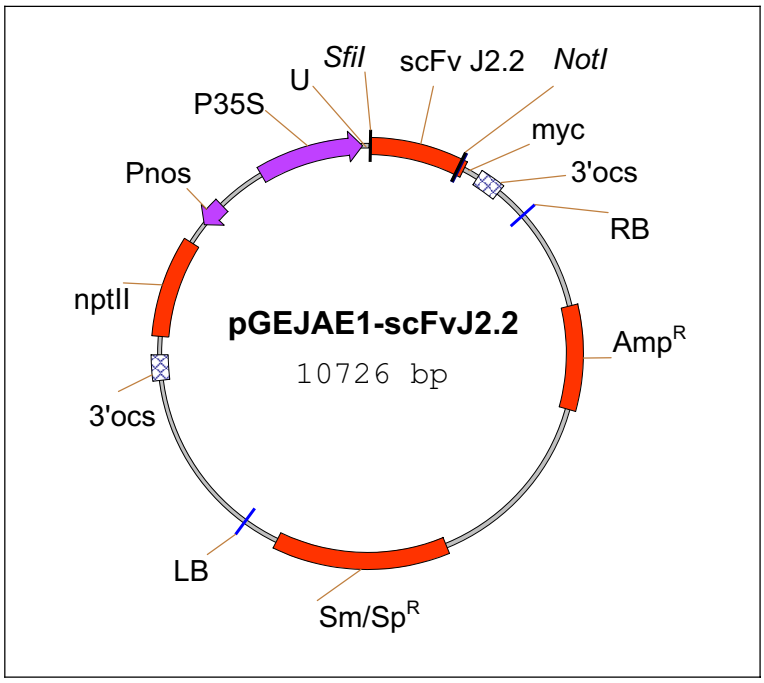

Figure 3-5: Schematic map of ready-to-transform recombinant co-integrative plant expression vector pGEJAE1 harboring scFv J2 coding sequence. LB, T-DNA left border; 3'ocs, 3' end of the octopine synthase gene as terminator; nptII, neomycin phosphotransferase gene, kanamycin resistance marker; Pnos, promoter of the nopaline synthase gene; P35S, 35S promoter of cauliflower mosaic virus; U, 5' untranslated omega-leader of tobacco mosaic virus; scFv, coding sequence of single-chain antibody fragment; myc, c-myc tag; SfiI and NotI, endonuclease sites for insertion of scFv sequence; RB, T-DNA right border; Amp^R and Sm/Sp^R, ampicillin and streptomycin/spectinomycin resistance marker genes

Due to simplicity and robustness *Agrobacterium tumefaciens*-mediated transformation of leaf disks is often used to transfer scFv genes to *Nicotiana tabacum.* Moreover, a suitable vector for straightforward scFv re-cloning from bacterial phage display to plant expression system, employing common SfiI and NotI endonuclease sites is available from Dr. Geert De Jaeger, (De Jaeger et al., 1999). In this vector (Figure 3-5) a strong constitutive cauliflower mosaic virus (CaMV) 35S promoter controlls the scFv expression and the 5' untranslated omega leader of tobacco mosaic virus (TMV) enhances transcription. Immunological detection of the expressed scFv is possible through the C-terminally fused c-myc tag and c-myc-specific mouse mAb. All these features are provided by the unmodified pGEJAE1 plant vector ensuring cytoplasmic expression of the inserted scFv gene. ATG start codon needs to be provided by scFv coding sequence while the vector supplies a stop codon. There are three marker genes within the vector. The marker nptII gene located between T-DNA borders confer kanamycin resistance of transformants necessary for selection. The ampicillin and streptomycin/spectinomycin resistance marker genes are necessary for plasmid maintenance in *E. coli* and selection of transconjugant *Agrobacterium* clones. This plant vector has been used in our experiments to express dsRNA-specific scFv in plants.

3.3.1. *Ex planta* applications of antibodies produced in plants

Antibodies synthesized in transgenic plants could be utilized in two ways (Figure 3-6). For *ex planta* applications pharmaceuticals are extracted, purified and used for diagnosis or therapy. Although diagnostic applications have predominated to date, there is a growing need for producing plant-made proteins for therapeutic purposes.

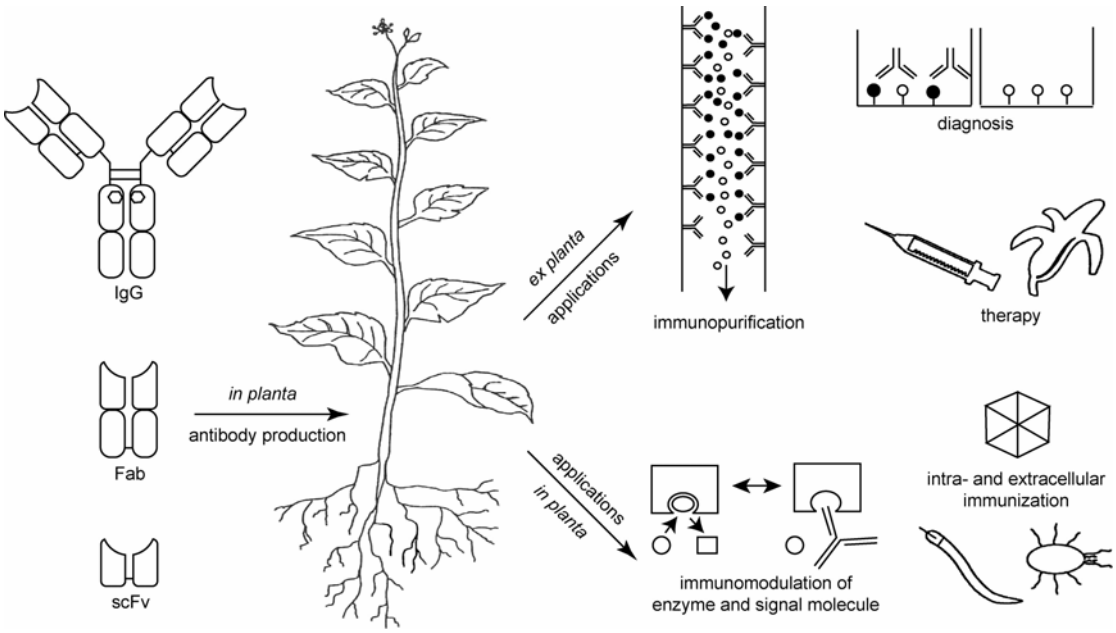

Figure 3-6: In planta *and* ex planta *applications of antibodies produced in plants (De Jaeger et al., 2000)*

Recombinant antibodies have been expressed in several plant species, including *Arabidopsis*, tobacco, potato, rice, wheat, alfalfa and pea (Morgun et al., 2004). The large-scale production of recombinant proteins in living cells, crops or domestic animals is termed molecular farming. Molecular farming of antibodies in transgenic plants or plant cell cultures is possible and offers many advantages over culturing mammalian cells. The cost of antibody production in plants is significantly lower. In the case of therapeutical applications the health risk from transmitting viruses and infectious agents is completely eliminated, while product quality and homogeneity remains high. The control of the production process is more complicated for transgenic plants, but this can be overcome by employing plant cell cultures for antibody synthesis (Schillberg et al., 2003).

Table 3-1: Selected examples for applications of recombinant antibodies ex planta. *The instances show different types of usage or genetic engineering approaches*

Antibody (antigen)	Host plant	Comments	References
IgM (neuropeptide hapten)	Tobacco	First IgM expressed in plants and protein targeted to chloroplast for accumulation	During et al., 1990
scFv-bryodin 1 immunotoxin (CD 40)	Tobacco	Production of pharmaceutical antibody fragment in plant cell-suspension culture	Francisco et al., 1997
IgG (Herpes simplex virus)	Soybean	Production of pharmaceutical immunoglobulin in soybean	Zeitlin et al., 1998
sIgA/G (*Streptococcus mutans* adhesion)	Tobacco	First secretory IgA expressed in plants; at present the most advanced plant-derived pharmaceutical protein	Ma et al., 1998

scFv (38C13 mouse B cell lymphoma)	Tobacco	Production of recombinant antibody-based tumour-specific vaccines	McCormick et al., 1999
Large single chain (Herpes simplex virus)	*Chlamydo-monas*	Antibody production in the chloroplast of green unicellular algae	Mayfield et al., 2003
IgG (hepatitis B virus)	Tobacco	Immunopurification of target antigen using a specific plantibody produced in large scale	Ramirez et al., 2003

3.3.2. *In planta* applications to modulate plant metabolism or to obtain pathogen resistance

In addition to protein expression of medical and industrial interests, immunologically relevant proteins are expressed in plants to study physiological effects and to immunomodulate plant and pathogen functions for agricultural purposes (Table 3-1). In case of such *in planta* applications, the aim of antibody expression is to immunomodulate enzyme and signal molecules to interfere with cellular metabolism or with pathogen infectivity in the host *in vivo*. The modulating effect of antibodies is based upon their specific binding to the target antigens. Because of the formation of antigen-antibody complex sites required for interaction with other molecules may be blocked, the diffusion rate and even the *in situ* localisation of the antigen may be altered, etc. These effects altogether have the consequence that the availability of the antigen for the usual physiological or regulatory processes will be reduced. This approach can only be effective if the antibodies are present at sufficient concentration, are correctly folded and localized in the same cellular compartment as the antigen. Since it is still difficult to express correctly folded active antibodies or antibody fragments in a particular cell compartment, especially in the cytoplasm, immunomodulation and immunoprotection approaches are challenging and not always successful (De Jaeger et al., 2000).

The molecular technique that allows to interfere with cellular metabolism or pathogen infectivity by the ectopic expression of genes encoding antibodies or antibody fragments is called immunomodulation (De Jaeger et al., 2000). The first successful immunomodulation strategy was achieved in relation to phytochrome function (Table 3-2). Immunomodulation has been adopted to investigate the action of signal molecules (e.g. jasmonates (ten Hoopen, 2002); receptors, phytohormones, enzymes, house-keeping proteins (e.g. heat shock proteins) and so on. From yet unpublished data of U. Conrad and co-workers we learned that transgenic plants have also been produced to express scFvs against a cyclic and biologically active compound of the jasmonic acid pathway ((9S,13S)-12-oxo-10,15(Z)-phytodienoic acid, cis(+)-OPDA)) and brassinosteroids. A further general field of application is the investigation of DNA–protein, RNA–Protein And protein–protein interactions in transcription control. Specific, high-affinity scFvs against a seed-specific transcription factor, FUS3, have been produced and characterized (G. Mönke and U. Conrad, unpublished). Both protein–DNA and protein–protein interactions could be studied *in vivo* depending on the epitope recognized by the specific recombinant antibody.

Table 3-2: Examples of immunomodulation in plant physiology as well as applications to plant protection

Antibody (antigen)	Host plant	Comments	References
scFv (phytochrome)	Tobacco	Immunomodulation of receptor activity	Owen et al., 1992
scFv (coat protein of artichoke mottled crinkle virus)	Tobacco	Antibody directed against essential viral protein. Attenuation of viral infection, reduction of infection incidence, delay in symptom development.	Tavladoraki et al., 1993
scFv (abscisic acid)	Tobacco Potato	Immunomodulation of phytohormone function	Artsaenko et al., 1995; Strauss et al., 2001
mAb (tobacco mosaic virus)	Tobacco	Reduced virus infectivity when a full size antibody is secreting	Voss et al., 1994
scFv (beet necrotic yellow vein virus coat protein)	*Nicotiana benthamiana*	Partial protection against the virus establishment and its pathogenic effects	Fecker et al., 1997
scFv (tobacco mosaic virus)	Tobacco	Resistance to viral plant-pathogen infection	Zimmermann et al., 1998
scFv (the major membrane protein of stolbur phytoplasma)	Tobacco	Blockade of a phytoplasma antigen that prevents the multicellular parasite from occupying transgenic tissue	Le Gall et al., 1998
scFv (dihydroflavonol 4-reductase)	*Petunia*	Attempt to immunomodulate enzyme function. Special attention for selection a target.	De Jaeger et al., 1999
scFv (gibberellins A19 and A24)	Tobacco	Immunomodulation of phytohormone function	Shimada et al., 1999
scFv (jasmonic acid)	Tobacco	Immunomodulation of signalling molecules	ten Hoopen, 2002
Single-domain variable region (starch branching enzyme A)	Potato	Immunomodulation of enzyme function by single-domain antibodies from camelids; more efficient than antisense approaches; successful targeting of plastids	Jobling et al., 2003
scFv (chlorpropham)	*Arabidopsis*	Development of herbicide-tolerance	Eto et al., 2003
scFv (small heat shock proteins)	Tobacco	Immunomodulation of function of heat shock proteins	Miroshnichenko et al., 2005

There are several molecular biological technologies available now for researchers to get a loss-of-function line, such as the production of mutants that cannot synthesize a distinct product or lack a specific functional protein or the antisense RNA, sense RNA and RNA-mediated interference. Neither of these strategies is compartment specific. Immunomodulation, however, beneficiary allows to interfere to compartment-specific functions of target molecule. U. Conrad argues on an instance of plant hormone study that specific antibodies trap only certain precursor or the final product of a hormone biosynthetic pathway without affecting the function of other components of the pathway. Plants with different levels of biologically active phytohormone can be investigated due to a different level of antibody expression. Another advantage is that the intracellular immunization or immunomodulation can be applied to any species that can be transformed. And finally, this technique does not require cloning of genes involved in the phytohormone biosynthesis (Conrad et al., 2001). The immunomodulation of regulatory processes in plant physiology and development is a modern versatile technique and yet powerful for cell biology. It could be used to design different immunomodulation approaches to produce useful phenotypes in order to enrich data obtained by other approaches.

Although several types of recombinant antibodies have been successfully expressed in transgenic plants, only single-chain Fv antibodies (scFv) could be accumulated as functional proteins in the cytosol. Antibody assembly is not necessary in the unfavorable environment because single-chain antibody fragments consist of two variable domains covalently linked by a flexible domain to one peptide chain (Bird et al., 1988). Efficient technologies for the isolation and characterization of specific, high-affinity scFvs from suitable phage display libraries have been developed (Winter et al., 1994; de Wildt et al., 2000; Hoogenboom et al., 2000). These tools can now be used to design different immunomodulation approaches to produce useful phenotypes for the study of plant physiology and development.

Up to date several strategies have been developed to apply the immunomodulation principles against pathogens. Great scientific help for the agriculture would be generation of transgenic plants that show enhanced resistance to pathogen infection. Big potential lays in modulation of biomolecules involved in plant-pathogen interaction by recombinant antibodies. Depending on the intra- or extracellular accumulation of the antibody or antibody fragment, this approach is termed intra- or extracellular immunization.

The first successful report in this field was published by Tavladoraki and co-workers in 1993. Transgenic tobaccos were produced to synthesize antibodies directed against a viral coat protein. These plants were resistant to artichoke mottled crinkle virus (Tavladoraki et al., 1993). Numerous examples of immunization against viral, or multicellular (Le Gall et al., 1998) pathogens or even against herbicides are shown in Table 3-2. Antibody-mediated virus resistance is seen as an attractive alternative to the various forms of pathogen-derived resistance. In the latter case, unintended side effects, such as heteroencapsidation and recombination of viral genomes, cannot always be excluded. When the immunomodulation approach should be used against plant viruses, it must be considered that most plant viruses replicate in the cytosol and are RNA viruses. Therefore, the primary task of a successful immunomodulation is the efficient expression of active antibody derivatives in this cell compartment.

3.4. Monoclonal antibodies to double-stranded RNA

Antibodies can specifically recognize not only proteins, but also nucleic acids. Based on this fact, an elegant tool to study and manipulate nucleic acid mixtures containing

double-stranded RNA was developed. A panel of mouse monoclonal antibodies that specifically recognizes A-helix structure of double-stranded RNA (dsRNA) independent of the nucleotide composition of the antigen was produced by N. Lukács and co-workers (Schönborn et al., 1991; Oberstraß, 1993). It was shown that these antibodies can be used to detect and characterize dsRNA even in unfractionated nucleic acid extracts. The antibody specifically reacts with long dsRNA helices, irrespective of their sequence. At the same time, there is no binding to single-stranded RNA, double-stranded DNA or RNA-DNA hybrids. Only background levels of binding were obtained on single-stranded RNA species, which contain short double-stranded helical secondary structures (e.g. rRNA, tRNA, viroid RNA). The J2 antibody was even shown to counteract the RNA duplex unwinding activity of alfalfa mosaic virus RNA-dependent RNA polymerase *in vitro* and to inhibit replicase activity on partially double-stranded template (de Graaff et al., 1995; Lukács, 1997).

Some of these antibodies, J2, K1 and K2 were used throughout the current PhD studies. We also used the P6 antibody, which interacts with viroid as well as with ribosomal RNA and dsRNA (Lukács, 1994) (Table 3-3). The heavy chains of all four antibodies belong to the same gene family, V23(J558) and differ only in a few amino acids. The light chains are from different gene families (Oberstraß, 1993).

Determination of preferential binding sites for anti-dsRNA antibodies on double-stranded RNA was carried out by scanning force microscopy (Bonin et al., 2000) while analysis of the importance of individual amino acids in H-CDR of J2 was investigated by site-directed mutagenesis (Kós et al., 1999).

Table 3-3: Features of dsRNA-specific monoclonal antibodies (Richter et al., 1991; Alexin et al., 2001)

Monoclonal antibody	**J2**	**K1**	**K2**	**P6**
Antigen used for immunization	dsRNA	dsRNA	dsRNA	Viroid-RNA
Antigen recognized	dsRNA	dsRNA	dsRNA	dsRNA and ssRNA
Isotype of parental antibody	IgG2a	IgG2a	IgM	IgG3
VH family	V23(J558)	V23(J558)	V23(J558)	V23(J558)
VL family	V_k8	aa4($V_k4/5$)	aa4($V_k4/5$)	aa4($V_k4/5$)

Specificity analyses suggest that the major binding interactions probably take place in the minor groove of dsRNA. Sequence comparison in addition to site-directed mutagenesis has pinpointed amino acids, which may be crucial for antigen recognition (Kós et al., 1999).

The known approaches to influence RNA-virus replication in plants on RNA level are antisense RNA technology, viral resistance mediated by dsRNA-specific RNases and expression of ribozymes. Most of the plant viruses are RNA-viruses and during their replication double-stranded replication intermediates arise. It was shown that expression of dsRNA-specific RNase III resulted in inhibition of virus multiplication in plants (Watanabe et al., 1995; Mitra et al., 1996; Zhang et al., 2001). Moreover, transgenic potato lines expressing the yeast-derived double-stranded RNA-specific ribonuclease pac1 were produced. After challenging with potato spindle tuber viroid (PSTVd) they suppressed PSTVd infection and accumulation, presumably because the pac1 gene

product digested double-stranded viroid regions and/or replicative intermediates (Sano et al., 1997). Besides, resistance to viral infection was obtained when interferon-regulated 2-5A system of higher vertebrates consisting of two enzymes, a 2-5A synthetase that produces 5'-phosphorylated, 2',5'-linked oligoadenylates (2-5A) in response to double-stranded RNA, and the 2-5A-dependent RNase L was expressed in transgenic tobacco plants. Infection of leaves, detached or in planta, of the coexpressing transgenic plants by tobacco mosaic virus, alfalfa mosaic virus, or tobacco etch virus resulted in necrotic lesions. This work indicates that expression of a mammalian 2-5A system in plants provides resistance to virus infections (Mitra et al., 1996). An example of ribozyme-mediated resistance could be expression of a hammerhead ribozyme targeting the minus strand RNA of potato spindle tuber viroid (PSTVd). Active ribozyme cleaved the PSTVd minus strand dimer RNA into three fragments. Transgenic potato plants expressing the active ribozyme showed high resistance to PSTVd and did not accumulate PSTVd after challenge inoculation (Lukács, 1997). Thus, it could be concluded that efficient anti-viral strategies may be developed by targeting viral RNA. In form of dsRNA all replicating genomic RNAs have a structure common to all RNA viruses. Therefore, dsRNA-specific antibodies may be expected to influence virus multiplication in all RNA-viruses by counteracting helicase activity and/or by influencing dsRNA-mediated gene silencing.

Due to the advances in plant molecular biology and antibody engineering expression of antibodies in plants became possible. Active antibodies and antibody fragments could be readily expressed in the apoplast or endoplasmic reticulum of plants (Lukács et al., 1994; Conrad et al., 1998; Morgun et al., 2004). However, the expression of active antibodies in the cytoplasm confronts with two obstacles. First, the assembly of heavy (H) and light (L) chains is very inefficient in the cytoplasm. Second, the reducing conditions hinder the formation of disulfide bridges, which are necessary for active and stable antibody structure. There are a few possible solutions to circumvent these difficulties although none of them is universal.

4. Aims of Studies

- To establish strategies for the expression of correctly folded active single-chain antibody fragments (scFv) in *Nicotiana tabacum* cv. Xanthi and to direct scFv to different cell compartments;
- To identify the frameworks, which allow stable expression of single-chain antibody fragments in the cytoplasm of higher plants;
- To use dsRNA-specific monoclonal antibodies to influence virus replication by stabilizing double-stranded replication intermediates;
- To find out whether broad virus resistance can be introduced in transgenic plants by expression of dsRNA-specific antibodies.

5. Materials and Methods

While I was working in Dr. Noémi Lukács's laboratory I learnt many methods. Some of the detailed protocols were already established in the laboratory, others I introduced of modified. This collection of methods reflects as accurately as possible those that were used successfully in the course of my work, described in sufficient detail to allow even a novice student to use them without difficulty.

5.1. Preparing nucleic acids

5.1.1. Preparation of primers and oligonucleotides

The design of primers was performed using Primer3 web hosted application located at Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Oligonucleotide synthesis was carried out at the DNA Sequencing Central Laboratory of the Biological Research Center, Szeged. Dry nucleotide pellet was solubilized in 10 mM Tris-Cl pH 7.4, 0.1 mM EDTA buffer at concentration 100 pmol/µl, aliquotted and stored at –20 °C. The oligonucleotides used during our studies are shown in the Appendix Table 12-1.

5.1.2. Recovery of digested DNA fragments from TAE agarose gels for cloning

The procedure was carried out with help of DNA Extraction Kit from Fermentas. The kit utilized the modified glass beads protocol of Vogelstein and Gillespie (Vogelstein et al., 1979). In the presence of chaotropic salts DNA bound to the specially prepared glass particles. The chaotropic salts and impurities were washed out from the glass particles containing adsorbed DNA. The washing steps were followed by elution of the DNA in pure water.

5.1.3. Double SfiI/NotI digestion of scFvs and vectors

This double DNA digestion was employed to prepare vectors pCANTAB 5E and pGEJAE1 as well as scFv-inserts for ligation. Usually 200 µl of preparative SfiI digestion was done in a special One-Phor-All Buffer Plus from Pharmacia Biotech followed by NotI digestion. Usage of this buffer was important as it allowed us to carry out subsequent NotI digestion in the same tube. SfiI digestion was incubated at 50 °C under some mineral oil to prevent liquid evaporation for 3-12 hour. To start NotI digestion, the tube was cooled to 37 °C. 22 µl of 1 % Triton X-100, 1 M NaCl and 10 units of endonuclease NotI were added under the oil. The aqueous phase was mixed properly and the tube was left at 37 °C for 3-12 hour. Afterwards, the covering mineral oil was aspirated, and completely removed with some 100 µl chloroform. After adding double volume of 6 x loading buffer, the digestion products were separated in agarose gel and purified for further cloning steps.

5.1.4. Purification of plasmid DNA: large-scale preparations and minipreps

The following procedure employed a standard alkaline cell lyses, including RNase digestion, phenol-chloroform extraction and alcohol precipitations (Sambrook et al., 1989). DNA yield depended on the copy number and size of the plasmid, cell density of the culture, and efficiency of the cell lyses. Typically, 2-4 µg of pUC18 DNA was

obtained per ml of bacterial culture. LB broth turned out to be the easiest to work with. Bacterial cultures of 500 ml for maxipreps and 2 ml for minipreps were processed. Finally, purified plasmid DNA was dissolved in TE buffer and stored at –20 °C.

5.1.5. Photobiotin labeling of RNA

Photobiotinylation was carried out as described by Theissen (Theissen et al., 1989). Light sensitive photobiotin was dissolved in pure water at 1 µg/µl concentration in dark. 5 µl of 1 µg/µl RNA solution was mixed with 10 µl of 1 µg/µl photobiotin solution in an eppendorf tube on ice. The light source was adjusted to have 5 cm distance between the top of eppendorf tube and the lamp. After opening the tube, the mix was irradiated in ice for 20 min. To remove unbound photobiotin, 185 µl of TE buffer and 200 µl of 2-butanol were added to the biotin-RNA solution and mixed. The sample was centrifuged in a microfuge at 5 000 rpm for 3-5 min. The upper organic phase was transferred to a fresh eppendorf tube and saved to monitor biotinylation efficiency later on if needed. Extraction procedure for the lower aqueous phase was repeated minimum three times. The RNA was precipitated following addition of 100 µl 7.5 M ammonium acetate and 900 µl cold 96 % ethanol. The tube was kept on ice for 30 min then centrifuged at 15 000 rpm 4 °C for 20 min. The bio-RNA pellet was washed with 70 % ethanol, air dried for a few minutes and dissolved in TE buffer. The concentration of bio-RNA was measured at A_{260}. Labeling efficiency was estimated in ELISA or dot blot in the range 0.1 - 30 ng/well or dot.

5.1.6. Genomic DNA quickprep from tobacco leaves for PCR

The method was adopted from Edwards (Edwards et al., 1991). 2-3 cm long first or second leaf or a leaf piece (some 0.2 g or 9 cm^2) of *N. tabacum* was used for extraction. If needed, leaves were stored frozen till extraction. Some quartz sand was added and tissue was ground to powder under liquid nitrogen in mortar and pestle. After mixing in 800 µl of fresh extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS and 0.1 % 2-mercaptoethanol (v/v) the last added just before use), the suspension was transferred to 1.5 ml eppendorf tube on ice. The tube was incubated at 65 °C for 20 min being inverted every 5 min. 250 µl of cold 5 M potassium acetate, pH 6.6 was added, immediately mixed by inverting the tube and incubated on ice for 5 min. After that extract was centrifuged in Hettich Universal 30RF centrifuge at 15 000 rpm 4 °C for 10 min. Cleared supernatant was transferred into a fresh 1.5 ml tube containing 600 µl isopropanol, mixed properly by inverting at least 10 times and spun at 15 000 rpm for 5 min. The DNA pellet was washed with 70 % ethanol, and completely dried by keeping inverted tube open for about 10 min on filter paper. Finally the pellet was dissolved in 50 µl TE buffer containing 100 µg/ml RNase and stored frozen. 1-2 µl of this total tobacco DNA solution was used for a 50 µl PCR reaction.

5.1.7. Total plant RNA purification from tobacco leaves

Total plant RNA was prepared with RNeasy Plant Mini Kit (50) from Qiagen. The method allowed us to obtain some 50-60 µg of RNA from 100 mg tissue. The only peculiarity was the necessity to use young leaves for isolation. They gave much higher yield of total RNA than mature leaves.

5.1.8. Total DNA extraction from *Agrobacterium tumefaciens*

Several clones were inoculated from master plate into 2 ml YEB broth containing 100 µg/ml spectinomycin and grown at 30 °C 250 rpm shaking overnight. Total

Agrobacterium DNA was isolated as described by Gelvin (Gelvin et al., 1994). 1.5 ml culture was centrifuged in a microfuge for 45 seconds. Bacterial pellet was re-suspended in 300 µl of autoclaved TEN buffer (10 mM Tris-Cl pH 7.8, 10 mM NaCl, 0.1 mM EDTA). 100 µl of 5 % sarcosyl was added and the solution was mixed by inverting. 10 µl of 50 mg/ml proteinase K was put in, mixed thoroughly and incubated at 37 °C for 15-30 min to lyze the cells. Ice-chilled lysate was pipetted until foam to reduce viscosity, extracted with phenol-chloroform once, and once with chloroform. Nucleic acids were precipitated with 3 M sodium acetate pH 5.0 and ethanol, washed with 70 % ethanol and air dried for 10 min. Total *Agrobacterium* DNA was solubilized in 50 µl of TEN buffer and analyzed by PCR for presence of scFv sequences.

5.2. Electrophoretic techniques

5.2.1. Polyacrylamide and agarose gel electrophoresis of DNA

Polyacrylamide gel electrophoresis of DNA (Sambrook et al., 1989; Ausubel et al., 1994; Gelvin et al., 1994) was occasionally used to verify the quality of oligonucleotides (especially when attaching KDEL) and to resolve short DNA fragments differing in a few nucleotides. 10 % or 20 % polyacrylamide gels in 1x TBE buffer were run for effective separation range of 50-300 bp and 6-100 bp, respectively. The 100 bp DNA Ladder from Fermentas or pBR322 plasmid digested with the endonuclease AluI were utilized as molecular weight standards. Gels were stained with ethidium bromide or silver according to Sammons-Schumacher (Sammons et al., 1981; Schumacher et al., 1983).

Agarose gels of 0.7 % were used to separate undigested plasmid molecules and long (3-10 kb) linear DNA fragments. Gels of 1-1.2 % were most routinely used for separation of 0.5-3 kb long DNAs. Short restriction and PCR fragments (0.1-0.5 kb) were separated in 1.7-2 % agarose purchased from Sigma.

5.2.2. Formaldehyde-denatured agarose gel electrophoresis of RNA

The integrity and size distribution of total RNA were checked by denaturing agarose gel electrophoresis essentially according to Memelink (Memelink et al., 1994). Total RNA was purified from young tobacco leaves with Qiagen RNeasy Kit. 12-cm long 1.5 % agarose gel containing 0.1 µg/ml ethidium bromide, MOPS buffer (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate pH 7.0) and formaldehyde was electrophoresed in the MOPS buffer at a constant voltage of 5 V/cm. Samples containing 25 - 30 µg RNA and MOPS buffer containing 6 % formamide were mixed at 1:1 and were then heated at 60 °C for 10 min, chilled on ice and mixed with 5 x loading buffer (12.5 % Ficoll 400, 2 mM EDTA pH 8, 0.25 % bromphenol blue, 0.25 % xylene cyanol). Finally, the gel was transferred to 1 l of 50 mM NaH_2PO_4 pH 6.5, 5 mM EDTA for 1 h to elute formaldehyde. Sharp ribosomal RNA bands colored with ethidium bromide of expected length indicated successful procedure.

5.2.3. Northern blotting of RNA

To prepare the probe, PCR amplified scFv sequence was eluted from 1 % agarose – 1 x TAE gel by using the Fermentas DNA Extraction Kit. 100 ng of purified DNA was used as a template for radioactive labeling through random priming (Feinberg et al., 1984) by Fermentas DNA polymerase I large fragment (Klenow fragment). 50 µCi [α-^{32}P]-dCTP FP-205 (3 000 Ci/mmol) was added to reaction. The probe was purified on Sephadex G75 column according to Memelink (Memelink et al., 1994). Approximately 200 µl (10

drops) fractions were collected and the radioactivity of each was measured in Delta 300, 6891 Liquid Scintillation System from Searle Analytic, Inc. Total activity of the 3-4 most active fractions usually reached 7 x 10^7 cpm. The probe was used at 10^6 cpm/ml for hybridization.

Separated RNA was transferred from denaturing agarose gel to nylon membrane (positively charged Biodyne B, Pall or Zeta-Probe, Bio-Rad) by capillary diffusion for 16-24 h. 2 layers of Whatman 3 MM paper were used to lead 50 mM NaH_2PO_4 pH 6.5, 5 mM EDTA transfer buffer to the gel pressed through a pad of towels of 0.5 kg weight. RNA species were photographed in UV light and cross-linked to the membrane in Stratalinker by 160 mJ/cm^2 UV.

Hybridization solution consisted of 50 % deionized formamide, 5 x SSPE (20x SSPE contained 3.6 M NaCl, 0.2 M NaH_2PO_4 pH 6.5, 20 mM EDTA), 1 % SDS, 5 x Denhardt's solution, and 50 µg/ml denatured salmon sperm DNA. Membrane was prehybridized in 0.13 ml/cm^2 hybridization solution at 42 °C for 2 h. Hybridization was carried out in the same solution containing radioactively labeled probe at 42 °C under continuous rotating. Washing was done twice for 10 min at room temperature and once at 60 °C in 15 mM NaCl, 1.5 mM sodium citrate, 0.1 % SDS. Membrane enclosed in Saran wrap was exposed to Agfa x-ray film for 3-7 days at –70 °C. The film was developed according to standard procedure.

5.2.4. SDS-PAGE of scFv in plant and bacterial protein extracts

SDS-polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (Sambrook et al., 1989). Separation occurred under reducing (due to the presence of 2-mercaptoethanol) or non-reducing conditions in 15 % polyacrylamide resolving gel at room temperature. Prior to separation protein extracts were boiled at 95 ºC for 5 min. For comparison of scFv expression levels total protein concentration of extracts was determined (Lowry et al., 1951; Bradford, 1976) and equal amounts, usually 20-50 µg protein per lane, were loaded. Resolved proteins were visualized by either silver or Coomassie Brilliant Blue R250 staining or blotted to nitrocellulose membrane for Western immunoblotting. Proteins were stained with Coomassie Brilliant Blue R25 or silver (Blum et al., 1987).

5.3. Cloning and transformation

5.3.1. Recombinant Phage Antibody System for expression of scFv in *E. coli*

The RPAS, **Recombinant Phage Antibody System** supplied by Amersham Biosciences (formerly Amersham Pharmacia Biotech, Inc.) was an integrated modular system designed for cloning recombinant antibody fragments from mice and expressing them in bacteria. It provided a complete system for cloning, expressing, detecting, and purifying single-chain fragment-variable (scFv) antibodies. The RPAS Mouse scFv Module was designed to generate a repertoire of single-chain variable fragment (scFv) genes in which the variable regions of the antibody heavy (VH) and light (VL) chain genes were joined by a flexible linker. The RPAS Expression Module was intended for cloning the antibody scFv genes, synthesized by using the Mouse scFv Module, into a phagemid for expression as phage-displayed or soluble recombinant antibodies. scFv J2, K1, K2 and P6 were partially produced by using components of these two kits.

5.3.2. Conditions of scFv assembly by PCR

For cloning scFv-encoding sequences we used degenerate PCR primers. The VH1FOR-2 and VH1BACK primer were used for amplification of VH region. Primer for incorporation SfiI restriction endonuclease site was VH1BACKSFI. Primers for amplification of DNA encoding VL region were MJK2FONX, VK2BACK. Primer for incorporation NotI restriction endonuclease site was JK2NOT10.

As templates we used cloned cDNAs coding for the complete H- and L-chains of the dsRNA-specific monoclonal antibodies J2 and K1 (Oberstraß, 1993). PCR amplification mix (McCafferty et al., 1996) consisting of 10 µl 10 times concentrated Fermentas PCR buffer, 2 µl 10 mM dNTP, 100 pmol/µl back and for primers each, 10-30 ng template plasmid DNA and deionized sterile water up to 100 µl was run in a Hybaid OmniGene MWG-Biotech thermocycler according to the following program: 95 °C for 5 min, 84 °C for 30 sec and than after addition Taq polymerase 30 cycles of 94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min.

Variable domain sequences of the heavy (VH) and light chain (VL) were linked together by PCR to give a complete single-chain Fv antibody sequence. The composition of the reactions was 25 ng VH fragment, 25 ng VL fragment, 25 ng DNA linker fragment, 10 µl 10 x PCR buffer, 2 µl 10 mM dNTP, Taq polymerase and deionized sterile water up to 100 µl. Twenty five PCR cycles were run at 94 °C for 1.5 min, 65 °C for 3 min. After assembly, scFv was amplified to incorporate SfiI and NotI endonuclease sites and to increase yield necessary for subsequent purification.

5.3.3. Ligation of scFv insert into bacterial plasmid vector

Ligation was carried out at 1:3 molar ratio of vector and insert, respectively, using T4 DNA ligase (Fermentas) according to the manufacturers instructions.

5.3.4. Preparation of competent *E. coli* cells

Inoue and co-workers described this elegant, simple and efficient method (Inoue et al., 1990) reevaluating conditions for preparing competent *E. coli* cells and plasmid transfection. Based upon calcium treatment, the method yielded high quality competent cells for transformation (1-3 × 10^9 cfu/microgram of pBR322 DNA) that were stored frozen for at least 40 days without loss of competence.

Frozen bacterial stock was streaked on minimal agar plate, and cultivated at 37 °C overnight to get single colonies. 250 ml of SOB broth (2 % bacto-tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 7.0, autoclaved) was inoculated with 10 - 12 large colonies in a big baffled flask, grown to A_{600} ~ 0.6 - 0.75 at 18 °C 250 rpm. At 18 °C it took two days to reach proper cell density (OD_{600} = 0.75), but it gave high competence in a wide range of cell concentration (OD_{600} = 0.4-1). Before centrifugation the culture was chilled on ice for 10 min, and then sedimented at 3 850 rpm in a Sorvall GS3 rotor for 10 min at 4 °C. The cells were re-suspended in 80 ml of ice-cold transformation buffer, incubated in ice-bath for 10 min, and then centrifuged again. The transformation buffer contained 10 mM PIPES (or HEPES instead), 55 mM $MnCl_2$, 15 mM $CaCl_2$ and 250 mM KCl. To prepare the buffer all components except $MnCl_2$ were mixed and the pH was adjusted to 6.7 with KOH. Then $MnCl_2$ was dissolved. The solution was sterilized by filtration through 0.45 µm filter and stored at 4 °C. Once the cell pellet was gently re-suspended in 20 ml of ice-cold transformation buffer, DMSO was added to a final concentration of

7 % while gently swirling the tube. Cells incubated in ice-bath for 10 min were dispensed by 0.6-1 ml into sterile eppendorf tubes, immediately frozen in liquid nitrogen and stored at –80 °C. The freezing in liquid nitrogen (cold shock) enhanced the transformation efficiency four to five fold.

5.3.5. Simple and efficient method of *E. coli* transformation

The competent cells (Inoue et al., 1990) were thawed on ice and dispensed by 200 µl per 1.5 ml eppendorf tube. 5 µl of the plasmid solution or inactivated at 65 °C ligation reaction were added to each tube, mixed and incubated in ice-bath for 30 min. The cells were heat pulsed at 42 °C for 30 seconds without agitation, and then immediately transferred to an ice-bath. After adding 800 µl of SOC (SOB containing 20 mM glucose), the cells were shaken at 250 rpm at 37 °C for 1 hour and finally plated on selective agar in serial dilutions. Transformed bacteria were propagated at 30 or 37 °C overnight depending on further applications.

5.3.6. Transformation of pGEJAE1-based constructs to *Agrobacterium tumefaciens* GV2260

Agrobacterium transformation was carried out by triparental mating (Walkerpeach et al., 1994). pGEJAE1 vector and its descending gene constructs were cointegrative plasmids, bearing just T-DNA and encoding no auxiliary enzymes necessary for *Agrobacterium* mediated plant transformation. This vector was able to replicate in *E. coli* only and was called the first mating partner. On the other hand, Ti plasmid of *Agrobacterium tumefaciens* strain GV2260 (the second mating partner) encoded the whole plant transformation machinery lacking T-DNA and was maintained just in agrobacteria (Deblaere et al., 1985). For successful plant transformation T-DNA had to be transferred to *Agrobacterium* with the assistance of the third mating partner (HB101 *E. coli* strain containing pRK2013 plasmid) and fused to Ti plasmid through site-specific recombination of ampicillin/carbenicillin coding gene present on the both plasmids.

To get single colonies *Agrobacterium* strain GV2260 was streaked on YEB agar plate containing 100 µg/ml rifampicin and 100 µg/ml carbenicillin and cultivated at 30 °C for 2 days. Stable temperature was very important for *Agrobacterium* cultivation! A fresh plate of pRK2013/HB101 with single colonies was prepared on LB agar containing 25 µg/ml of kanamycin. In addition, a fresh plate of pGEJAE1 construct in XL1-Blue *E. coli* strain was obtained on LB agar containing 100 µg/ml spectinomycin and 100 µg/ml ampicillin. All three liquid bacterial cultures were started in corresponding media with antibiotics. On the first day 0.5 ml GV2260 culture was transferred to a 1.5 ml eppendorf tube. 0.5 ml pRK2013 together with 0.5 ml pGEJAE1 culture were put into another tube. Both tubes were spun for 30 s in a microfuge. The supernatants were discarded. Work was carried out under sterile conditions. The *Agrobacterium* pellet was suspended in 1 ml sterile distilled water, transferred to *E. coli* pellet and re-suspended. After spinning the suspension for 40 s in a microfuge, 0.9 ml of supernatant was aspirated and the remaining liquid was used to re-suspend the cell pellet. The germs were transferred on a YEB agar plate into one 2-cm wide disc. One such disc avoiding bubbles was made in a separate plate for each pGEJAE1-scFv construct. When excess liquid dried out the plate was closed, inverted, sealed as it was usually done and left at 30 °C overnight. On the second day selection of transconjugants pGEJAE1/pGV2260 in C58C1Rif^R was done on YEB agar plates containing 0.1 mg/ml rifampicin, 0.1 mg/ml carbenicillin and 0.1 mg/ml spectinomycin. Mixed bacteria from mating disc were

streaked on the entire surface of one plate and cultivated at 30 °C for 2–3 days. The selected clones were re-streaked twice to get pure *Agrobacterium* culture. Several transconjugant lines were collected in one master plate. Liquid cultures were started from the master plate to purify total bacterial DNA and to analyze the presence of desired sequence by PCR.

5.3.7. Detection of transformed *Agrobacterium* clones by PCR

Agrobacterium transconjugants were screened for occurrence of recombined scFv sequence within T-DNA by PCR. Back and forth primers hybridized to the edges of scFv coding sequence. Appropriate negative and positive controls were always included in total DNA preparation and PCR test to ensure reliability of results.

Table 5-1: The setup of PCR for detection of the scFv gene in total DNA of transconjugant agrobacteria

Solutions	Volume, µl
100 pmol/µl VH1BACK primer	0.5
100 pmol/µl MJK2FONX primer	0.5
10 mM dNTP	1.0
10x Dupla Taq buffer	5.0
25 mM $MgCl_2$	5.0
5x cresol	10.0
Milli-Q water	25.6
5 units/µl Dupla Taq polymerase	0.4
Total DNA prep	2.0

Three balls of wax were added to plug aqueous phase. The tube was spun briefly and the following program was run: 95 °C for 2 min, one cycle; 60 °C for 1 min, 72 °C for 1 min, 94 °C for 45 sec, 30 cycles; 35 °C for 10 sec, one cycle. Reaction products were separated in agarose gel by electrophoresis and visualized in UV light with ethidium bromide. Positive samples possessed a fat amplified band at about 700 bp.

5.3.8. Tobacco leaf disc transformation

Positive *Agrobacterium* clones were inoculated from the master plate into 20 ml YEB medium containing MgSO4 and spectinomycin (20 ml broth, 40 µl of 1 M MgSO4 and 40 µl of 50 mg/ml spectinomycin) and grown at 30 °C, 250 rpm overnight or until OD600 was 0.7 - 1.0. On the next day bacterial glycerol stock was made and the remaining culture was centrifuged at 5 000 rpm for 5 min at room temperature. The pellet was resuspended in 30 ml MS2 broth to bring OD600 to about 0.7 - 1.0.

Young sterile tobacco leaves were cut into 1 cm^2 pieces and wounded in several places under sterile conditions. The leaf discs were soaked in the *Agrobacterium* suspension in Petri dish for 2 min, transferred one by one onto sterile filter paper discs to remove excess of bacteria, and then were placed on MS2 agar plates stoma upward at about 20 leaf discs per dish. Ten MS2 plates were processed for one transformation construct. After 2 day incubation in illuminated thermostat room, leaf disks were transferred onto MS3 agar plates putting 10 discs per one MS3 plate. The plates were left for 3 weeks in

the thermostat room. Regenerating shoots were replanted into jars with MS4 agar for rooting.

5.4. Screening for antibody expression

5.4.1. Screening of recombinant *E. coli* clones by colony PCR

Colony PCR is a fast method for screening transformed *E. coli* colonies for recombinant plasmids. Work was performed on ice in sterile hoods having gloves on, trying not to contaminate the bacterial cultures. A single bacterial colony was picked from the plate with a toothpick, spread on the surface of a new selective LB agar plate in a numbered section. Then the toothpick tip was washed into 6 µl Millipore Milli-Q water in a numbered 500 µl thin wall Costar PCR tube by twisting, twirling 5-10 times while pressing to the wall of the tube. Finally the toothpick was dropped into 2 ml liquid LB broth containing antibiotic to obtain bacterial cultures for plasmid isolation and digestion. PCR Master Mix was prepared according to Table 5-2. 20 µl of Master Mix was added to each tube as well as two-three wax (paraffin) balls on top. The tubes were briefly spun to get liquid drops from the walls to bottom. Reaction was launched by transferring tubes directly from ice to a preheated PCR device and running the following program: 94 °C for 2 min; 55 °C for 1 min, 72 °C for 1 min, 92 °C for 30 sec 30 cycles; 35 °C for 30 sec.

Table 5-2: Composition of PCR solution for colony PCR. Dupla Taq polymerase is a commercial brand name of Taq polymerase sold by Zenon Biotechnology Ltd., Szeged, Hungary

Solutions	Volume, µl
100 pmol/µl forward primer	0.2
100 pmol/µl reverse primer	0.2
10 mM dNTP mix	0.5
10 x Dupla Taq buffer	2.5
25 mM $MgCl_2$	2.5
60 % sucrose, 1 mM cresol red	5.0
Milli-Q water	8.9
5 unit/µl Dupla Taq polymerase	0.2
Bacterial cells in Milli-Q water	5.0

Once the reaction was over, the tubes were heated to 60 °C for three minutes to melt paraffin and laid on their side to be able to punch the thinned wax layer and aspirate the samples (Hoppe et al., 1992). 10 µl were directly loaded in a 1.2 % agarose – 1 x TBE gel. Amplified DNA fragments were documented with an Eagle Eye II Still Video System from Stratagene.

5.4.2. Induction of scFv expression in *E. coli* periplasm and extract preparation

A single bacterial colony was inoculated to 2 ml SOB medium (Sambrook et al., 1989) containing 100 µg/ml ampicillin, 0.11 M glucose and shaken at 37 °C 300 rpm overnight. The following day 200 µl of the saturated culture was used to inoculate 10 ml

of 2 x YT containing 100 μg/ml ampicillin. The bacteria bearing pCANTAB 5E-scFv gene construct were grown at 37 °C 250-300 rpm until A_{600} reached 0.8 - 0.9. The lac promoter for scFv production was induced by 1 mM IPTG final concentration and 3 h propagation as above. The cells were pelleted in a benchtop centrifuge (Universal 30 RF Hettich, FRG) at 4 °C 5 000 rpm for 10 min. The pellet was resuspended in 0.5 ml 30 mM Tris-Cl pH 8.0, 20 % sucrose. After 10 μl of 1 M EDTA pH 8.0 was added, the suspension was chilled in ice for 10 min. The cells were centrifuged in a microfuge (Eppendorf Centrifuge 5414, Germany) for 30 sec. When the pellet was resuspended in 100 μl 5 mM $MgSO_4$, the cells were again chilled in ice for 10 min, and centrifuged in the microcentrifuge for 2 min. The supernatant, i.e. the periplasmic extract was stored at -20 °C and used for recombinant protein detection.

5.4.3. Western blotting to detect expressed scFv

Proteins were separated by polyacrylamide gel electrophoresis and transferred to Bioblot-nitrocellulose membrane (Costar, Canada) with pore size 0.45 nm in a semi-dry transfer cell (from Bio-Rad) at 15 V for 30 min or in some cases for 2 hours. Free binding sites on the membrane were saturated with 1 % (w/v) BSA solution in PBS (0.15 M NaCl, 10 mM potassium phosphate buffer pH 7.2) for 1 h at room temperature or at 4 ºC overnight at 40 rpm. Between steps the membrane was washed with 0.1% Triton X-100 in PBS. Bound scFvs were probed with the E-tag specific rabbit serum or mouse ET2 monoclonal antibody diluted in 1 % BSA, followed by incubation with alkaline phosphatase-conjugated goat secondary IgG antibodies in 1 % BSA. The membrane was developed in 0.5 mM $MgCl_2$, 0.5 M $NaHCO_3$ pH 9.5 containing 0.1 mg/ml BCIP and 0.2 mg/ml NBT.

For expression analysis of plant-produced scFvs, where scFv was labeled with c-myc tag instead of an E-tag, 3 000 x diluted monoclonal mouse c-myc tag specific antibody was used for probing.

5.4.4. Anti-E-tag colony blot

This method was used to screen single bacterial colonies expressing scFv. Usually 50-100 colonies could be examined on one 80 mm diameter membrane.

A disk of hydrophilic Millipore Durapore 0.22 μm GV filter was placed aseptically onto a fresh SOBAG agar plate. This is the bacterial membrane. Single bacterial colonies were inoculated on it with sterile toothpicks. Colonies of well-known positive (expressing scFv) and negative (not expressing scFv) controls were also used. The bacteria were grown overnight at 30 °C. Next day 2YTAI plate (containing 2YT agar, 100 μg/ml ampicillin and 1.1 mM IPTG) and a nitrocellulose membrane disk were prepared. After assembling a sandwich of 2YTAI agar, NC membrane, bacterial membrane, it was incubated at 37 °C for 3 h for bacteria to produce scFv. Then membranes were punctured for correct identification, the sandwich was disassembled, and bacterial membrane was saved on SOBAG plate at 4 °C. The NC membrane was washed 3 times with 0.5 % Tween 20, 0.2 % NaN_3 in PBS and free binding sites were saturated with 1 % BSA in PBS at room temperature shaking for 30 min. 2 ml of hybridoma supernatant of mouse mAb ET2 was diluted with 10 ml 1 % BSA, poured over the NC membrane and shaken at room temperature for another hour. After the usual washing procedure, the membrane was treated with alkaline phosphatase-conjugated GAM IgG (H+L)-AP solution at 5 000 x dilution in 1 % BSA for 1 h on a shaker. Membrane was developed with BCIP and NBT in ELISA substrate buffer at room temperature in dark without agitation.

5.4.5. Modified AN-ELISA to detect scFv activity

The original method of anti-nucleic acid ELISA described by Oberstraß (Oberstraß, 1993) was altered in a way that biotinylated RNA was prepared and used. Polyvinyl chloride microtiter plates (Microtest III, Falcon) were precoated at 4 °C with goat anti-mouse IgG (H+L) antibody and saturated with 1 % BSA in PBS (0.15 M NaCl, 10 mM potassium phosphate buffer pH 7.2). After binding anti-E-tag or anti-c-myc antibody diluted in 1 % BSA, periplasmic extracts from *E. coli* or plant soluble protein extracts, respectively were added to immobilize scFv on the solid surface. After 2 h the plate was washed and 15-30 ng/well biotin labeled dsRNA in STE buffer was loaded. After 1 h incubation at 37 °C, the plate was washed and then 25-50 ng/well alkaline phosphatase conjugated streptavidin in 1 % BSA was added. The plate was kept at 37 °C for 1 h, washed three times and filled with 100 µl/well 1 mg/ml p-nitrophenyl phosphate in 0.5 mM $MgCl_2$, 0.5 M $NaHCO_3$ pH 9.5. Absorption was measured in a MR 700 Microplate Reader (Dynatech) at 405 nm.

5.4.6. Preparation of protein extracts from tobacco leaves

This procedure was adopted from Vaquero (Vaquero et al., 1999). Extraction buffer contained 200 mM Tris-Cl pH 7.5, 5 mM EDTA, 0.1 mM DTT and 0.1 % Tween 20. When investigating plasma membrane attached antibody fragments 1 % of Triton X-100 was added to the buffer. Just before use 30 µl of 0.1 M PMSF and 35 µl of Protease Inhibitor Cocktail (Sigma P9599) were added to 2 ml of the extraction buffer to extract soluble proteins from one gram of leaf material. Tobacco leaves were ground under liquid nitrogen to a fine powder with mortar and pestle. Cell debris were removed by two rounds of centrifugation at 20 000 x g 4 °C for 5-10 min and the supernatant was used for expression analyses.

5.4.7. Small-scale antibody extraction from tobacco leaves

This protocol was used to prepare plant extracts from small (< 2 g) amounts of tobacco leaves for analytical purposes. After removing the middle ribs, leaves were homogenized under liquid nitrogen using mortar and pestle. 100-200 µl 3 x extraction buffer (60 mM Tris-HCl pH 8.0, 450 mM NaCl, 1 mM PMSF) per gram leaf was added to get a thick pulp. (If only small amounts of plant material were available, we use 0.5 ml 2 x extraction buffer per gram of leaves.) The suspension was transferred to an eppendorf tube and centrifuged in a bench-top centrifuge (Universal 30 RF from Hettich Zentrifugen, Germany) at 4 °C, 12000 rpm (14500 x g) for 15 min. The supernatant was used for ELISA immediately. Since phenylmethanesulfonyl fluoride (PMSF) is not stable in aqueous solution, it had to be added fresh to the extraction buffer from the 0.1 M PMSF stock stored at -20 °C.

5.4.8. Detection of plant-produced IgG antibodies by ELISA

Polyvinyl chloride microtiter plates (Microtest III, Falcon) were precoated at 4 °C with 4 µg/ml goat anti-mouse IgG (H+L) in PBS overnight (optionally goat anti-mouse IgG 1-3 (H-chain) could be used instead). Usually 100 µl was distributed into plate wells. To saturate free binding sites, plates were incubated at 4 °C with 2 % BSA in PBS overnight or at 37 °C for 2 h and then washed three times with PBS containing 0.5 % (v/v) Tween 20. 100 µl leaf extract per well was loaded, incubated at 37 °C for 2 h and washed as above. Bound plantibody was detected by 100 µl/well alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) diluted adequately in 2 % BSA in PBS. After 2

h incubation at 37 °C the plate was washed and filled with substrate solution (1 mg/ml 4-nitrophenyl phosphate in 0.5 M $NaHCO_3$ pH 9.5, 0.5 mM $MgCl_2$). Absorption was measured with MR 5 000 Microplate Reader (Dynatech) at 410 nm. The highest absorption signal for the best plants reached 1.2 absorption units within an hour.

5.4.9. Extraction and ammonium sulphate precipitation of plantibody

Mature leaves were harvested from plants of the same genotype, weighed and homogenized with a kitchen mixer at 4 °C in 1 ml of 1 x PBS buffer, 1 mM PMSF per gram of plant material. After filtering the suspension through 2 layers of Miracloth (Calbiochem Corporation, La Jolla, CA), the filtrate was centrifuged at 10 000 x g 4 °C for 20 min. Soluble proteins of the supernatant were precipitated with 2 M (50 % saturated or 313 g/l) ammonium sulphate. While solution was continuously mixed, salt was gradually added. After 30-min incubation on ice, the solution was centrifuged at 10 000 x g 4 °C for 20 min. Pellet was dissolved in a minimum amount of 0.1 M phosphate buffer pH 8.0 and centrifuged as before. The supernatant was dialyzed against 0.1 M phosphate buffer pH 8.0 at 4 °C overnight. Antibody solution was centrifuged and immediately used for Protein A affinity chromatographic purification or stored frozen.

5.5. General laboratory techniques

5.5.1. Plasmids, bacterial strains and plants

For expression of scFv by phage display in *E. coli* the **pCANTAB 5E** phagemid vector (Figure 6-13), **TG1** and **HB2151** *Escherichia coli* K-12 strains were used form Amersham Biosciences (http://www4.amershambiosciences.com/) within the Recombinant Phage Antibody System (RPAS). scFv genes were cloned through SfiI and NotI endonuclease sites. Recombinant protein overexpression took place in HB2151 strain. The genotype of *E. coli* **TG1** strain was K12 Δ(lac-pro), supE, thi, hsdΔ5/F'[traD36, proAB, lacIq, lacZΔM15]. The genotype of *E. coli* **HB2151** strain was K12 Δ(lac-pro), ara, nalr, thi/F'[proAB, lacIq, lacZΔM15].

pGEM3Z was a specialized *E. coli* sequencing, transcription vector obtained from Promega Corporation, Madison WI and used for intermediate cloning steps.

pGEJAE1 was a cointegrative *E. coli* vector (De Jaeger et al., 1999) containing T-DNA flanked with the Left and Right Borders and antibiotic marker genes for maintenance in *E. coli, Agrobacterium tumefaciens* and higher plants (Figure 3-5). The plasmid was replicated in *E. coli*, then transferred to *A. tumefaciens* via triparental mating and fused to Ti plasmid by site-specific recombination. Dr. Geert De Jaeger elegantly designed this vector for transformation of dicotyledonous plants with scFv sequences selected from phage display libraries. It had unique SfiI and NotI restriction sites between CaMV P35S promoter and 3' octopine synthase terminator. These restriction sites being uncommon in antibody genes were intelligently employed for cloning scFvs also in phage display pCANTAB 5E phagemid. The vector along with the **pRK2013/HB101** helper *E. coli* strain for *Agrobacterium* transformation (Ditta et al., 1980; Thomas, 1981a; Thomas, 1981b), and the *A. tumefaciens* **C58C1Rif^R (pGV2260)** (Deblaere et al., 1985) strain for tobacco transformation were kindly provided by Dr. De Jaeger.

Stratagene **XL1-Blue MRF'** strain proved to be an optimal host for propagation of plasmids. This strain permitted blue/white screening. The EndA – phenotype allowed us to prepare high-quality plasmid DNA. The F' episome carried antibiotic resistance, eliminating the need to select on minimal media plates. XL1-Blue strain genotype was

recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F'proAB, lacI^{q}ZDM15, Tn10 (Tet^{r})].

Nicotiana tabacum L. cultivar **Xanthi** (Family Solanaceae) plants were used to generate antibody-expressing transgenic plants.

5.5.2. DNA sequencing and sequence analysis

The Central DNA Sequencing Laboratory of the Biological Research Center, Szeged carried out sequencing of plasmids and amplified isolated DNA fragments using fluorescent dye-labeled (BigDye) terminators and cycle sequencing method. The runs of sequencing reactions were done on an ABI Prism 3100 Genetic Analyzer capillary sequencer via the 50 cm capillaries. One sequencing reaction usually required 1 µg of template DNA dissolved in 10 µl of Milli-Q water. DNA was homogeneous and free from RNA, proteins, phenol, and salts. Primers other then SP6, T7, T3, M13 forward and reverse were supplied in Milli-Q water at concentration 10 pmol/µl. As a rule, the first 50 nucleotides of the sequences were not clearly read, while the next 500 - 550 bases were well distinguishable. Resulting chromatogram files in ABI Prism format were analyzed with a small but powerful DNA sequence editing and analysis program for Windows 98, DNA for Windows written by David Dixon from Department of Biological Sciences, University of Durham, UK.

5.5.3. Sterilization of tobacco seeds with sodium hypochlorite

This simple and robust method allowed us to sterilize seeds with household bleach (sodium hypochlorite, NaOCl). First of all, seeds were treated with approximately 5 ml of 70 % ethanol for 1 min in a small sterile baker and then solution was exchanged to 10 % sodium hypochlorite by aspiration with 1 ml pipette. After 3 min incubation at most, the seeds were thoroughly washed with some 5 ml of sterile distilled water five times. Seeds should be taken in excess, as some of them will be washed away with changing liquid. 30-50 sterile seeds were evenly distributed on sterile MS medium in a 10 cm Petri dish. Germination occurred in the cultivation room at standard conditions.

5.5.4. Cultivation of tobacco plants

Whenever it was necessary to get sterile tobacco plants or it was difficult to germinate seeds in open soil we sterilized dry tobacco seeds in 10 % sodium hypochlorite and grow plantlets under sterile conditions. Germinated 0.5-cm long shoots with short roots were transferred aseptically to fresh MS agar (3 - 5 plantlets per half-liter jar). Sterile tobaccos for transformation were cultivated *in vitro*. Transgenic plants were transferred in soil when they had 4 leaves. Their roots were washed under tap water to remove traces of agar and plantlets were potted in general composition soil. For the first 3 – 5 days they were covered with Saran wrap to retain high humidity and were then transferred to the greenhouse. 16-hour-long light was maintained in the greenhouse as well as in the cultivation room at 24 - 27 °C.

The plants were regularly watered, fertilized and sprayed against infection and pests (e.g. whiteflies). Right before flowering, the flowers were put into light paper bags to prevent cross-pollination and environment contamination. Seeds were collected and stored in paper bags.

5.5.5. Preparation of dialysis tubes

Dialysis tubes from Serva were boiled (Sambrook et al., 1989) in 2 % $NaHCO_3$ pH 8.0, 1 mM EDTA for 10 min. After thorough wash, it was boiled in distilled water and stored in the cold room in 0.2 % sodium azide.

5.5.6. Media for propagation of *E. coli* and *A. tumefaciens*

LB medium for *E. coli* contained 1 % tryptone, 0.5 % yeast extract, 1 % NaCl and 1.5 % bacto-agar for plates.

SOB medium for *E. coli* contained 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.625 ml of 4 M KCl per 1 l of tap water. The solutes were dissolved and pH was adjusted to 7.0 with 1 M NaOH. 15 g of bacto-agar per 1 l of medium was added for agar plates. Medium was autoclaved for 20 minutes on liquid cycle (Sambrook et al., 1989). To get **SOBAG**, glucose, $MgCl_2$ and ampicillin from sterile stocks were added at the following amounts 56 ml of 2 M filter sterilized glucose; 1 ml of 100 mg/ml ampicillin; 10 ml 1 M $MgCl_2$ per 1 l of SOB.

2 x YT medium for *E. coli* contained 16 g tryptone, 10 g yeast extract, 5 g NaCl per 1 l of tap water. After dissolving the substances, the pH was adjusted to 7.0 with 1 M NaOH. 15 g of bacto-agar per 1 l of broth was added to get solid medium for agar plates. The medium was autoclaved on the same day. To make **2 x YTAG** medium 56 ml of 2 M filter sterilized glucose and 1 ml of 100 mg/ml ampicillin from the sterile stocks were added per liter of broth right before use.

YEB medium for cultivation *Agrobacterium tumefaciens* cells contained 0.5 % beef extract, 0.1 % yeast extract, 0.5 % peptone and 0.5 % sucrose. After adjusting the pH to 7.2, 1.5 % bacto-agar was added. Right before use filter sterilized $MgSO_4$ was added to final concentration 2 mM.

5.5.7. Plant cultivation media

Murashige-Skoog (MS) medium (Murashige et al., 1962) was the basic medium for *Nicotiana tabacum* propagation and transformation. The 10 x stock solution of macroelements, **MS Macro** contained 19 g/l KNO_3, 16.5 g/l NH_4NO_3, 3.7 g/l $MgSO_4$ x 7 H_2O, 1.7 g/l KH_2PO_4. It was stored at –20 °C without sterilization.

The 1 000 x stock solution of microelements, **MS Micro** contained 22.3 g/l $MnSO_4$ x 4 H_2O, 8.6 g/l $ZnSO_4$ x 7 H_2O, 6.2 g/l H_3BO_3, 0.25 g/l Na_2MoO_4 x 2 H_2O, 0.025 g/l $CuSO_4$ x 5 H_2O, 0.025 g/l $CoCl_2$ x 6 H_2O. It was stored frozen at –20 °C without sterilization.

The **Ca stock solution** was 100 mg/ml $CaCl_2$ x $2H_2O$, autoclaved and stored at room temperature. 200 x stock of **Fe solution** contained 5.56 g/l $FeSO_4$ x 7 H_2O, 7.46 g/l Na_2EDTA x 2 H_2O and was stored at 4 °C in dark. 455 x stock of **KI solution** contained 0.377 g/l KI and was stored at 4 °C in dark.

The 500 x stock of **B5 vitamins** contained 50 g/l myo-inositol, 0.5 g/l pyridoxine-HCl, 0.5 g/l nicotinic acid, 5 g/l thiamin-HCl. The solution was filter-sterilized and kept at –20 °C.

The basic medium to propagate plants, the **MS agar** contained 200 ml 10 x MS macro, 2 ml 1 000 x MS micro, 10 ml 200 x Fe, 4.4 ml 455 x KI and 60 g sucrose per 2 l Milli-Q water. Once pH 5.6 – 5.7 was adjusted with NaOH, KOH or HCl, 3.2 g of plant agar (or 0.8 g Gelrite) was added to 400 ml liquid broth in half-liter bottle. Filled bottles

were sterilized by autoclaving at 120 °C for 20 min and stored in the cold room. Right before use 400 ml of medium in a bottle with loosen cap was melted in the microwave oven cooled to about 50 °C and then 1.76 ml of 100 mg/ml $CaCl_2$ x 2 H_2O, 800 µl of 500 x B5 vitamins, antibiotics and hormones (depending on medium utilization) were added.

The **MS 2** medium to cocultivate explants and *Agrobacterium* consisted of 100 ml MS broth, 200 µl 500 x B5 vitamins, 100 µl 1 mg/ml NAA (1- naphthaleneacetic acid), 100 µl 1 mg/ml BAP (6-benzylaminopurine) and 440 µl 100 mg/ml $CaCl_2$ x 2 H_2O. The **MS2 Agar** contained 400 ml MS agar, 800 µl 500 x B5 vitamins, 400 µl 1mg/ml NAA (1- naphthaleneacetic acid), 400 µl 1 mg/ml BAP (6-benzylaminopurine) and 1.76 ml 100 mg/ml $CaCl_2$ x 2 H_2O. The **MS3 Agar** was prepared to regenerate explants and to eliminate agrobacteria. The following additives were added per 400 ml of melted MS agar before pouring the plates or jars: 800 µl 500 x B5 vitamins, 40 µl 1 mg/ml NAA, 400 µl 1 mg/ml BAP, 8 ml 10 mg/ml kanamycin, 1.5 ml 100 mg/ml augmentin, 1.5 ml 100 mg/ml claforan, 1.76 ml 100 mg/ml $CaCl_2$ x 2 H_2O. Plates or jars were stored in dark in the cold room.

The **MS4 medium** was used for root induction. It contained less sucrose than MS3 agar. Thus, 200 ml 10 x MS macro, 2 ml 1 000 x MS micro, 10 ml 200 x Fe, 4.4 ml 455 x KI were added per 2 l Milli-Q water. 3.2 g plant agar (or 0.8 g Gelrite) was added to 400 ml liquid broth in 0.5 l glass bottles and autoclaved at 120 °C for 15 min. Medium was stored in the cold room and whenever necessary melted in a microwave oven, cooled down to about 50 °C and then the following additives were added per 400 ml medium: 1.76 ml 100 mg/ml $CaCl_2$ x 2 H_2O, 800 µl 500 x B5 vitamins stock, 8 ml 10 mg/ml kanamycin, 1.5 ml 100 mg/ml augmentin, 1.5 ml 100 mg/ml claforan. The ready medium was used immediately.

5.5.8. Other solutions used during the study

Phosphate Buffered Saline (PBS) consisted of 10 mM potassium phosphate buffer pH 7.0 and 150 mM NaCl.

BSA solution contained 1 % bovine serum albumin, 0.2 % sodium azide (NaN_3) in PBS and was stored at 4 °C.

ELISA washing buffer consisted of 0.5 % Tween 20, 0.2 % NaN_3 in PBS and was stored at room temperature.

ELISA substrate buffer was composed of 0.5 mM $MgCl_2$, 0.5 M $NaHCO_3$ pH 9.5 and stored at room temperature.

BCIP (5-bromo-4-chloro-3-indolyl phosphate disodium salt) was prepared as 200 x stock in N,N-dimethylformamide (DMF) and stored at -20 °C. The concentration of 200 x stock was 20 mg/ml. The final concentration of BCIP in ELISA substrate buffer for membrane development was 0.1 mg/ml.

40 mg/ml **NBT** (nitroblue tetrazolium) was dissolved in 70 % N,N-dimethylformamide (DMF) and stored at -20 °C. The final concentration of NBT in ELISA substrate buffer for membrane development was 0.2 mg/ml.

5.5.9. Computer programs: biological and general

BLAST® (Basic Local Alignment Search Tool) was a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the

query was protein or DNA. It was a free web service offered by the National Center for Biotechnology Information (NCBI) located in the Internet at www.ncbi.nlm.nih.gov.

Primer3 was a free service located in the internet at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi and maintained by Whitehead Institute for Biomedical Research. It was used to design primers for PCR reactions.

The Kabat Sequence Database (www.kabatdatabase.com), the first created retrievable collection of protein sequences with immunological interest, was used to classify scFvs.

The ImMunoGeneTics Database (IMGT) located at http://imgt.cines.fr/ Montpellier, France was the international immunogenetics information system, a high-quality integrated knowledge resource specialized in the immunoglobulins (Ig), T cell receptors (TcR), major histocompatibility complex (MHC), immunoglobulin superfamily and related proteins of the immune system (RPI) of human and other vertebrate species, created in 1989 by Marie-Paule Lefranc. The database consisted of sequence databases, genome database, structure database, Web resources and interactive tools and was very helpful for characterizing CDRs, scFv and IgG sequences.

Clone Manager version 4.0 by Scientific & Educational Software was applied to inventory different vectors, constructs, sequences, as well as to plan cloning and screening recombinant molecules with restriction enzymes. A small but powerful DNA sequence editing and analysis program for Windows 98, **DNA for Windows** version 2.2 by David Dixon (1999) from Department of Biological Sciences, University of Durham, UK was used to read sequence chromatograms in ABI format, and to assemble and align sequences.

5.5.10. Preparation of potato virus Y stock

The Hungarian isolate of potato virus Y^{NTN} strain (Thole et al., 1993) used along the study was propagated in *N. tabacum* cv. Xanthi. Leaves displaying infection symptoms but still green were collected from several plants, homogenized under liquid nitrogen with mortal and pestle, and some quartz sand. After addition of one volume 20 mM potassium phosphate buffer pH 7.0, the suspension was centrifuged at 5 000 rpm, 4 °C for 20 min in benchtop Hettich Universal 30RF centrifuge twice to clear up sap containing virus particles. Having been aliquotted the supernatant was kept at –80 °C. This viral stock was considered as undiluted, when titration was made to determine infection power.

5.5.11. Infecting tobacco plants with PVY virus

Approximately 25 cm high *N. tabacum* cv. Xanthi plants of different genotypes, grown in the greenhouse in the same pots, soil and conditions were used for tests. Usually infection took place in a separate laboratory in the afternoon. Virus stock was diluted with 20 mM potassium phosphate buffer pH 7.0 200 – 6 000 times. The third, counting from the top, half-developed leaf was slightly powdered with abrasive material, celite. 200 µl of freshly diluted virus suspension was pipetted on the dorsal leaf side, and was evenly, gently distributed with a special glass flattened spoon. Usually 5 – 10 plants were infected from each genotype. When the inoculum dried on the leaves, the plants were transferred to the greenhouse under standard growth conditions and were monitored for symptom development.

5.5.12. Electron microscopy of plant samples

Electron microscopy investigation of plant samples was carried out by László Mustárdy at the Biological Research Centre. Pieces of young half-developed leaves were cut from greenhouse plants. They were fixed by 1 % glutaraldehyde for 1 h, dehydrated with graded series of ethanol (30 – 50 – 70 – 90 – 95 – 100 %) and embedded in Lowicryl K4M resin. Thin sections were sliced on a Leica UCT microtome and mounted on a nickel grid. The sections were stained with uranyl acetate for 20 minutes and studied under an electron microscope Tesla BS540. Images were taken with the same electron microscope.

6. Results

In earlier experiments done by Andreas Richter two constructs, HLM and HLO, of the dsRNA-specific monoclonal antibody J2 were expressed in transgenic *N. tabacum* cv. Xanthi (Lukács et al., 1994; Richter, 1995).

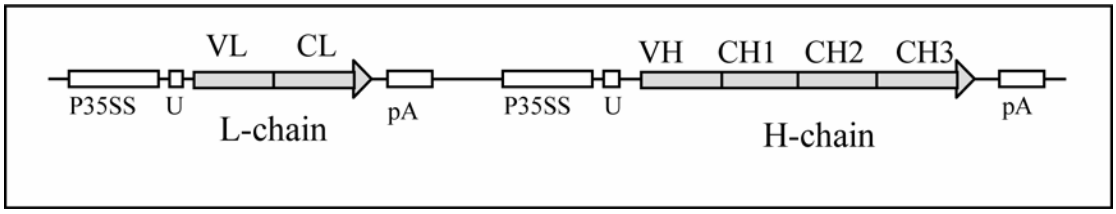

Figure 6-1: Schematic map of IgG J2 genes cloned into plant expression vector for intracytoplasmic expression in N. tabacum *(Richter, 1995). Transformed plants were designated as HLO. P35SS, double 35S promoter; U, 5' untranslated omega-leader of tobacco mosaic virus; pA, terminator, polyadenylation site. The original mouse signal peptides were deleted from the sequence to ensure intracytoplasmic expression*

In HLM plants the cDNAs encoded the full-length heavy and light antibody chains together with their natural murine leader peptides at the N-terminal ends. On the contrary, in HLO plants these leader peptide sequences were deleted (Figure 6-1). It was shown that the HLM plants secreted correctly assembled and active IgG antibodies in the apoplast. In several plants the concentration of J2 reached 1 - 3 % of the total soluble protein. In contrast, the absence of the leader peptide was supposed to provide cytosolic localization of the translated HLO antibody chains. It was shown that the H-chains were present at detectable concentrations in several transformants (Richter, 1995). H-chains are known to play a major role in determining antibody specificity. They can spontaneously associate with L-chains *in vitro* even under oxidizing conditions. Therefore, we wanted to investigate whether H-L assembly takes place in transgenic plants and if so, whether detectable binding of the plantibody to dsRNA is possible *in vivo*.

6.1. H- and L-chains are expressed at different concentrations in the cytoplasm

In the beginning, Andreas Richter's observations on dissimilar expression levels of the cytoplasm and ER targeted IgG J2 plantibody (Richter, 1995) were verified. Due to the expected low expression levels of the HLO J2, we employed more sensitive ELISA technique rather than Western blotting. Initially, we intended to select high expressers and then thoroughly analyze them.

Each individual plant was uniquely numbered. Its label reflected HLO or HLM genotype that it belonged to. The first number (i.e. 37 or 40) always indicates the genotype originated from a single leaf disc. The second number written after a slash (i.e. -3 or -1) shows the self-pollinated plant in the second generation from which the seeds were collected. The third number, standing after a full stop (i.e. .1 or .5), marked the individual plant of the third generation investigated in a particular experiment. Usually seeds have not been collected from such plants but their biomass was solely used for laboratory tests. For example, HLO 37-3 and 40-1 were independent transformants and probably not homologous. As a rule, we were checking several individual plants from each genotype.

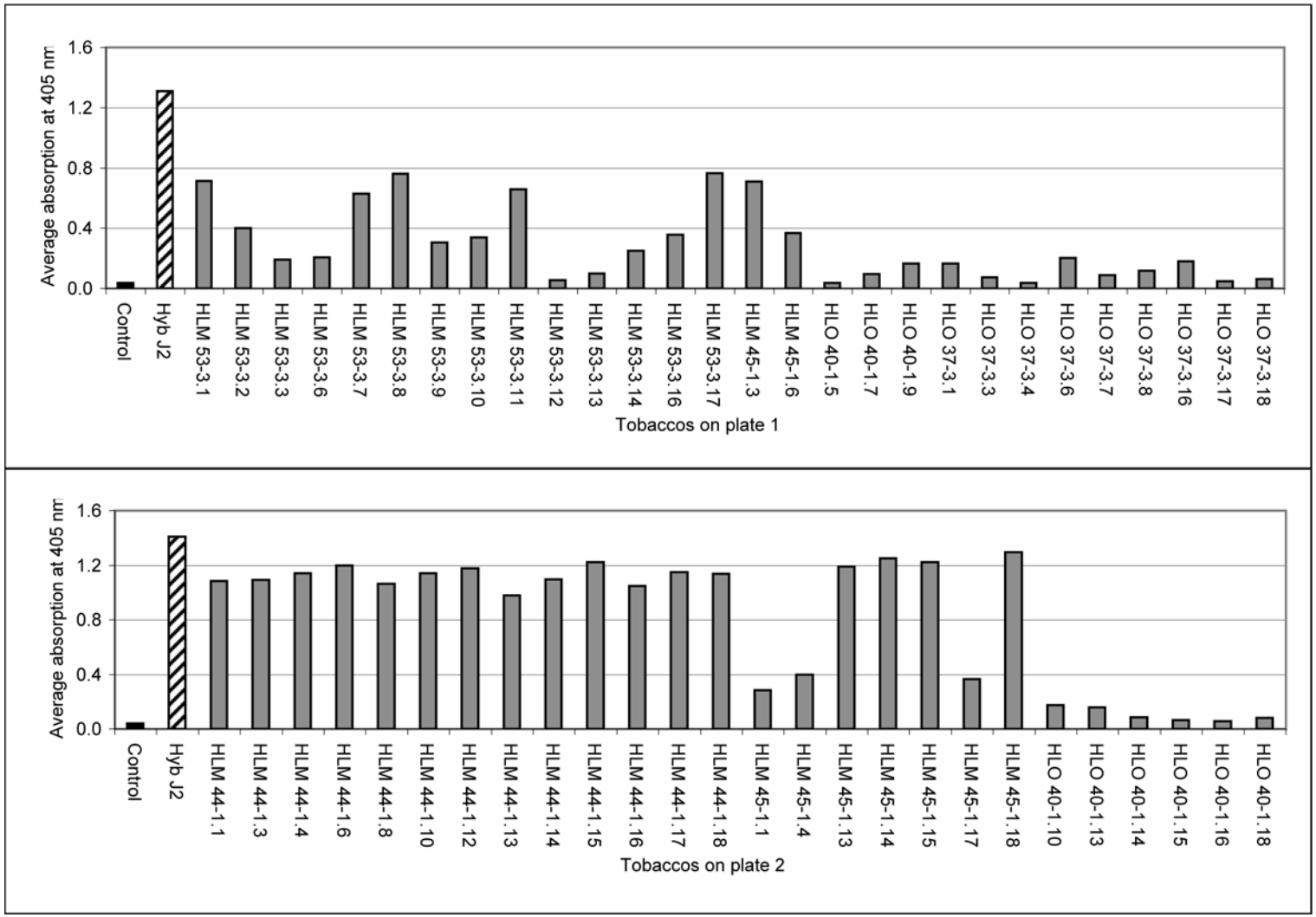

Figure 6-2: The murine IgG J2 targeted to the cytoplasm (HLO) and to the ER (HLM) in transgenic tobacco can be detected by the ELISA. Five transgenic genotypes were screened for the best expressors. The plantibody was immobilized with mouse IgG (H+L) and detected with an alkaline phosphatase-conjugated goat anti-mouse IgG (H+L). The negative control was a non-transgenic tobacco, while the positive control was the native hybridoma-produced IgG J2. The plate was read in 45 min

In order to detect plantibody polypeptides we used three types of antibodies for immobilization and detection. These are the goat anti-mouse IgG (H+L) for the recognition of both murine chains of IgG J2; the goat anti-mouse γ for recognition of the H-chain only and finally, the goat anti-mouse κ for recognition of the L-chain only. For the first assay the ELISA plate was coated with the goat anti-mouse IgG (H+L). The HLO J2 plantibody as well as its separate polypeptide chains were detected with an alkaline phosphatase-conjugated goat anti-mouse IgG (H+L). Three HLM genotypes (44-1, 45-1 and 53-3) know from earlier experiments to be high expressers were compared to two HLO genotypes (37-3 and 40-1) (Figure 6-2). As the positive control for the plant expression, we used the standard monoclonal antibody J2 prepared in hybridoma cell culture. The measurement indicated that the signal of HLO J2 (the average OD = 0.1) was about 10-fold lower than that of HLM J2 (the average OD = 1.0).

When the H-chain specific antibody was applied for immobilization and detection, the transgenic lines HLO 37-3.6, 37-3.16, 40-1.9, 40-1.10 and 40-1.13 were identified as positive (Figure 6-3). At the same time, the ELISA plates had to be incubated quite long with the substrate solution. This suggested that the H-chain concentration was low in the plant extracts but still some 10 times higher than the concentration of the L-chains. Moreover, the distribution of the individual signals was uneven and very contrast.

Similar test was made for the L-chains produced in the HLO J2 tobacco (Figure 6-4). Being of the mouse origin, the polypeptide was captured with the goat anti-mouse IgG κ L-chain specific antibody and detected with the same antibody that was conjugated with the alkaline phosphatase. The signals were obtained only after prolonged incubation and were quite low. Almost all signals were in the same range and hardly exceeded the negative control two fold. This was rather in contrast to the H-chain scheme.

Thus, the T1 generation of both types of transgenic tobacco plants was screened for expression of IgG J2. All together 65 plantlets were tested by ELISA and only plants displaying the highest expression level (about 50 % plantlets) were kept.

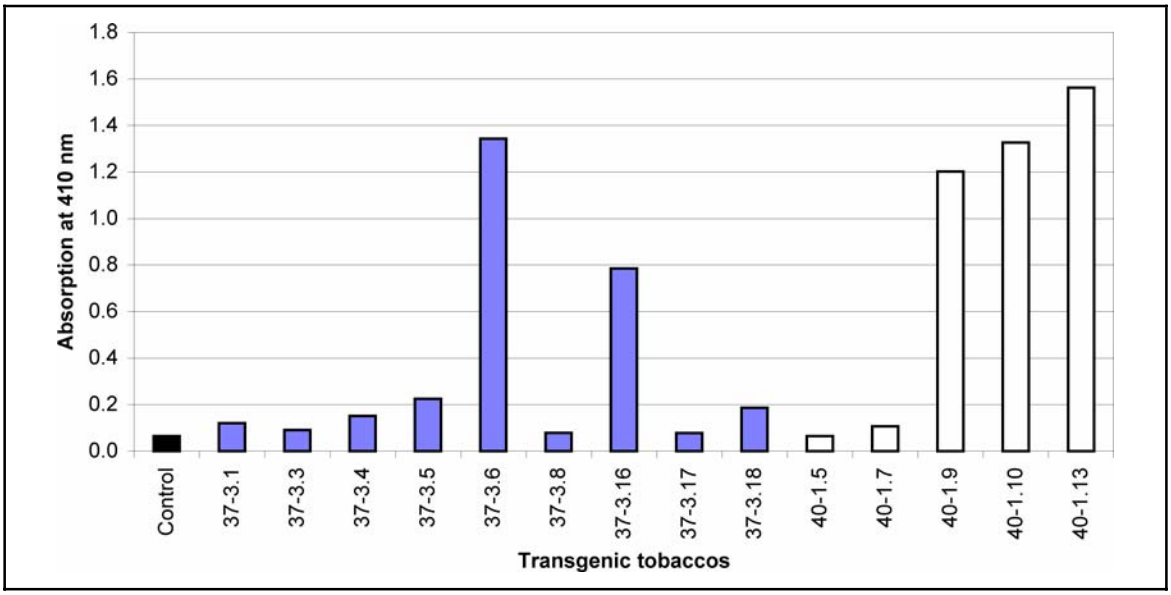

Figure 6-3: Some HLO genotypes show well detectable levels of the H-chain expression. The plantibody chains were immobilized and detected with GAM IgG (H)-specific reagent. The plate was read in 11 h 20 min

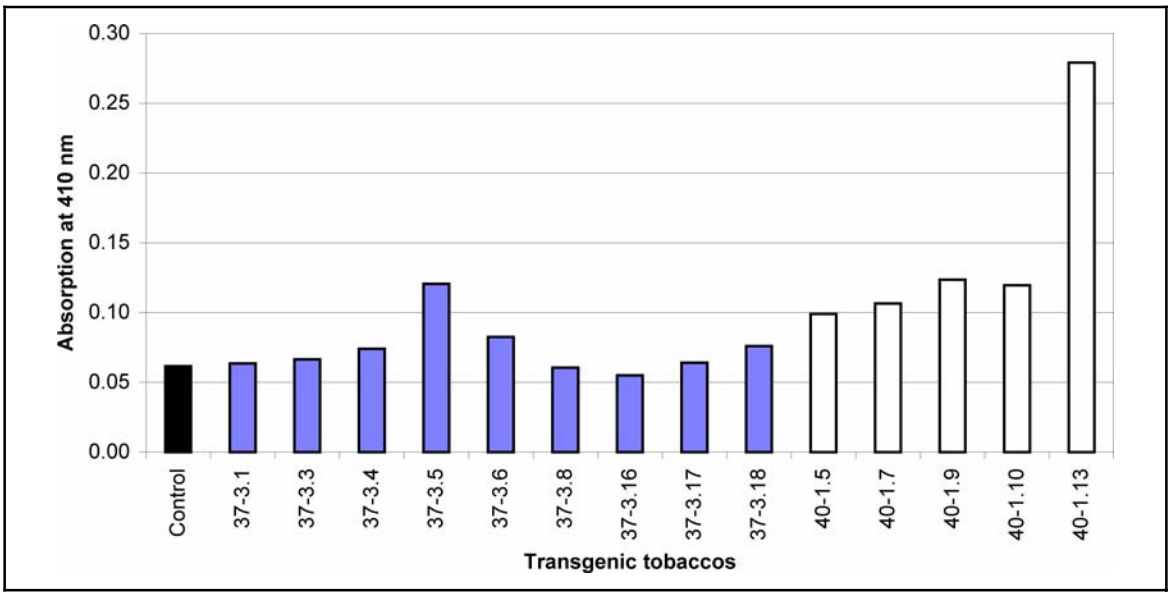

Figure 6-4: The L-chain expression seems to be at noise level when κ-specific secondary antibodies (GAM (κ)) are used to immobilize and to detect plantibodies. Reading was after approximately 12 h of substrate incubation

6.1.1. Detection of expressed mouse dsRNA-specific IgG J2 in transgenic tobaccos

If the H- and L-chains are present at one location, they may associate or even form correct structures. In such case it should be possible to detect these complexes by immobilizing one chain to the ELISA plate and detecting the other with a specific immunoreagent, i.e. a secondary antibody. The reciprocal so called cross-detections are shown in Figure 6-5 and Figure 6-6.

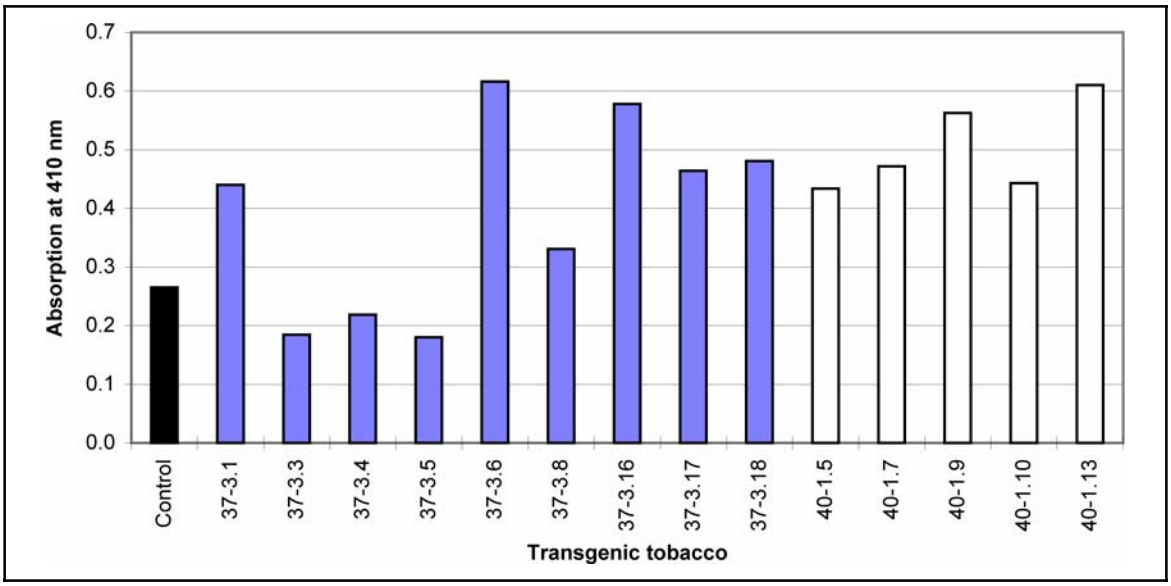

Figure 6-5: The J2 L-chains were detected in HLO samples following immobilization by the H-chain specific secondary antibody. The alkaline phosphatase-conjugated GAM (κ) was used for the cross-detection. The plate was read in 11 h 30 min

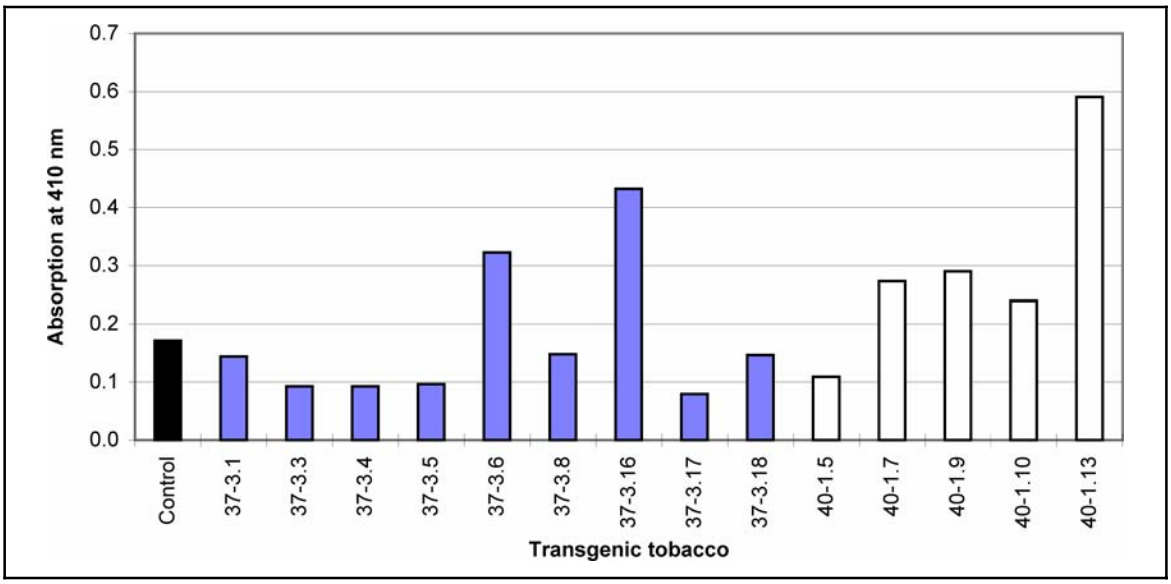

Figure 6-6: The J2 H-chains were detected in some HLO samples after immobilization with the L-chain specific GAM (κ). The alkaline phosphatase-conjugated GAM (γ) was employed for visualization. The plate was read in 11 h 30 min

Although the ELISA plates were incubated overnight, the absorption signals were quite consistent. In both cases there were several samples that gave twice as high signal as the negative control. These were for example HLO 37-3.6, 37-3.16, 40-1.13. They performed equally well in both assays. This indicated that their expression levels were the highest within the studied group of plants.

Thus, we successfully confirmed the previously obtained results (Richter, 1995) that the amount of HLO antibody was extremely low compared to the HLM J2. Nevertheless, the expression level of the H-chains in HLO plants was detectable (Figure 6-2 – Figure 6-6). The association between H- and L-chains did occur in the cytoplasm but was low. To understand the physiological state and especially binding characteristics of the H-L associations in the HLO J2 we had to purify them. The plantibodies were precipitated with $(NH_4)_2SO_4$ followed by the liquid affinity chromatography. Since Protein A specifically binds to the constant domains of H-chains, we expected to isolate H-chains as well as L-chains, which were associated with them. As positive control, IgG j2 was also purified in the same way from HLM plants. Detection of the HLO J2 H- and L-chains in the eluted fractions was done according to the established earlier ELISA assays and additionally with SDS-PAGE and Western blotting. The binding of plantibodies to dsRNA *in vitro* was estimated with AN-ELISA.

The IgG J2 chromatographically purified from HLM tobacco was fairly pure when the most concentrated antibody elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The HLM J2 samples were run under reducing and nonreducing conditions (Figure 6-7) in 10 % polyacrylamide gels. After the electrophoresis under reducing conditions, two sharp major bands were visible. Their molecular weights were estimated as 50 and 25 kDa. The molecular weights of IgG J2 chains were deduced from the nucleotide sequences. They were 49.1 kDa (447 aa) for the heavy chain and 24.2 kDa (219 aa) for the light chain not considering the glycosylation. Since the calculated and observed sizes were in the same range and quite comparable, we believed that the electrophoresed bands represented the H- and L-chains of the targeted to the ER IgG J2. Under non-reducing conditions a major protein band and some smear, probably indicating non-specific degradation products were observed

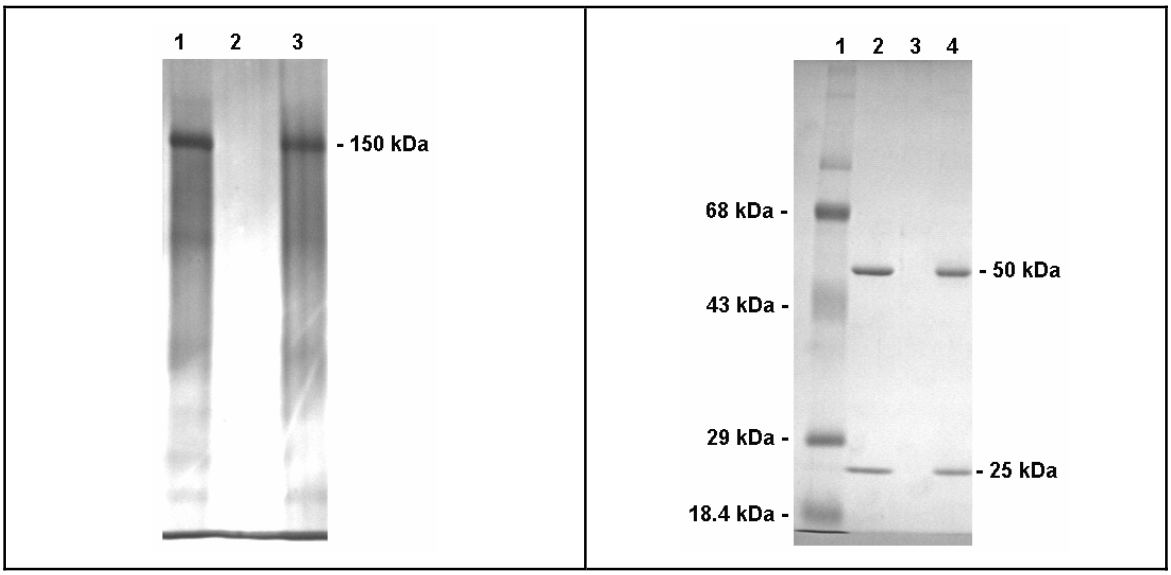

Figure 6-7: SDS-PAGE gels of the affinity purified IgG J2 from HLM plants. ***Left****: The samples were electrophoresed under non-reducing conditions and stained with silver nitrate. Lane 1, 0.5 μg hybridoma-produced IgG J2; lane 2, an empty lane; lane 3, 0.5 μg plant-produced IgG J2.* ***Right****: The samples were electrophoresed under reducing conditions and stained with Coomassie Brilliant Blue. Lane 1, BRL molecular weight standard; lane 2, 1 μg hybridoma-produced IgG J2; lane 3, an empty lane; lane 4, 1 μg plant-produced IgG J2*

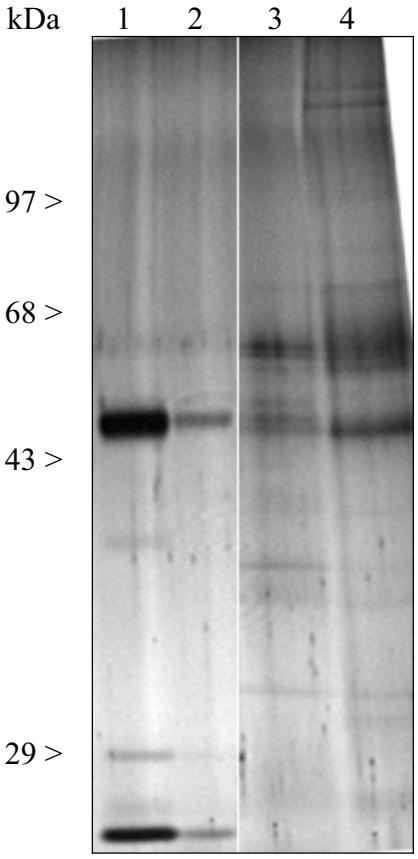

Figure 6-8: 10 % SDS-PAGE of cytoplasmically expressed and affinity purified HLO J2. Samples were electrophoresed under reducing conditions and stained with silver. Lane 1 and 2, 100 and 10 ng of hybridoma-produced IgG J2, respectively; lane 3 and 4, affinity purified non-concentrated fraction 4, and 1, respectively

The identification of the targeted to the cytoplasm IgG J2 chains in HLO transgenic tobaccos was much harder. When affinity-purified plantibody was separated by SDS-PAGE under reducing conditions and loaded on a Western blot, not even H-chain specific secondary antibodies could detect the H-chains. At the same time on the same blot both antibody chains of HLM or hybridoma produced J2 were specifically detected with different secondary antibodies (not shown). Nevertheless, we believe that the H-chain is produced by the HLO tobacco because it was detected by the H-chain-specific secondary antibody in above mentioned ELISAs (Figure 6-3, Figure 6-5, Figure 6-6); it was purified on the Protein A column; it was seen though very faintly on the silver-stained SDS-polyacrilamid gel (Figure 6-8); and finally as we would later on it would bind the dsRNA in AN-ELISA (Figure 6-9). It was also detected by dot blot (not shown).

Although the heavy chain was indeed present in the chromatographically purified HLO J2, the light chain band was not seen after silver staining of polyacrylamide gels (Figure 6-8). The fact that the L-chain expression level was very low was also supported by ELISA of total plant extracts. Here the L-chains were immobilized on the plate and detected with anti-L-chain antibodies (Figure 6-4). We even tried to concentrate the eluted chromatographic fractions by ultra centrifugal filtration. The Ultrafree-MC Millipore 10 000 NMWF filter unit brought the volume from 0.4 ml to 0.04 ml, but still there was no L-chain band detected by SDS-PAGE.

The estimated yield of J2 plantibody from the Protein A affinity chromatography ranged from 3.1 – 5.3 mg/kg fresh HLM leaves in different experiments. Thus, the purified plantibody composed 0.41 % of total soluble proteins. This is in good agreement with the ELISA results, where the antibody content in HLM plants was approximately 1 % of total soluble proteins. At the same time the expression level of the HLO plantibody was always lower than 0.2 %.

The purification yield of the HLO J2 was nominally 0.4 mg/kg when isolated on the column, but the fractions contained a high proportion of some strange probably non-IgG proteins (Figure 6-8).

6.1.2. Estimation of H:L ratio in plantibodies

To estimate the H:L ratio of plantibody chains in chromatographically purified fractions a dot-blot immunoassay was performed. The calculations argued that the H-chain specific signal for 10 000-fold diluted hybridoma produced J2 had the same intensity as 30 x diluted J2, purified from the HLO plants. Consequently, the $H_{hybridoma}$:H_{HLO} ratio was 330. The L-chain specific signal for 3 000 x diluted hybridoma J2 was the same as for the undiluted affinity purified fraction HLO J2. Therefore, the ratio for the L-chains was 3 000. On the basis of these data we conclude, that expression of H- and L-chains in the cytoplasm was imbalanced and the HLO plants contained at least ten times less L-chains than the H-chains. Similar calculations for the HLM J2 resulted in the same 1:1=H:L ratio as observed for the hybridoma-produced J2 (data are not shown).

6.1.3. Detection of dsRNA-binding to purified plantibodies

Whichever technique was used for enrichment of J2 antibody chains from HLO plants, the L-chains were always hardly detectable and the concentration of H-chains remained low too. Since the intradomain disulfide bonds are known to hardly form in the cytoplasm due to unfavorable redox potential, we believe that the misfolded polypeptides become quickly degraded in the cytoplasm. Nevertheless, H-chains seemed to be enriched by Protein-A affinity chromatography, therefore we investigated

whether they are able to bind to dsRNA. The activity of the purified plant produced HLO J2 antibody was analyzed by anti-nucleic acid ELISA. The test showed that the main fractions bound dsRNA albeit the activity was much lower than that of correctly assembled J2 plantibody (Figure 6-9). A possible interpretation could be that H-chain itself was responsible for the binding or there was a spontaneous assembly of heavy and light chains, albeit at a very low degree.

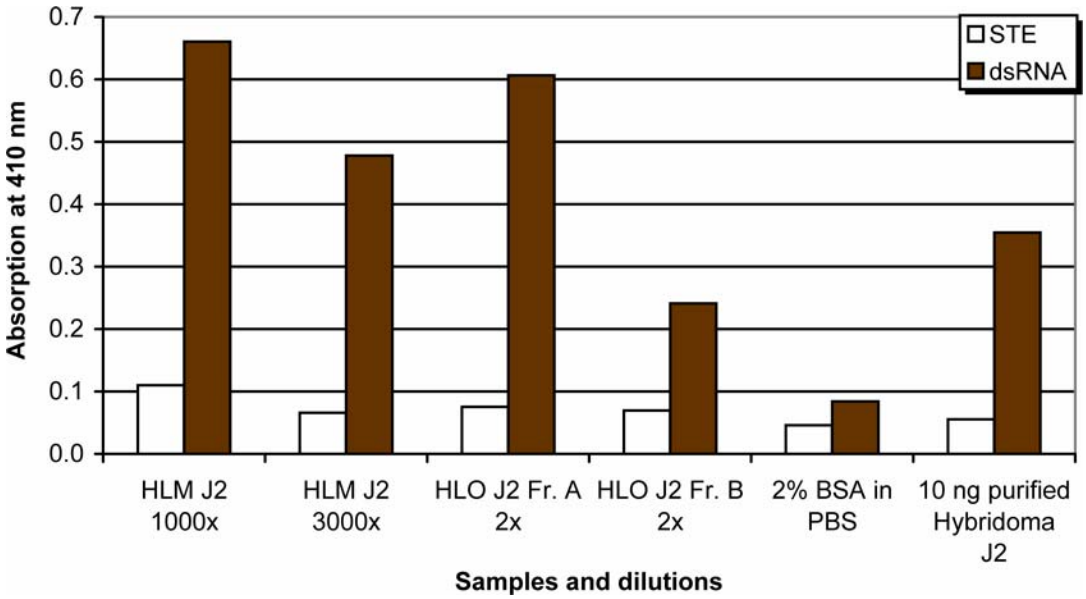

Figure 6-9: The activity of purified plantibodies in the anti-nucleic acid ELISA. The antibodies were produced in HLM or HLO plants. The most enriched chromatographic fractions were tested for antigen binding to the killer dsRNA. 2 % BSA served as a negative control. 10 ng of hybridoma-produced IgG J2 was used as positive control

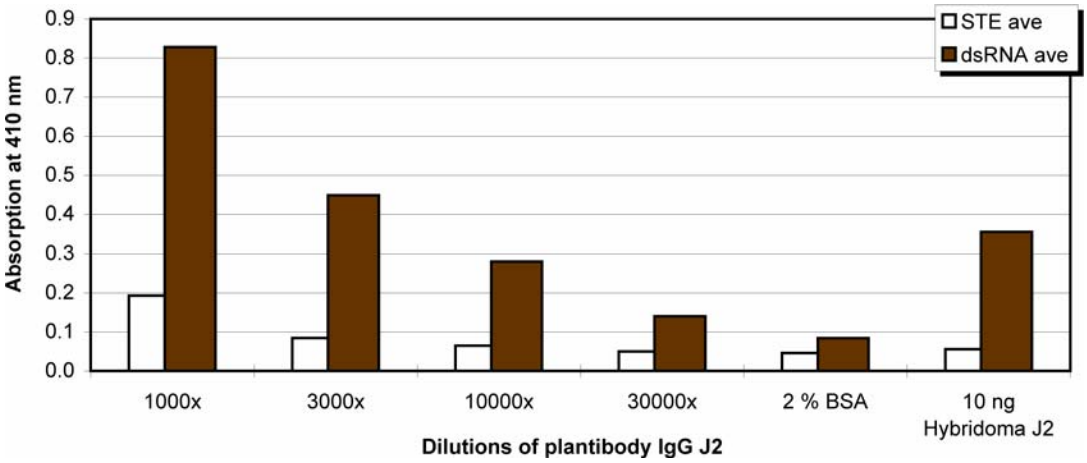

Figure 6-10: Specific antigen binding activity of the plant produced and chromatography purified IgG J2 from HLM J2 tobaccos. 2% BSA was the negative control in this AN-ELISA, while hybridoma-produced IgG J2 was the positive control

Purified HLM J2 at the optimal dilution was as active in binding to dsRNA as J2 synthesized by hybridoma cells (Figure 6-10).

6.1.4. Virus challenge of HLO-type transgenic tobacco plants with CMV and PVY viruses

Potato Virus Y (PVY) and *Cucumber Mosaic Virus* (CMV) are important crop pathogens belonging to positive single-stranded RNA viruses. They infect several crop species including potato, tobacco, pepper, and tomato, and lead to significant yield losses and quality degradation (Shukla et al., 1994). We hypothesized that dsRNA-specific antibodies, which were shown to stabilize double-stranded RNA replication intermediates *in vitro* (de Graaff et al., 1995) may hinder the replication of viral RNA when expressed in the cytoplasm of transgenic plants. To find out whether at the low expression level found in HLO plants young HLO J2 transgenic tobaccos as well as non-transgenic negative controls of variety Xanthi were infected. Ninety-one transgenic plants from two genotypes (HLO 40-1 and HLO 37-3) were first checked for plantibody expression and then infected with PVY. Its multiplication was measured by sandwich ELISA where the coat protein was under evaluation. My visual impression was that the transgenic plants suffered from systematic virus infection less than the control plants. HLO J2 plants looked stronger, higher, better but at the same time they displayed disease symptoms and produced strong ELISA signals as did the non-transgenic controls (Figure 6-11).

Tobacco plants infected with CMV were observed only visually for virus resistance. There was no difference detected between transgenic and control plants.

Taking together the results show that the expression of the mouse monoclonal antibody J2 was not successful in the cytoplasm. The concentrations of H- and L-chains were low, and L-chains were especially unstable. There was hardly any regular assembly of antibody chains into a stable functional complex. Considering these obstacles we decided to develop a new strategy - to express recombinant antibody in form of a single-chain antibody fragment.

6.2. Construction and analysis of single-chain antibody fragments from hybridoma-derived cDNAs

In Chapter 6.1 we analyzed the expression of the full-length monoclonal IgG J2 antibody in the cytoplasm of *Nicotiana tabacum* cv. Xanthi, as well as its *in vivo* effect on virus replication. Expression of antibodies in the cytoplasm is very attractive, as its success would allow researchers to immunomodulate the metabolism of viral dsRNA and possibly protect plants against a broad range of viruses. Besides, there are a number of other important antigens that could be targeted in the cytoplasm, if a reliable strategy to generate stable antibody scaffolds for cytosolic expression could be developed.

The low level or possibly non-existent assembly of J2 chains in the cytosol (see previous chapter) prompted us to engineer monovalent single-chain antibody fragments (scFv) from murine monoclonal dsRNA-specific antibodies. scFv is comprised from one polypeptide and is about one fifth of IgG mass. By expressing scFv one can circumvent the problem of assembling four antibody chains, but the problem of stabilizing the structure by disulfide bridges remains to be solved. No generally applicable solution to this issue was available in the literature. It was reported that even slight differences in the sequence or the addition of short stabilizing sequences would greatly alter the expression level (Nolke et al., 2003). Therefore, we used several dsRNA-specific antibodies to construct scFvs in *E. coli*, and made plant constructs containing a stabilizing KDEL-peptide at the C-terminus for cytosolic expression.

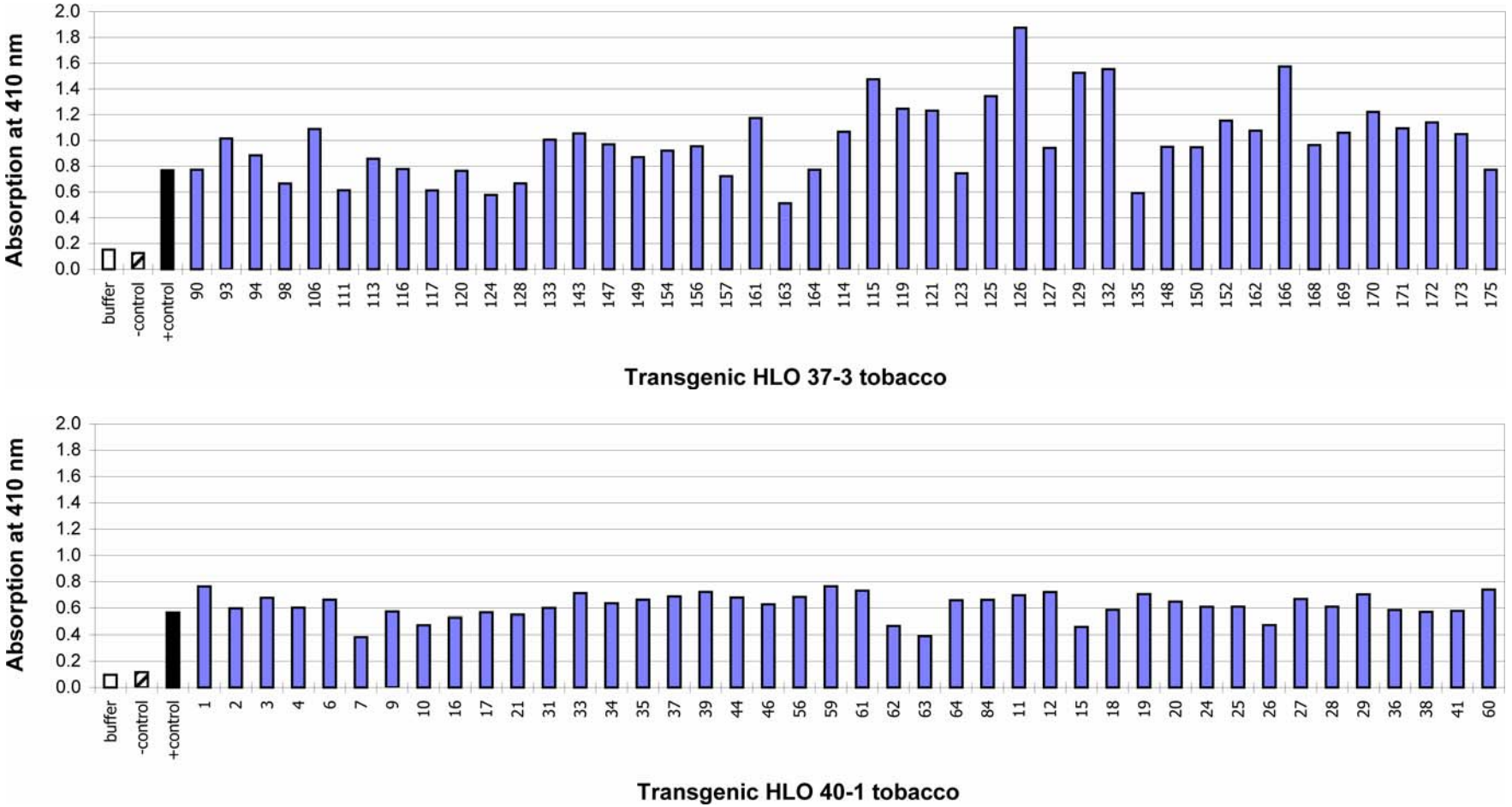

*Figure 6-11: Detection of virus coat protein in individual HLO tobaccos after PVY infection by ELISA. The signals for HLO 37-3 plants (**upper**) were read after 1 h incubation with the substrate. The signals for HLO 40-1 genotype (**lower**) were read after 30 min incubation. "-control" and "+control" stand for the negative and positive control, respectively. These were a non-transgenic, non-infected tobacco plant and a non-transgenic, PVY-infected tobacco, respectively. The "buffer" was the sample extraction buffer (0.05 % Tween 20, 0.25 % skimmed milk, 2 % polyvinilpyrrolidone (PVP-10) in PBS)*

The murine monoclonal anti-double stranded RNA antibodies J2, K1, K2 and P6 possess similar antigen specificity but differ in their sequence composition (Schönborn et al., 1991). They were chosen for antibody engineering experiments, and first four scFvs (J2, K1, K2, and P6) were constructed from these immunoglobulins utilizing the phage display technique. In previous experiments of our group scFv constructed exactly from the J2 sequence turned out to be inactive (Post, 1994). Therefore, we altered our approach, and right after the scFv assembly and cloning to bacteria, we screened numerous recombinant colonies for expression of active scFv fragments. The active clones were sequenced and thoroughly analyzed. We expected some heterogeneity in recombinant scFvs, because apart from mutations introduced by PCR amplification, we used degenerate library primers.

6.2.1. Isolation and cloning of scFv-encoding cDNA

All four scFvs derived from mouse monoclonal antibodies. The sequences of IgG J2 and K1 chains were known from earlier experiments and cloned cDNA of H- and L-chains was also available by the time we started to construct scFvs. Consequently, the variable domains (VH and VL) of scFv J2 and K1 were amplified from these plasmids.

To construct scFv we followed a strategy shown in Figure 3-3. After cDNA synthesis and primary PCR amplification of variable region sequences, the DNA fragments were purified. Assembly of scFv from VH, VL and the linker was done by a fill-in PCR reaction. The DNA linker fragment from Pharmacia Biotech encoding a 15-amino-acid flexible hydrophilic peptide $(Gly_4Ser)_3$ between the variable domains of heavy and light chains was employed. To obtain full-length scFv-cDNA and to add SfiI and NotI endonuclease sites to its ends reaction products were amplified by PCR using flanking primers.

Amplified sequences and assembled scFv-cDNAs exemplified by scFv-J2 are shown in Figure 6-12. The sharp bands are purified PCR products of heavy and light chain variable domain cDNA sequences. Beside, assembling the original variable domains of J2 we were able to produce scFv-hybrids (Figure 6-12) where one variable domain belonged to one monoclonal antibody and the other variable domain to another.

The other two immunoglobulins, K2 and P6 had not been cloned and sequenced earlier. To obtain cDNA of the variable domains of K2 and P6, $polyA^+$ mRNA was isolated from hybridoma cells and reverse transcribed (Alexin, 2001). VH and VL sequences of scFv K2 and P6 were amplified from cDNA by PCR. In the given case we had no prior information which degenerate primer from the scFv-library primers to use for amplification, so we had to evaluate them all to select a pair that gives the strongest amplification products. For construction of scFv K2 the VH1BACK and VH1FOR-2 primers for VH, and VK2BACK and MJK5FONX primers for VL were used. The major PCR products had the expected length approximately 340 bp and 325 bp for H- and L-chain respectively. To construct scFv P6 we also used primers suggested by the Recombinant Phage Antibody technique established by Pharmacia Biotech. The VH1FOR-2, VH1BACK degenerate primers were applied to amplify DNA encoding VH region. The primers for amplification of DNA encoding VL region were MJK2FONX and VK2BACK. The primer for incorporation NotI restriction endonuclease site was JK2NOT10.

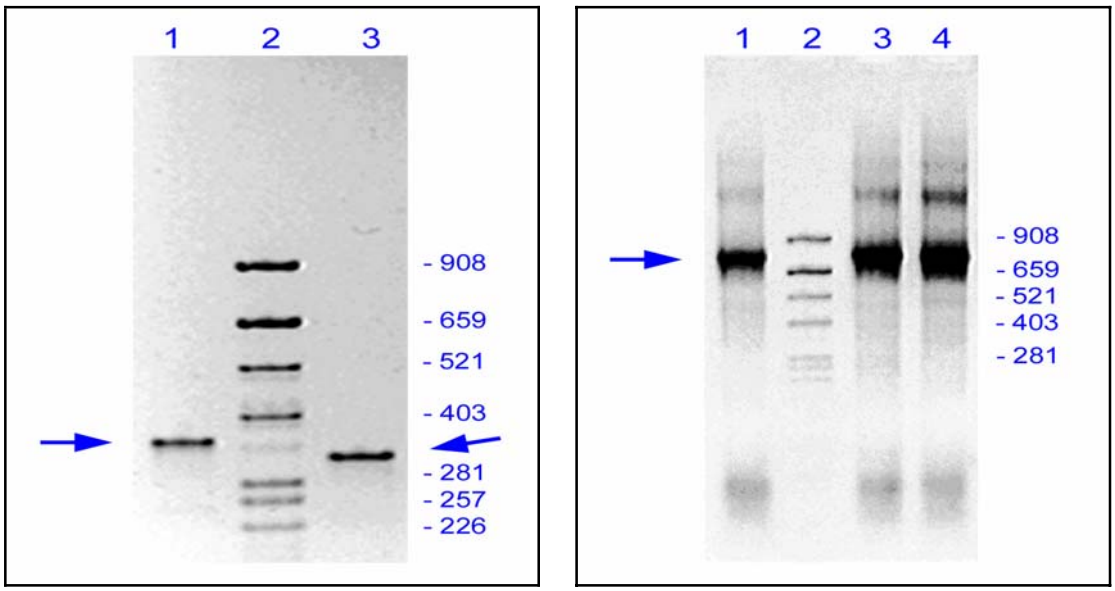

*Figure 6-12: Agarose gel electrophoresis of amplified and purified scFv VH and VL domain sequences (**left**). 5 µl aliquots of the PCR reactions were run in 1.5 % agarose gel in 1 x TBE buffer and ethidium bromide-stained products were visualized in UV light. Lane 1, variable domain of heavy chain (VH) of IgG J2; lane 2, molecular weight standard, pBR322 digested with AluI (the sizes of the DNA fragments are in base pares, shown on the right); lane 3, variable domain of light chain (VL) of IgG J2. On the **right**, agarose gel electrophoresis of amplified products after scFv assembly reaction. Lane 1, scFv J2; lane 2, 80 ng of molecular weight standard, pBR322 digested with AluI (the sizes of the DNA fragments are in base pares); lane 3, scFv VH(J2)-VL(K1) hybrid; lane 4, scFv VH(K1)-VL(J2)*

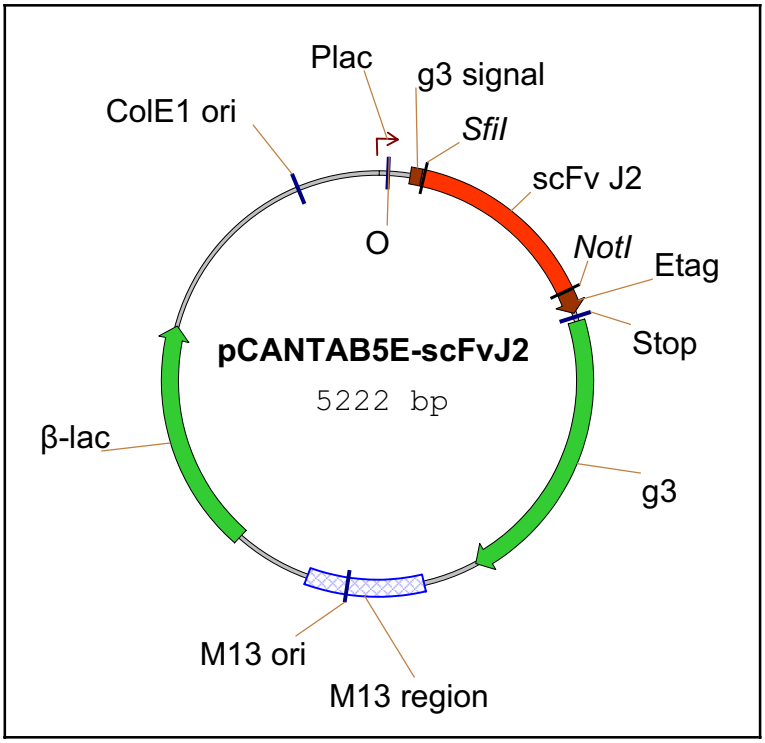

Figure 6-13: Schematic map of pCANTAB 5E phagemid harboring scFv J2. Stop stands for amber stop codon. This is the scFv construct for expression in E. coli periplasma

After SfiI and NotI digestions and subsequent purification all four scFv DNA fragments were ligated into pCANTAB 5E phagemid vector (Figure 6-13) and transformed into *E.*

coli TG1 strain. Isolated recombinant plasmid DNA from selected single bacterial colonies was digested with different restriction endonucleases and analyzed by gel electrophoresis for correct size and integrity of the DNA insert. The expected lengths of scFvs are shown in Table 6-5. Approximately 50 recombinant colonies harboring the correct DNA constructs of each of the four antibodies were screened for scFv expression by Western blotting and ELISA by using E-tag specific polyclonal or monoclonal antibodies produced in our group.

No scFv expression could be detected in periplasmic extracts by electrophoresis and staining with Coomassie Brilliant Blue (Figure 6-14), but in some cases scFv were visible after silver staining (Figure 6-18). Protein expression was frequently observed on Western blots (see Figure 6-15 and Figure 6-16) as well as on colony blots (Figure 6-17). For analysis of antigen binding activity and specificity of scFvs periplasmic extracts were extracted from colonies showing the highest scFv-expression levels.

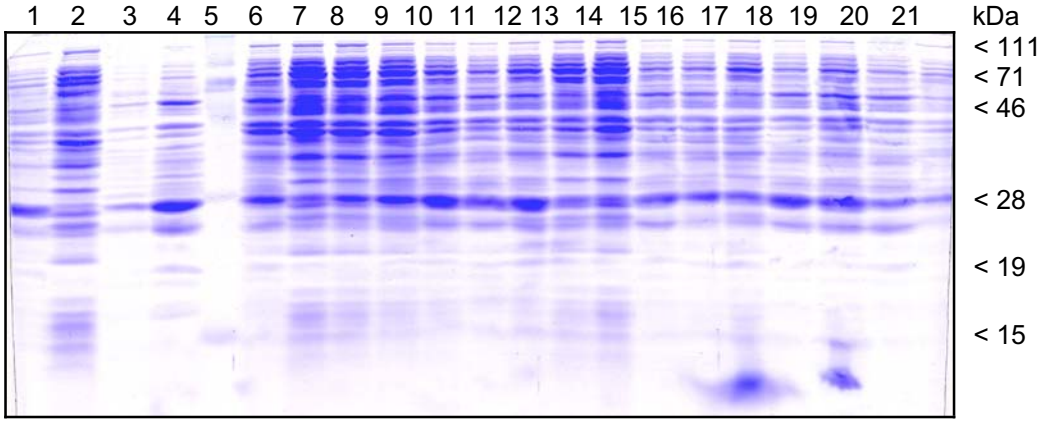

Figure 6-14: SDS-PAGE of periplasmic proteins stained with Coomassie Brilliant Blue. Samples are the same as in the next figure

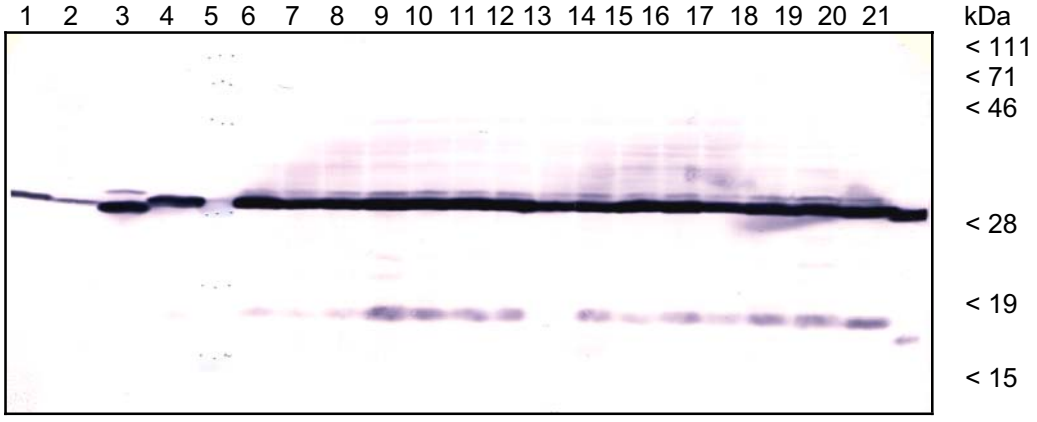

Figure 6-15: Western blot of periplasmic extracts. Lane 1, pCK2.44; lane 2, pCK2.1; lane 3, pCP6.84; lane 4, pCP6.54; lane 5, BRL molecular weight standard (sizes are shown on the right); lane 6, pCK1.38.5; lane 7, pCK1.38.4; lane 8, pCK1.38.3; lane 9, pCK1.38.2; lane 10, pCK1.38.1; lane 11, pCK1.24.5; lane 12, pCK1.24.4; lane 13, pCK1.24.3; lane 14, pCK1.24.2; lane 15, pCK1.24.1; lane 16, pCK1.21.5; lane 17, pCK1.21.4; lane 18, pCK1.21.3; lane 19, pCK1.21.2; lane 20, pCK1.21.1; lane 21, pCJ2.104

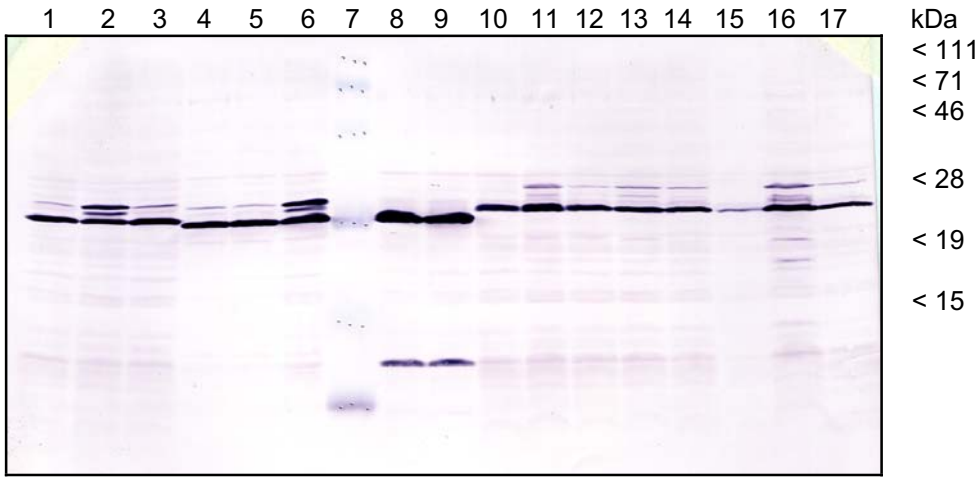

Figure 6-16: Western blot immunoassay of separated periplasm proteins extracted from the recombinant single bacterial colonies, probed with a rabbit anti-E tag antibody. Lane 1, pCP6.54.1; lane 2, pCP6.54.2; lane 3, pCP6.54.3; lane 4, pCP6.84.1; lane 5, pCP6.84.2; lane 6, pCP6.84.3; lane 7, BRL molecular weight standard (sizes are shown on the right); lane 8, pCJ2.104.1; lane 9, pCJ2.104.2; lane 10, pCK2.1.1; lane 11, pCK2.1.2; lane 12, pCK2.1.3; lane 13, pCK2.1.4; lane 14, pCK2.44.1; lane 15, pCK2.44.2; lane 16, pCK2.44.3; lane 17, pCK2.44.4

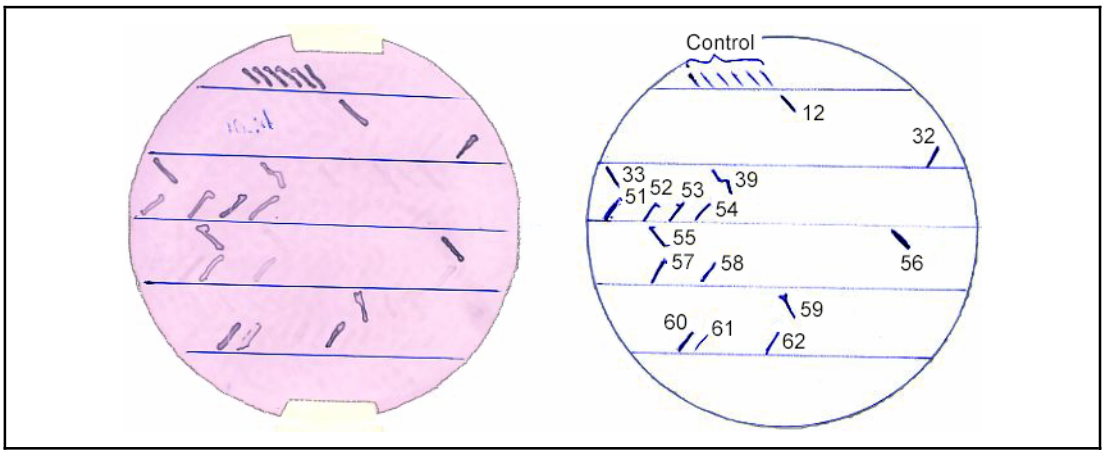

Figure 6-17: Colony blot for pCP6 single bacterial colonies, probed with a rabbit anti-E tag antibody. The original membrane is shown on the left, while a schematic sketch to identify the colonies is on the right

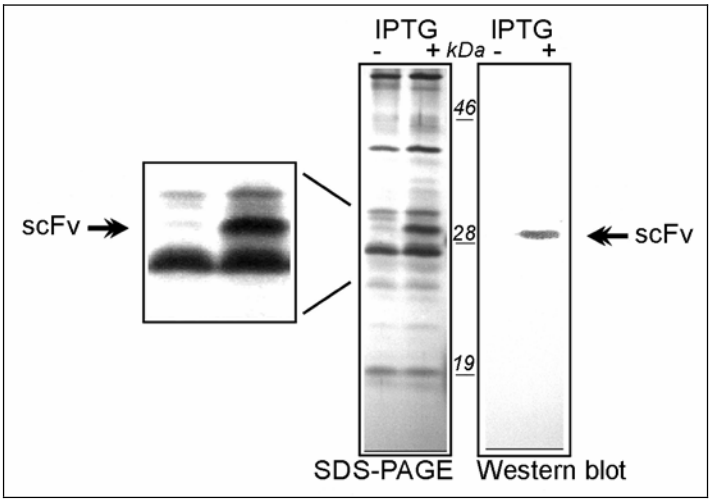

Figure 6-18: The expressed scFv K1 is detected by silver stained SDS-PAGE and Western blot

6.2.2. Detection of dsRNA binding activity in periplasmic scFv extracts

Inheriting only one antigen binding site, scFv K1, J2, and P6 were shown to retain antigen-binding activity when expressed in *E. coli* periplasma (Figure 6-21 - Figure 6-22). However, although the bacterially expressed scFvs were active, we observed a broad variation in antigen binding activity of the same scFv clone depending on the batch and probable subtle differences in preparation and in the ELISA procedure. The highest dsRNA binding was always detected in case of the J2-scFvs, the activity of which stayed more or less at the same level. While the intact K1 antibody shows similar affinity to dsRNA antigens as J2, the affinities of the corresponding scFvs were clearly different: The ELISA signals obtained with K1-scFv were not only lower, but showed very large differences between subclones/isolations (see Figure 6-21).

The sequence of the K2 antibody was nearly identical to that of K1 (Figure 6-23). Periplasmic extracts were prepared from K2-colonies displaying positive signals on colony blot. They were analyzed by AN-ELISA for scFv activity using dsRNA as antigen. The OD405 reading after 3 hours of substrate incubation is shown in Figure 6-21. Same active clones like pCK2.1, pCK2.4, pCK2.34, pCK2.36 and pCK2.44 were investigated in several preparations, but they did not always performed as positive in ELISA. To exclude inhomogeneity of cells several clones (namely pCK2.1, pCK2.44, pCK2.34 and pCK2.36) expressing active scFv K2 were subcloned and re-investigated. It was impossible to reproduce exactly the scFv K2 activity in standardized and repeated experiments. Nevertheless, clone 1, 34, 36 and 44 assayed in AN-ELISA were positive in several assays. scFv K2 protein was detectable by Western blot and scFv K2 sequence was confirmed by sequencing.

The original P6 monoclonal antibody differs from the other three monoclonals investigated, because it reacts not only with dsRNA, but also with single-stranded RNAs. To analyze the antigen-specificity of scFv P6 27 scFv-expressing pCP6 clones were tested in AN-ELISA. At this time bio-rRNA was used as an antigen. Only 3 positive clones were found, pCP6.54 and pCP6.84 being the most active ones. The assay was repeated two times and gave identical results (Figure 6-20). An additional AN-ELISA test with bio-dsRNA antigen revealed that pCP6.54 and .84 hardly bound dsRNA (Figure 6-21).

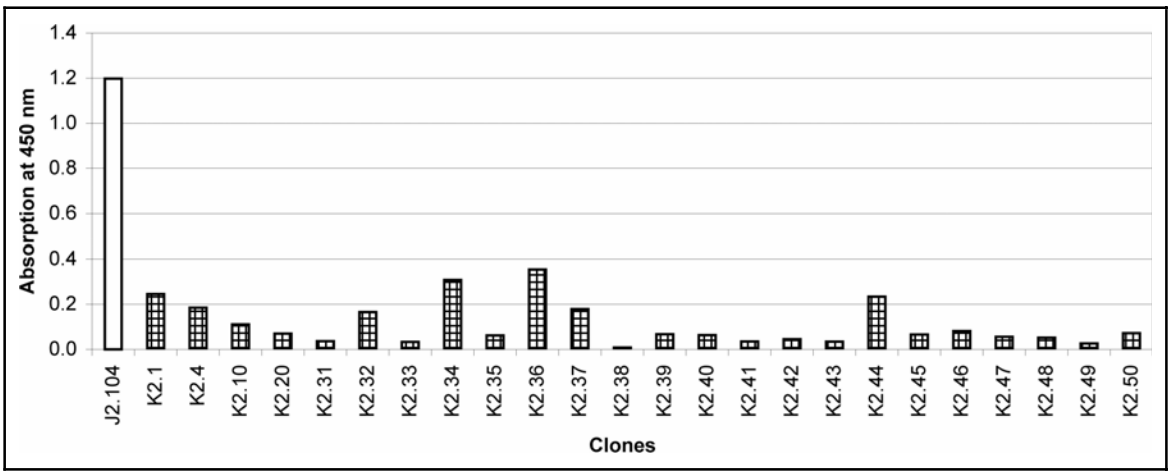

Figure 6-19: Antigen binding ELISA to screen for activity of bacterially-produced scFv K2 in pCK2 clones. The plate was read after 6 h of substrate incubation. Difference between average dsRNA signal derived from 2 parallels and background signal produced by STE buffer is shown on Y axis as absorption at 450 nm

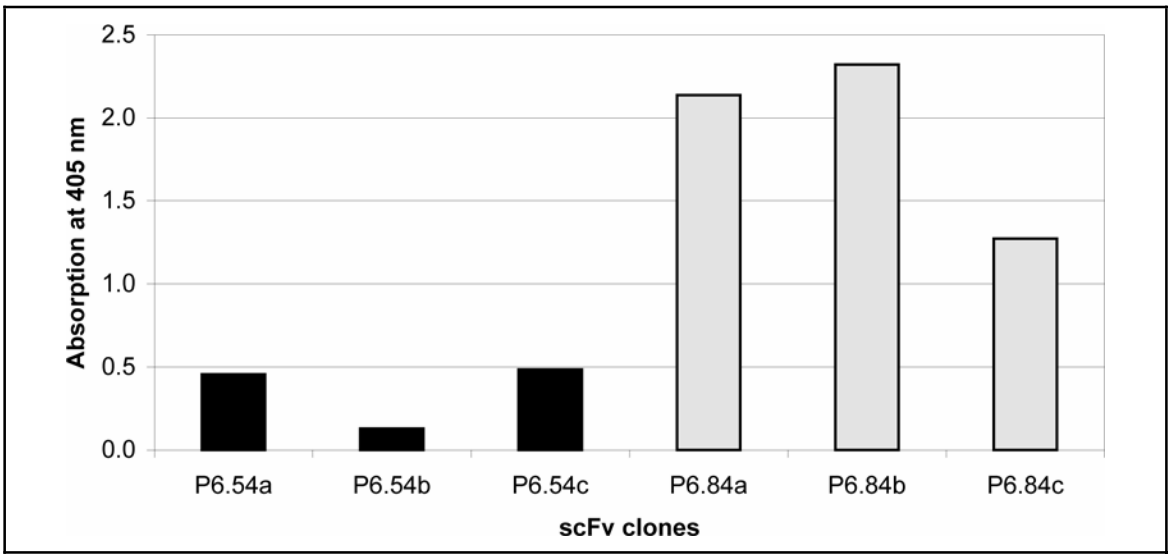

Figure 6-20: Activity of bacterially-produced scFv P6 towards bio-rRNA

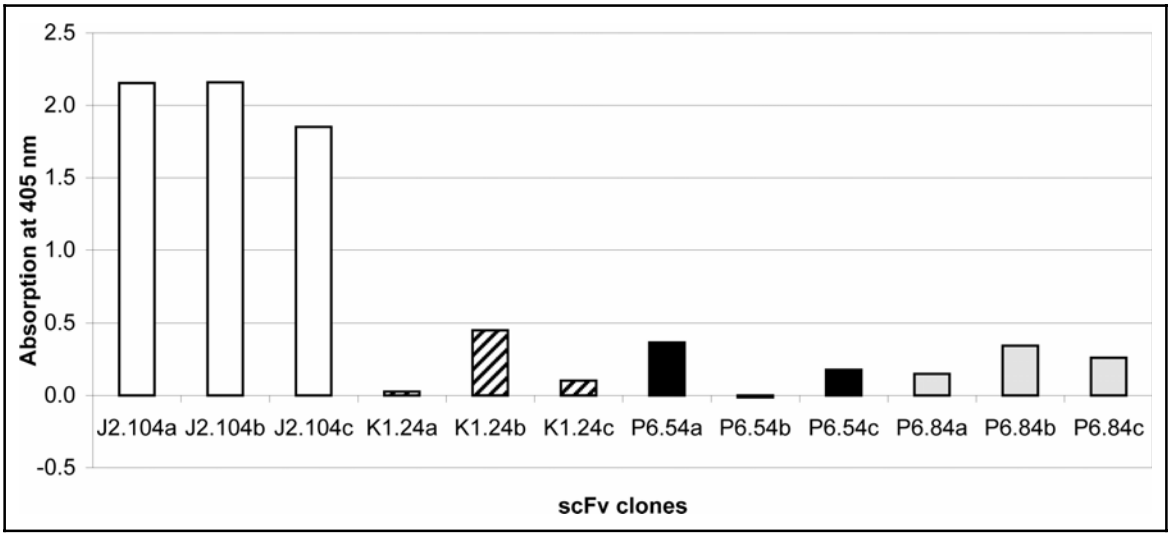

Figure 6-21: Activity of bacterially-produced scFv P6 towards bio-dsRNA

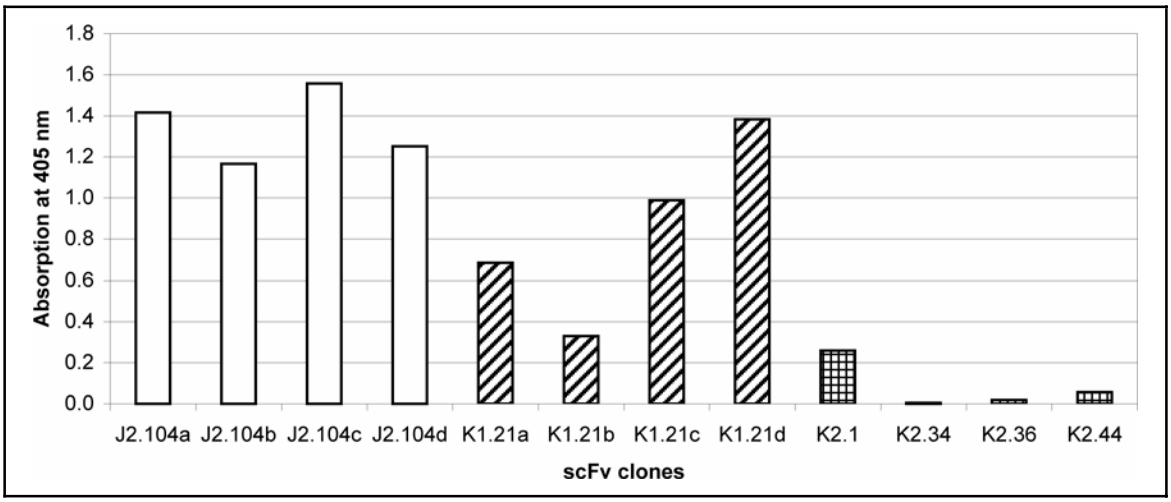

Figure 6-22: Activity of bacterially-produced scFv J2, K1 and K2 towards bio-dsRNA. Here the intensity of signals could be compared among dsRNA-binding scFvs

6.2.3. Sequencing and alignments of scFv cDNAs

To confirm gene identity several of the most active scFv clones from each genotype were sequenced. Purified double-stranded DNA plasmids were sequenced using fluorescing dye-labeled terminators and cycle sequencing method from both ends at the Central Laboratory of Biological Research Center, Szeged.

scFv J2 and K1 sequences were compared to cloned cDNA sequences of IgG J2 and K1 (Oberstraß, 1993), while P6 and K2 were compared to amplified and sequenced cDNA of VH and VL. cDNAs sequences of P6 and K2 antibodies were obtained and studied by Tamás Alexin and Volodymyr Stepanyuk. According to the sequencing results the length of scFv coding sequences without signal sequences and immunological tags was the following: scFv J2.104 - 750 bp; scFv K1.21 equals to K2.32 – 725 bp; and scFv P6.84 – 731 bp. Predicted molecular weight of scFv polypeptides was in the range 25.5-26.5 kDa (Table 6-5). The expected length was calculated from original antibody sequence plus artificially incorporated linker, leader peptide, and E-tag. These calculations were carried out using computer program Vector NTI.

Table 6-1: Mutations in amino acid sequences of active scFv clones versus the sequences of variable domains of monoclonal antibody J2. FR stands for framework region. Numbering starts from the 20 amino acid long g3 signal sequence

	23	**25**	**231**
scFv J2.104	Glu	Gln	Asp
scFv J2.115	Glu	Lys	Asn
IgG J2 cDNAs	Gln	Gln	Asp
Location	*FR*	*FR*	*FR*

Table 6-2: Mutations in amino acid sequences of active scFv clones versus the sequences of variable domains of monoclonal antibody K1

	25	**47**	**133**	**153**	**155**
scFv K1.18	Lys	Phe	Val	Asp	Glu
scFv K1.21	Lys	Ser	Val	Asp	Glu
scFv K1.24	Gln	Ser	Val	Asp	Glu
scFv K1.38	Lys	Ser	Val	Asp	Glu
scFv K1.39	Gln	Ser	Val	Asp	Glu
IgG K1 cDNAs	Gln	Ser	Leu	Gln	Val
Location	*FR*	*FR*	*FR*	*FR*	*FR*

Table 6-3: Mutations in amino acid sequences of active scFv K2 clones versus the sequences of variable domains of monoclonal antibody K2

	25	**28**	**30**	**50**	**68**	**85**	**233**	**236**	**252**	**257**
scFv K2.1	Lys	Glu	Gly	Thr	Val	Lys	Asp	Thr	R	Ile
scFv K2.32	Lys	Glu	Gly	Thr	Glu	Lys	Asp	Thr	Gly	Ile
scFv K2.36	Lys	Glu	Gly	Thr	Glu	Glu	Asp	Ile	Gly	Leu
scFv K2.44	Gln	Gln	Glu	Ile	Glu	Lys	Asn	Thr	Gly	Ile
IgG K2 cDNAs	Gln	Glu	Gly	Thr	Glu	Lys	Asp	Thr	Gly	Ile
Location	*FR*	*FR*	*FR*	*FR*	*FR*	*HCDRII*	*FR*	*FR*	*FR*	*FR*

Table 6-4: Mutations in amino acid sequences of ELISA-active scFv P6 clones versus the sequences of variable domains VH and VL cDNAs of monoclonal antibody P6. L stands for linker, FR stands for framework region. LC1 and LC3, CDR1 and CDR3 of light chain, respectively

	23	25	28	140	153	165	166	167	180	182	229	230	244	245	249	253	259
scFv P6.54	Gln	Gln	Gln	Cys	Gly	Ile	Ser	Ala	Ser	Ala	Ser	Ile	Trp	Ser	Leu	Ala	Ile
scFv P6.84	Lys	Lys	Gln	Gly	Ala	Met	Phe	Val	Asn	Thr	Ile	Ser	Trp	Ser	Leu	Ala	Leu
IgG P6 cDNAs	Lys	Gln	Glu	Gly	Gly	Met	Ser	Ala	Ser	Thr	Ser	Ile	Cys	His	Phe	Ser	Ile
Location	FR	FR	FR	L	L	FR	FR	FR	LC1	LC1	FR	FR	LC3	LC3	LC3	FR	FR

```
                      1                                                  50
scFv J2.104     (1)   MAEVQLQQSGPELVKPGASVKMSCKASGYTFANHVMHWVKQKPGQGLEWI
 scFv K1.21     (1)   MAQVKLQQSGPELVKPGASVKMSCKASGYTFANHVMHWVKQKPGQGLEWI
 scFv K2.32     (1)   MAQVKLQESGPELIKPGASVKMSCKASGYTFANHVMHWVKQKPGQGLEWI
 scFv P6.84     (1)   MAKVKLQQSGPELVKPGASVQMSCKASGYTFTSYVMHWVKQKPGQGLEWI

                      51                                                 100
scFv J2.104    (51)   GYIYPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLASEDSAVYYCAR
 scFv K1.21    (51)   GYIYPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLASEDSAVYYCAS
 scFv K2.32    (51)   GYIYPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLASEDSAVYYCAS
 scFv P6.84    (51)   GYINPYTDTTKYNEKFKGKATLTSDKSSSTAYMELSGLTSEDSAVYYCAL

                     101                                                 150
scFv J2.104   (101)   GGNPAWFAYWGQGTLVTVSAGGGGSGGGGSGGGGSNIMMTQSPSSLAVSA
 scFv K1.21   (101)   N---YYFDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPAIMSASP
 scFv K2.32   (101)   N---YYFDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPAIMSASP
 scFv P6.84   (101)   RSR-YYFNYWGQGTTVTVSSGGGGSGGGGSGGGASDIELTQSPAIMFVSP

                     151                                                 200
scFv J2.104   (151)   GEKVTMSCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYWASTRESGVP
 scFv K1.21   (148)   GEKVTMTCSASSSVSY-------MHWYQQKSGTSPKRWIYDTSKLASGVP
 scFv K2.32   (148)   GEKVTMTCSASSSVSY-------MHWYQQKSGTSPKRWIYDTSKLASGVP
 scFv P6.84   (150)   GEKVTISCSANSTVSY-------MYWYQQKPGSSPKPWIYRTSNLASGVP

                     201                                                 250
scFv J2.104   (201)   DRFTGSGSGTDFTLTISSVQAEDLAVYYCHQYLSS-YTFGGGTKLEIKRA
 scFv K1.21   (191)   ARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPYTFGGGTKLEIKRA
 scFv K2.32   (191)   ARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPYTFGGGTKLEIKRA
 scFv P6.84   (193)   VRFSGSGSGTSYSLTIISMEAEDAATYYCQQWSSYPLTFGAGTKLELKRA
```

Figure 6-23: Multiple alignment of amino acid sequences for selected scFv J2, K1, K2, P6 clones. Common for all the g3 signal sequence that is cleaved in the periplasm and the E-tag sequence are not shown. The sequence of the linker is GGGGSGGGGSGGGGS

Table 6-5: The expected length and molecular weights of scFvs expressed in E. coli *periplasm. The nucleotide sequence includes the g3 signal and immunological E-tag sequences as well as original or cloned mAb cDNA. The molecular weight was calculated based upon this hypothetical nucleotide sequence*

scFv	J2	K1	K2	P6
Length, base pairs	862	832	832	838
Length, amino acids	287	277	277	279
Weight, kDa	30.5	29.4	29.4	29.9

The results of scFv sequencing showed, that scFv J2 and K1 were precisely amplified except for some point mutations that were incorporated into framework regions by the degenerate phage display primers. The CDRs have not been influenced by those changes. These mutations are Q changed to E at position 3 for VH of scFv J2; Q changed to K at position 5 for VH of scFv K1; Q changed to D at position 1 for VL of scFv K1 and V changed to E at position 3 for VL of scFv K1 (Table 6-1 and Table 6-2). The cause for these mutations is that the primers for amplification of the variable fragments of heavy and light chain sequences contain some degenerated and mismatching nucleotides. This primer design leads to their universal character suitable for cloning of the variable fragments from different antibodies. Consequently, the amplified VH or VL sequences may slightly differ from the original sequences. Nevertheless, the incorporated changes are thought to be harmless for scFv performance because they are located not in the CDRs but in the framework regions at the termini of antibody variable domain.

Table 6-6: Complementarity Determining Regions (CDRs) of four scFvs that were intended for expression in higher plants. The CDRs are aligned to show the homologues stretches

	CDRI of VH	CDRII of VH	CDRIII of VH
scFv J2.104	NHVMH	YIYPYNDGTKYNEKFKG	GGNPAWFAY
scFv K1.21	NHVMH	YIYPYNDGTKYNEKFKG	N---YYFDY
scFv K2.32	NHVMH	YIYPYNDGTKYNEKFKG	N---YYFDY
scFv P6.84	SYVMH	YINPYTDTTKYNEKFKG	RSR-YYFNY

	CDRI of VL	CDRII of VL	CDRIII of VL
scFv J2.104	KSSQSVLYSSNQKNYLA	WASTRES	HQYLSS-YT
scFv K1.21	SASSSVSY-------MH	DTSKLAS	QQWSSNPYT
scFv K2.32	SASSSVSY-------MH	DTSKLAS	QQWSSNPYT
scFv P6.84	SANSTVSY-------MY	RTSNLAS	QQWSSYPLT

Sequence comparison of the VH-domains revealed the complete identity of scFv K2 and K1 sequences, although K2-scFv incorporated a much larger number of primer derived mutations than scFvs K1 (Table 6-3). Because of the identity of K1 and K2 sequences no further analysis was carried out for scFv K2. Both positive clones (54 and 84) of scFv P6 were slightly different from IgG P6 VH and VL cDNAs and incorporated mutations in the L-CDRs (

Table 6-4).

To express scFv in plants one clone from each genotype was selected. We always choose the clone showing high ELISA activity and the lowest number of mutations. Among the scFv J2 clones clone 104 showed the least altered sequence. It contains only one mutation at position 23 Q mutated to E. Thus, it was chosen for further detailed studies and expression in higher plants. The two scFv P6 clones both differed from the original VH- and VL-sequence, therefore clone P6.84 was arbitrarily chosen. The scFv K1 sequences of clone 24 and 39 have no unexpected mutations and the smallest number of expected alterations. Thus, the scFv K1 sequences of clone 24 and 39 are the closest sequences to the original IgG K1 sequence. Since, however scFv K1.21 and scFv K2.32 amino acid sequences were identical except two mutations at 27 Q-E, 33 V-I and K1.21 differed only in position 25 from clone K1.24, K1.21 was chosen for expression in tobacco.

Sequence comparison of the selected scFv clones revealed the almost complete identity of scFv K2 and K1 sequences, and 87.5 % identity of scFv P6 and K1 sequences. Identity of the heavy chain variable domain sequences of scFv J2 and K1, except the complementarity determining region (CDR3), was known before. This sequence uniformity of dsRNA binding antibodies shows that all four heavy chain variable domain sequences belong to the same germline sequence. The light chain variable domain sequences belong to two different groups: 1) scFv J2, and 2) scFv K1, K2, P6.

6.3. Constructs to target dsRNA-specific scFv to different cellular compartments in transgenic plants

The aim of expression dsRNA-specific scFv in plants was to influence virus replication. Most of the plant viruses are known to replicate in the cytoplasm, usually in association with cellular membranes (Buck, 1996). Unfortunately, the cytoplasmic environment for antibodies, naturally passing through the endoplasmic reticulum and requiring an oxidizing environment to form disulfide bridges, is not favorable. Thus, the cytoplasmic expression is still cumbersome and there is no general solution for it. That's why we pursued several approaches to protect or target single-chain antibody fragments. It is known that stability and performance of heterologous proteins greatly depend upon the primary sequence and their intrinsic properties. Since it is barely possible to predict *a priori* which antibody sequence will be stably expressed inside the cytoplasm, we tried to express different scFv sequences possessing similar antigen-binding specificities.

To target scFvs to the cytosol no signal sequences were added (Figure 6-24 A). Coding sequences of scFv J2, K1 and P6 were cut from phagemid pCJ2.104, pCK1.21 and pCP6.84, respectively with SfiI and NotI endonucleases. Gel purified DNA fragments were ligated into pre-digested pGEJAE1 vector and transformed into XL1-Blue *E. coli* strain.

It was shown in the literature that the C-terminal KDEL sequence increases the expression level of a single-chain antibody in the cytosol (Schouten et al., 1996). Therefore, we also generated constructs containing the DIKDEL (asp-ile-lys-asp-glu-leu) hexapeptide, an endoplasmic reticulum retention signal. Their nucleotide sequence was optimized for tobacco expression according to the Codon Usage Database built by Kazusa DNA Research Institute in Japan. A 62-mer PCR primer, Oligo 12, encoding DIKDEL was used for amplification of a 187 bp DNA fragment from pGEJAE1. Following purification from agarose gel, it was cloned back to the plant expression vector via HindIII and SalI endonuclease sites. A success of operation was confirmed by endonuclease analysis and sequencing. Consequently scFvs J2, K1 and P6 were

recloned from phagemid pCJ2.104, pCK1.21 and pCP6.84, respectively, in a similar way. scFv ending with KDEL sequence (Figure 6-24 B) was expected to accumulate stably in the cytoplasm.

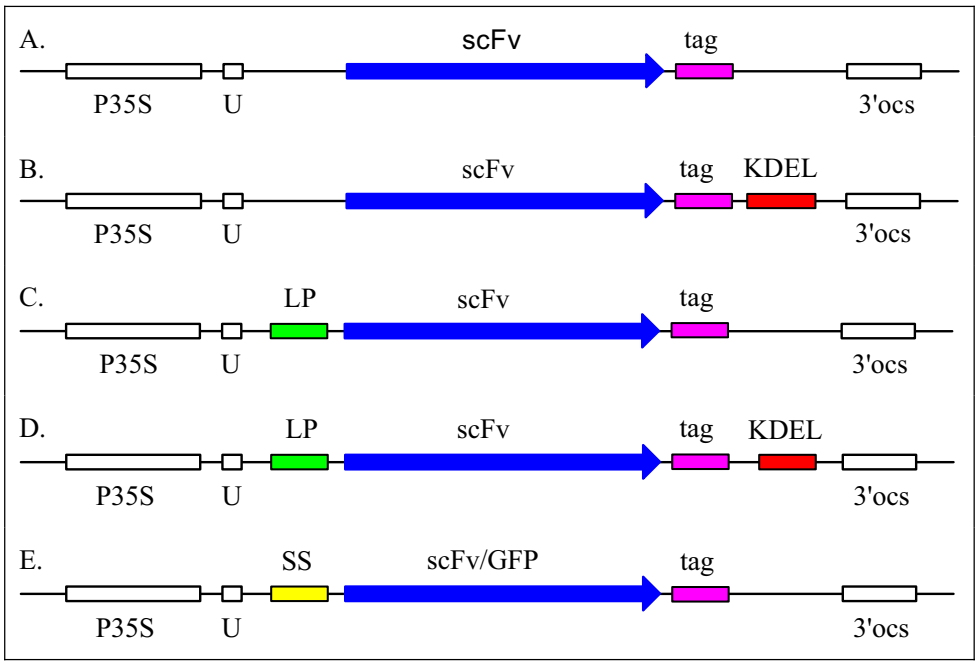

*Figure 6-24: Gene constructs used to express dsRNA-specific scFv at different cellular locations. The simplest, basic (**A**) and KDEL protected constructs (**B**) were intended for expression in the cytosol. The ones of type **C** were designed for scFv secretion to the apoplast, of type **D** for retention in the endoplasmic reticulum, while those of type **E** for anchoring in the plasma membrane. P35S, 35S promoter of Cauliflower mosaic virus; U, 5' untranslated omega-leader of* Tobacco mosaic virus*; LP, leader peptide of mouse J2 heavy chain; SS, signal sequence; scFv, coding sequence of single-chain antibody fragment or green fluorescent protein (GFP); tag, c-myc tag; 3'ocs, 3' end of the octopine synthase gene; KDEL, carboxy-terminal endoplasmic reticulum retention signal*

It is necessary to mention that the numbering of clones all along the studies was done according to the following rules. The pC or pG letters stood for plasmid pCANTAB 5E or pGEJAE1 respectively. The J2 or K1 or K2 or P6 stood for monoclonal antibody or scFv being dealt with. Finally, numbers after the full stop (e.g. 104 or 21 or 84) designated the number of clone obtained after *E. coli* transformation with recombinant scFv-cDNA.

In previous experiments of our research group it was shown that the leader peptide of the heavy chain of mouse monoclonal IgG J2 antibody is able to direct expressed polypeptides to the ER and then into the intercellular space in *N. tabacum* (Lukács et al., 1994; Richter, 1995). Relying on this finding we used the same heterologous signal to target scFvs to the apoplast (Figure 6-24 C). Utilizing the unique endonuclease DraIII site in VH domain we cloned scFv K1.21 from pCK1.21 phagemid to pAJ53H plasmid (Oberstraß, 1993). As a result, 5' end of scFv K1 was elongated with antibody leader. Finally, scFv LK1 was amplified by PCR using FORSFIL (introducing SfiI site and start codon) pared with pCANTAB5-R2 primers, digested with SfiI and NotI and ligated into pGEJAE1. Fortunately this strategy worked out equally well for J2 and P6 too.

The oxidative environment inside the endoplasmic reticulum is optimal for disulfide bridge formation. That's why high and stable expression was expected for scFv LKJ2, LKK1 and LKP6 constructs directed into and made resident in the ER (Figure 6-24 D). They, along with constructs passing through the ER to the apoplast, may serve as positive controls for cytosolic scFv expression. Thus, for transport into the endoplasmic reticulum (ER) the heavy chain leader peptide of J2 antibody was attached to the N-terminal end and for retention inside the compartment DIKDEL signal was added to the C-terminal end. Like to scFv LK1, elongated scFv was amplified by PCR using FORSFIL (introducing SfiI site and start codon) and pCANTAB5-R2 primers, digested with SfiI and NotI and ligated into pGEJAE1 vector already containing KDEL signal.

Replication of ssRNA viruses is usually closely associated with membrane complexes derived from the ER or perhaps nuclear membranes. Plant virus genomes also move from cell to cell via plasmodesmata or complex membrane-lined channels that penetrate the cell wall (Rybicki, 2000). Replication sites of plant RNA viruses are usually associated with cell membranes (Noueiry et al., 2003; Lee et al., 2003). If scFv J2 antibody fragment could be anchored at the cytoplasmic side of plasma membrane (PM), retaining its antigen-binding site free for interaction, the proximity to the PM, the posttranslational modification and the presence of viral dsRNA antigens might stabilize the structure of artificial antibody fragments. Thus, to enhance stability and local concentration of recombinant antibody fragments in the plasma membrane fatty acylation signals were used to anchor scFv at the cytoplasmic side of the plasma membrane. In order to verify the reliability of heterologous but natural signal sequence, a control cytosolic reporter (Davis et al., 1998), Green Florescent Protein (GFP) was also modified with the same signals and expressed like dsRNA-specific scFv J2 (Figure 6-24 E). 11-amino-acid-long membrane targeting signal from N-terminal end of protein-tyrosine kinase Fyn (Fyn-PTK) was used in its unaltered form. The signal is responsible for myristoylation and dual palmitoylation, two main types of protein fatty acylation. Myristoylation is a permanent co-translational linkage of the 14-carbon fatty acid myristate to the N-terminal glycine, while palmitoylation is a reversible posttranslational linkage of 16-carbon fatty acid palmitate to variably located cysteine residues. Fyn-PTK is a conservative protein naturally present in mammalians, plants and other living organisms. Constructs for membrane anchoring as well as transgenic plants expressing these constructs were made by Sachin Deshmukh. Analysis of the transgenic plants was performed within the current PhD project.

The integrity and accuracy of all constructs was confirmed by endonuclease digestion of recombinant molecules and sequencing. Coding sequences of three antibody fragments, scFv J2.104, K1.21 and P6.84, were cloned into the plant expression vector pGEJAE1. scFv K2 was left out due to its almost absolute identity to scFv K1. All plasmids were transferred to C58C1Rif^{R} (pGV2260) *Agrobacterium tumefaciens* strain and eventually used for leaf disc transformation of tobacco.

6.4. Expression of dsRNA-specific scFvs in transgenic *Nicotiana tabacum*

6.4.1. Preparation of *Agrobacterium* transconjugant clones for tobacco transformation

Gene constructs engineered in *E. coli* were transferred first to *Agrobacterium* and then to tobacco. The *Agrobacterium* strain containing pGV2260 Ti plasmid was transformed by tri-parental mating. This special system uses pRT2013 helper plasmid in HB101 *E.*

coli strain to enhance transformation efficiency. Recombinant agrobacteria were screened for desired genetical background. Total DNA was prepared from pure homogeneous *Agrobacterium* clones and subjected to PCR amplification to verify presence of scFv sequences in transconjugant clones. Specific primes annealing within scFv (such as VH1BACK and MJK2FONX) along with necessary controls were employed for reactions. Usually some 6-12 clones were prepared for each antibody construct and in most cases 50-80 % of them were positive. An example of the results is shown in Figure 6-25.

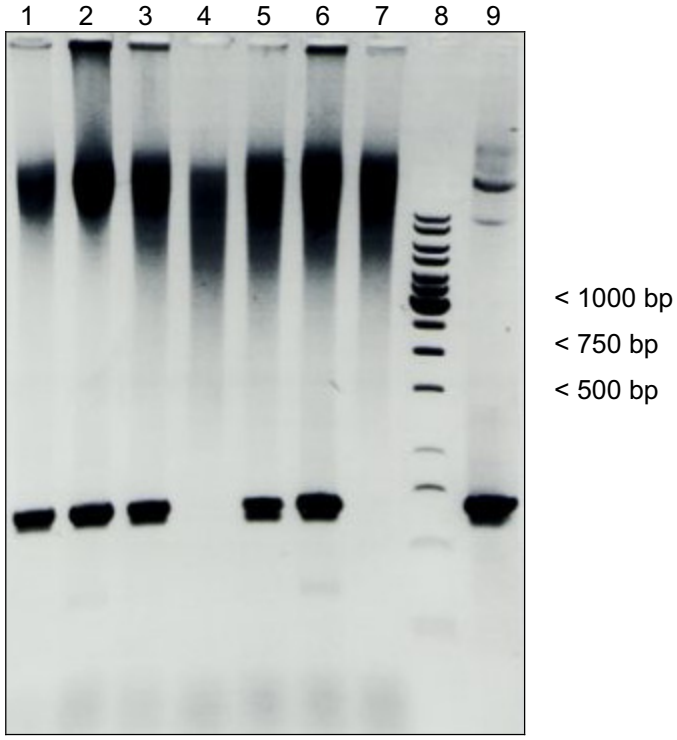

Figure 6-25: PCR products of selected Agrobacterium *clones were resolved in 1 % agarose gel containing ethidium bromide. The expected fragment size was about 700 bp. Lane 1, pGKP6.2; lane 2, pGKP6.4; lane 3, pGKP6.6; lane 4, pGKP6.7; lane 5, pGKP6.8; lane 6, pGKP6.9; lane 7, pGKP6.11; lane 8, 400 ng of the 1 kb DNA Ladder; lane 9, positive control pGP6.84.4. The length of markers is indicated on the right. The results show that five of the seven clones contained the expected insert*

Glycerol stocks were prepared from positive clones and stored at –80 °C. Whenever necessary, bacteria were revived at 30 °C in YEB broth containing rifampicin and used for tobacco transformation.

6.4.2. *Agrobacterium*-mediated transformation of *Nicotiana tabacum*

Leaf discs from aseptically grown *N. tabacum* cv. Xanthi plantlets were transformed by submersion into *Agrobacterium* suspension. After transformation bacterial growth was suppressed with antibiotics (claforan together with augmentin) and regenerating transformants were selected on kanamycin-containing MS agar. Usually about 200 leaf disks were used to transform one scFv construct. One regenerating disk sometimes produced numerous shoots but at most four of them were taken (Figure 6-26). Each shoot was uniquely numbered to follow single transformation event. For example, plant labeled as LKJ2/48/1 meant that it was transformed with scFv LKJ2 gene construct,

obtained from the 48th leaf disk, and being the first shoot cut from that leaf disk. The number after the full stop indicated a particular plant.

Table 6-7: Summary on produced scFv constructs and their promotion into Agrobacterium *and tobacco. The quantity of regenerated and evaluated plants is indicated (n.d. stands for not determined)*

Intracytoplasmic expression	**K1**	**J2**	**P6**
scFv construct in pGEJAE1 plant expression vector in *E. coli*	+	+	+
Transconjugant *Agrobacterium* clones	+	+	+
Transgenic plantlets *in vitro*	156	133	103
Transgenic plants in soil	57	69	24
Number of studied plants in T1 generation	5	7	24
Intracytoplasmic with KDEL	**KK1**	**KJ2**	**KP6**
scFv construct in pGEJAE1 plant expression vector in *E. coli*	+	+	+
Transconjugant *Agrobacterium* clones	+	+	+
Transgenic plantlets *in vitro*	n.d.	n.d.	n.d.
Transgenic plants in soil	27	85	23
Number of studied plants in T1 generation	n.d.	11	16
Targeted to apoplast	**LK1**	**LJ2**	**LP6**
scFv construct in pGEJAE1 plant expression vector in *E. coli*	+	+	+
Transconjugant *Agrobacterium* clones	+	n.d.	n.d.
Transgenic plantlets *in vitro*	n.d.	n.d.	n.d.
Transgenic plants in soil	27	25	n.d.
Number of studied plants in T1 generation	n.d.	10	6
Targeted to ER	**LKK1**	**LKJ2**	**LKP6**
scFv construct in pGEJAE1 plant expression vector in *E. coli*	+	+	+
Transconjugant *Agrobacterium* clones	+	+	+
Transgenic plantlets *in vitro*	n.d.	n.d.	n.d.
Transgenic plants in soil	4	12	n.d.
Number of studied plants in T1 generation	n.d.	11	6

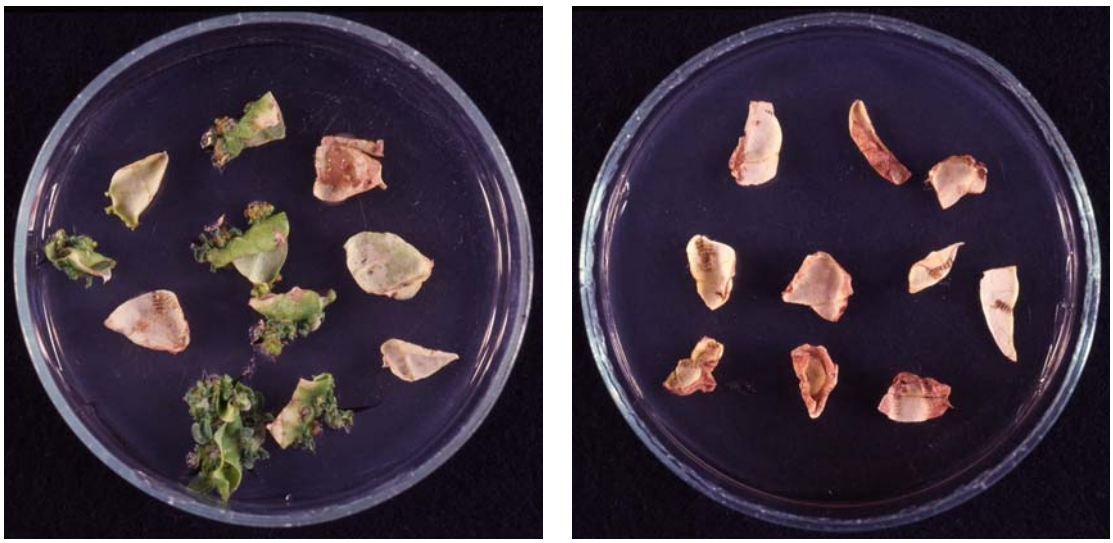

*Figure 6-26: Regenerating tobacco leaf disks of sample (**left**) and negative control (**right**) on agar plates containing kanamicyn as a selective agent 2-3 weeks after transformation*

The summary of transformation results is shown in Table 6-7. The detailed analysis of transgenic plants is described in the following chapters.

6.4.3. Transgene detection in T1 generation by DNA amplification

scFv sequences in T1 plants were usually detected by polymerase chain reaction. In contrast to PCR analysis of transconjugant agrobacteria, for T1 plants we used primers located in pGEJAE1 vector, usually at 3' end of scFv gene on one side and inside scFv on the other side (see Table 12-1 for primer sequences and applications). This allowed us to screen not only for genetically modified plants but also to predict scFv integrity. The results of a typical amplification are shown in Figure 6-27, where fragments amplified from total tobacco DNA (lane 3 and 4, scFv K1 plants) were compared to the positive control, DNA amplified from purified plasmid (lane 7). It was clearly demonstrated that fragments of the same length (800 bp) were obtained from transformants as from the control plasmid. HLM plants (lane 2), strongly expressing intact J2 antibody (IgG) served as negative controls.

Different antibody fragments as well as GFP required various primer pairs for reliable amplification. The different scFv J2 constructs were all analyzed with primers J2-LT and pG-rev03, which annealed to the coding sequence common to all constructs and led to production of a 486-bp PCR fragment (Figure 6-28).

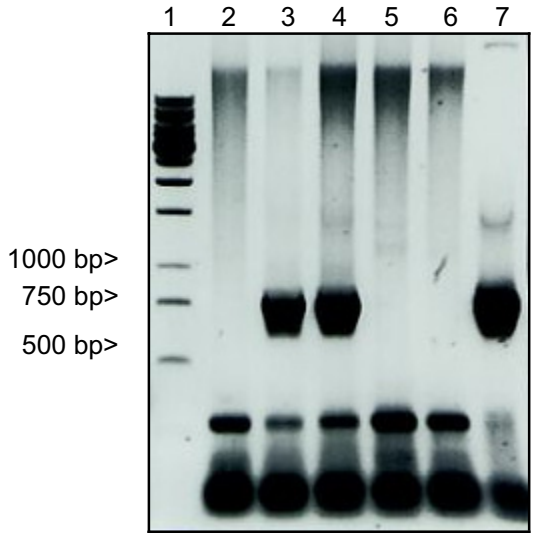

Figure 6-27: PCR products specific for scFv K1 obtained with primers SSF1 and Oligo12. Lane 1, 500 ng of the 1 kb DNA Ladder; lane 2, HLM2; lane 3, plant K1/38/1; lane 4, plant K1/13/2; lane 5, negative control Xanthi.5; lane 6, negative control Xanthi.6; lane 7, positive control, scFv K1 amplified from plasmid pGK1.2

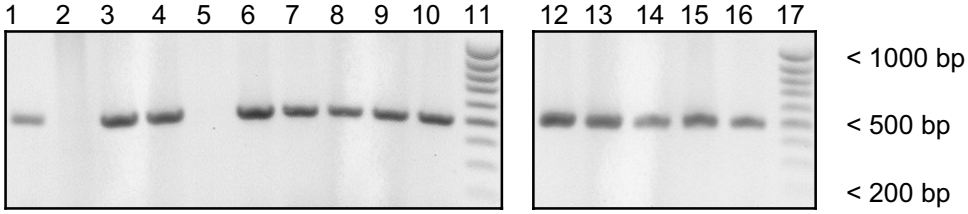

Figure 6-28: Detection of J2-specific PCR products from tobacco using primers J2-LT and pG-rev03. Transgenic plants were obtained by transformation with LKJ2 (lane 1), Fyn-J2 (lane 3 - 6), J2 (lane 7 - 9), KJ2 (lane 10, 12, 13), and LJ2 (lane 14 - 16) constructs. Lane 2 is the non-transgenic control; lane 11 and 17, the 100 bp DNA ladder. PCR products were separated in 1.2 % agarose gel and stained with ethidium bromide

6.4.4. Detection of transgenes by Northern hybridization

To verify the expression of scFv mRNA and to compare mRNA-levels in transgenic plants Northern blot analysis was carried out. Since scFv proteins are known to be highly unstable at some intracellular locations, successful plant transformation as well as sufficient transcription of scFv encoding sequences can be demonstrated at the same time by RNA hybridization. In Figure 6-29 hybridization results of four scFv J2 gene constructs are shown. The highest signals were observed for LKJ2 and LJ2. Not all J2 plants transcribed scFv J2 mRNA though there were a few positives with different expression level. Among these plants J2/69/4 (lane 8) showed the highest expression level. Similar results were obtained for tobaccos transformed with scFv K1 and P6 (Figure 6-30). The following K1 plants were Northern blot positive: K1/38/1, K1/21/4, K1/32/2, K1/6/2 out of several investigated. Among the P6 plants P6/14/5, P6/2/1, P6/16/1, P6/6/2, and P6/24/2 were positive. We did not analyze every single plant, as it was evident that the majority of them were positive and transformation occurred efficiently.

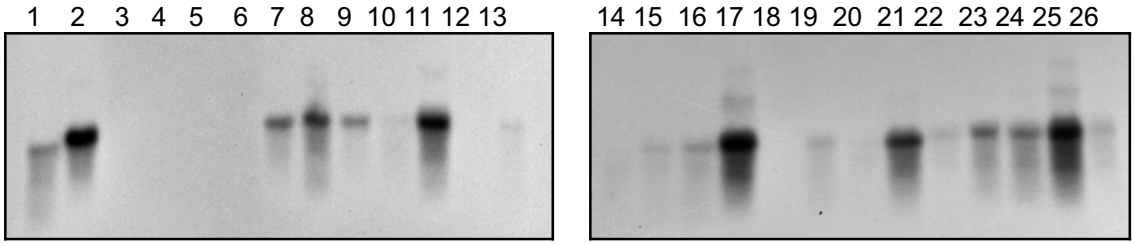

Figure 6-29: Northern blot analysis of tobaccos transformed with different scFv J2 constructs. Lane 1, J2/17/4; lane 2, LKJ2/48/1; lane 3, non-transgenic plant DX4; lane 4, J2/2/1; lane 5, J2/49/1; lane 6, J2/52/1; lane 7, J2/55/1; lane 8, J2/69/4; lane 9, J2/79/2; lane 10, KJ2/21/1; lane 11, KJ2/40/1; lane 12, KJ2-1; lane 13, KJ2-10; lane 14, KJ2-2; lane 15, KJ2-4; lane 16, KJ2-7; lane 17, LKJ2/56/1; lane 18, non-transgenic plant DX4; lane 19, KJ2-8; lane 20, KJ2-9; lane 21, LJ2/58/3; lane 22, LJ2/39/1; lane 23, LJ2/48/1; lane 24, LJ2/48/2; lane 25, LJ2/5/1; lane 26, LJ2/59/1

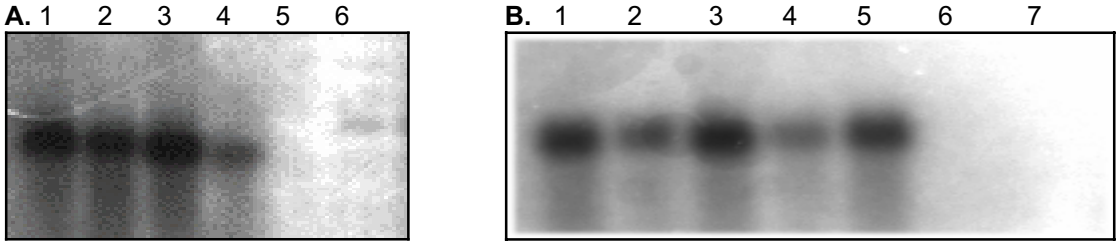

Figure 6-30: Northern blot analysis of scFv K1 (part A) and scFv P6 (part B) expression in transgenic tobaccos. ***A.*** *Lane 1, K1/38/1; lane 2, K1/21/4; lane 3, K1/32/2; lane 4, K1/6/2; lane 5, non-transgenic tobacco X1; lane 6, non-transgenic tobacco X2.* ***B.*** *Lane 1, P6/14/5; lane 2, P6/2/1; lane 3, P6/16/1; lane 4, P6/6/2; lane 5, P6/24/2; lane 6, non-transgenic tobacco X1; lane 7, non-transgenic tobacco X2*

6.4.5. Visualization of scFv proteins by Western blotting

In order to demonstrate the ability of transformed tobaccos to synthesize antibody fragments at different sites, plants were screened by Western blotting. Total soluble protein extracts were prepared with or without ionic detergent, also from different plant organs and separated in polyacrylamide gels under reducing or non-reducing conditions.

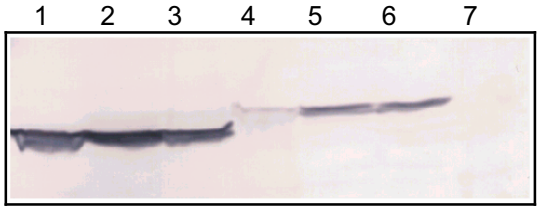

Figure 6-31: scFv LKJ2 and LKP6 targeted to the endoplasmic reticulum can be detected by Western blotting. Lane 1, LKJ2/39/3; lane 2, LKJ2/48/1; lane 3, LKJ2/56/3; lane 4, LKP6/10/1; lane 5, LKP6/17/1; lane 6, LKP6/17/2; lane 7, non-transgenic tobacco

The highest expression levels were detected in LKJ2 and LKP6 plants, i.e. when proteins were made ER-resident by attaching a C-terminal KDEL sequence (Figure 6-31). Nearly all of these plants expressed scFvs. In fact, 10 of 11 tobaccos for LKJ2 and 5 out of 6 plants for LKP6 were positive. Seemingly, the high accumulation of the scFv proteins in the ER is the result of both the formation of covalent disulphide bonds and adopting a stable conformation.

Table 6-8: Expected molecular mass of different scFvs produced in tobacco as calculated from the expected amino acid (aa) sequence

	J2	**LJ2**	**KJ2**	**LKJ2**	**P6**	**LP6**	**KP6**	**LKP6**
Length, bp	796	847	805	856	775	826	784	835
Length, aa	265	282	268	285	258	275	261	278
Molecular mass, kDa	28.2	30.2	28.7	30.7	27.7	29.6	28.2	30.2

The expected molecular weight of LKJ2 is slightly higher (30.7 kDa) than that of LKP6 (30.2 kDa) (Table 6-8). However, the actual data obtained by Western blotting show the opposite (Figure 6-31): LKJ2 proteins migrate always faster than LKP6 scFvs. Since we did not find any insertions in the LKP6 sequence, probable explanation could be some posttranslational modification taking place in the ER.

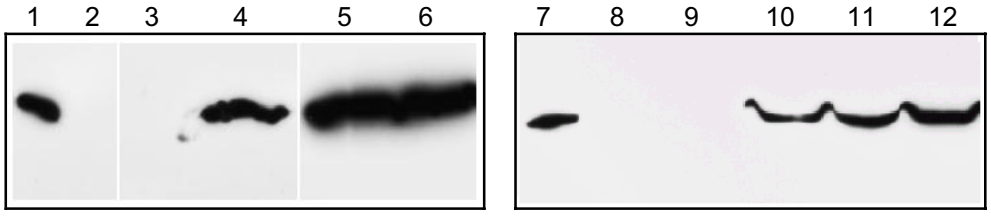

*Figure 6-32: Immunoblotting analysis of scFv Protein Accumulation in tobacco plants expressing scFv P6 (on the **left**) and KP6 (**right**). Lane 1, LKJ2/48/1; lane 2, non-transgenic tobacco; lane 3, P6/16/1C; lane 4, P6/16/1D; lane 5, P6/6/2A; lane 6, P6/6/2B; lane 7, LKJ2/48/1; lane 8, non-transgenic tobacco; lane 9, KJ2-9; lane 10, KP6/7/2; lane 11, KP6/7/1; lane 12, KP6/58/1*

Plant scFv KP6 and P6 targeted to the cytosol were also detected by immunoblotting in tobacco soluble protein extracts. In contrast, no scFv protein could be shown in KJ2 and J2 transformants. To detect P6 and KP6 we used more sensitive visualization system.

The secondary antibody was conjugated with horseradish peroxidase and the substrate was SuperSignal West Pico Chemiluminescent Substrate (Figure 6-32). We found, that out of 23 transgenic KP6 tobaccos eight were positive and out of 38 tested transgenic scFv P6 tobaccos just 12 showed the expected signals. In all cases the scFvs were seen as single, undegraded band migrating at approximately 30 kDa. The deduced molecular weight for various scFvs is shown in Table 6-8. As to cytosol-targeted scFv J2 constructs then there were no positives from 7 tested J2 tobaccos or from 11 KJ2 plants. However, as shown earlier the majority of these plants transcribed scFv mRNAs at high levels.

```
                          1                                          40
   tob scFv P6.4     (1) --------------------MAKVKLQQSGPELVKPGAS…
  tob scFv LP6.2     (1) ---MEWSWIFLFLLSGTAGVHSKVKLQQSGPELVKPGAS…
tob scFv LKP6.62     (1) ---MEWSWIFLFLLSGTAGVHSKVKLQQSGPELVKPGAS…
  tob scFv KP6.1     (1) --------------------MAKVKLQQSGPELVKPGAS…
 bact scFv P6.84     (1) VKKLLFAIPLVVPFYAAQPAMAKVKLQQSGPELVKPGAS…

                          241                                        280
   tob scFv P6.4   (221) …QQWSSYPLTFGAGTKLELKRAAAEQKLISEEDLNGAA---
  tob scFv LP6.2   (238) …QQWSSYPLTFGAGTKLELKRAAAEQKLISEEDLNGAA---
tob scFv LKP6.62   (238) …QQWSSYPLTFGAGTKLELKRAAAEQKLISEEDLNDIKDEL
  tob scFv KP6.1   (221) …QQWSSYPLTFGAGTKLELKRAAAEQKLISEEDLNDIKDEL
 bact scFv P6.84   (241) …QQWSSYPLTFGAGTKLELKRAAAGAPVPYPDPLEPRAA--
```

Figure 6-33: N- and C-terminal ends of different tobacco and E. coli *expressed scFv P6 to compare the difference in targeting signals. The middle part, of antibody fragment coding sequence is identical for all and removed for easiness*

To evaluate scFv expression in different organs, protein extracts were prepared from mature leaves and roots of transgenic as well as non-transgenic control plants. Equal amounts of protein (usually about 25 µg) were loaded into each lane and the intensity of 30 kDa band was compared on Western blots visually. The analysis revealed difference in expression levels for scFv P6 and scFv KP6 genotypes, which both had more plantibody in leaves than in roots. The amount of LKJ2 was equal in leaves and roots (Figure 6-34). scFv LKJ2 was equally expressed in all leaves and we did not notice any influence of PVY infection on its expression. scFv LKP6 showed the same expression pattern as scFv LKJ2 (see summarizing Table 6-9). However, when plants were infected with PVY virus the amount of scFv LKP6 was reduced in leaves comparing to that one in roots (Figure 6-34).

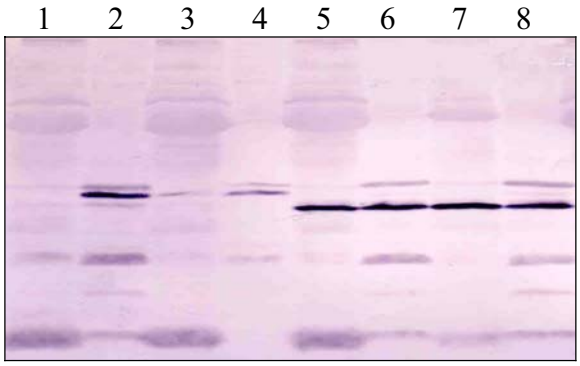

Figure 6-34: scFv LKP6 is expressed at higher levels in roots than in leaves in PVY infected tobaccos. scFv LKJ2 expression remains equal in both organs as detected by Western blot of soluble protein extracts. Lane 1 and 2, LKP6/10/1.491 leaf and root, respectively; lane 3 and 4, LKP6/10/1.492 leaf and root, respectively; lane 5 and 6, LKJ2/48/1.550 leaf and root, respectively; lane 7 and 8, LKJ2/48/1.555 leaf and root, respectively

Table 6-9: Summarized results on scFv expression levels in different organs in non-infected tobaccos. Strong expression levels are indicated as +++, while weak expression levels as +

scFv	Leaf	Root
P6	+++	+
KP6	+++	+
LKP6	+++	+++
LKJ2	+++	+++

When single-chain antibody fragments were targeted to the apoplast with the heterologous mouse leader peptide derived from the IgG J2 heavy chain, no scFv-protein was detected. Eleven transgenic plants transformed with scFv LJ2 gene construct were investigated but in none of them was antibody protein visualized by immunoblotting even using a very sensitive chemiluminescent substrate. Out of tested six scFv-LP6 tobaccos there were also no positives (results not shown). Nevertheless, scFv LJ2 tobaccos possessed high mRNA levels as it was shown earlier (see Figure 6-29 e.g. lines LJ2/5/1, LJ2/48/1, LJ2/48/2, and LJ2/58/3). Previous studies at our research group indicated high expression levels of intact IgG J2 antibody in the apoplast. Moreover, the very same leader peptide worked out for scFv targeting to the ER in case of LKJ2 and LKP6 constructs.

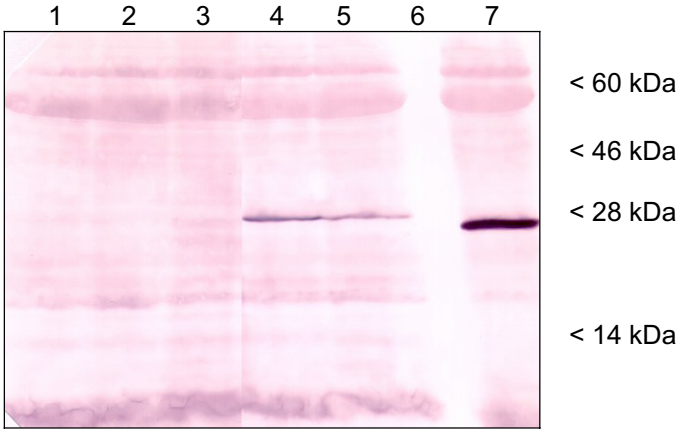

Figure 6-35: Membrane-associated Fyn-GFP was detected by immunoblotting in T2 generation of transgenic tobaccos in contradiction of scFv Fyn-J2 expression. Soluble proteins were extracted with buffer containing 1 % Triton X-100. Lane 1, Fyn-J2 56/16/1p.8; lane 2, Fyn-J2 56/16/1p.9; lane 3, Fyn-J2 56/16/1p.11; lane 4, Fyn-GFP 46/5/1p.25; lane 5, Fyn-GFP 46/5/1p.43; lane 6, empty; lane 7, LKJ2/48/1.1 as a positive control. Molecular weight standards are shown on the right

Membrane-associated expression was evaluated in *N. tabacum* plants transformed with Fyn-scFv J2 and Fyn-GFP constructs (Figure 6-35). The gene constructs (Figure 6-24) contained signal for protein fatty acylation taken from Fyn protein tyrosine kinase (PTK) of *Arabidopsis thaliana*.

While for detecting soluble scFv in the cytosol or in the ER the extraction buffer did not contained any detergents, the extraction buffer for the membrane targeted proteins had to contain ionic detergent. Usually the proteins were prepared in a buffer containing 1 % Triton X-100. Whenever the detergent was omitted no signals for Fyn-GFP 46/5/1p.25

and .43 were detected. PCR positive lines for Fyn-GFP (e.g. 46/5/1p.25; 46/5/1p.43) were positive in Western blotting though a Fyn-GFP-specific band on the blot was observed only after extended exposure. This finding indicated an association of reporter protein with membranes confirming results obtained by Sachin Deshmukh studying PM targeting with Fyn- and modified Src-signals in BY2 cell line. Thus, Arabidopsis derived Fyn-signal sequence targeted GFP to the PM in intact tobacco plants.

Using the same targeting signals for J2 scFv (Fyn-J2) we could not detect any protein expression by immunoblotting, although PCR-positive lines 56/16/1p.8; 56/16/1p.9 and 56/16/1p.11 were analyzed (Figure 6-35). Therefore we conclude that the targeting signals used in our experiments were not sufficient to stabilize scFv structure and to elevate its concentration to detectability.

6.4.6. Is plant-expressed scFv active?

dsRNA-specific scFv LKJ2 expressed at high levels in tobacco leaves and containing disulfide bonds was tested for its ability to bind double-stranded RNA by anti-nucleic acid ELISA (Schönborn et al., 1991). For comparison *E. coli* produced scFv J2 from periplasmic extracts was utilized as positive control. Figure 6-36 shows results of the test.

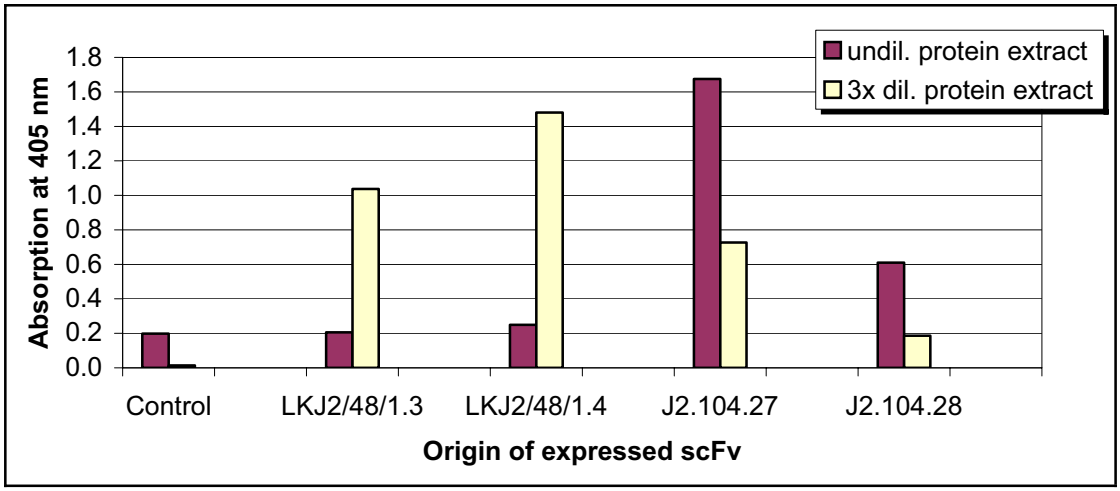

Figure 6-36: Plantibody scFv LKJ2 targeted to the endoplasmic reticulum bound dsRNA in AN-ELISA. 100 ng per well of biotin-labeled L-A dsRNA from yeast was applied as antigen along with STE buffer as negative control. Following plate wash, 100 ng of alkaline phosphatase conjugated streptavidin was put on per well. In 30 minutes light absorption at 405 nm was measured. Control, non-transgenic tobacco; LKJ2/48/1.3 and .4, two transgenic tobaccos expressing scFv LKJ2; J2.104.27 and .28, two E. coli *periplasmic extracts. Applied extracts were undiluted or three times diluted with 1 % BSA in PBS*

The preparative amount (usually 50 ml) of bacterial periplasmic extracts was produced from single colonies and dialyzed against PBS buffer overnight. In contrast, fresh soluble protein extracts from developed tobacco leaves were used without dialysis or purification. To detect scFv activity all extracts were diluted with 1 % BSA in PBS and were cleared by centrifugation. scFv was captured through its immunological tag (E-tag or c-myc) with tag-specific monoclonal antibody and bound to the microtiter plate leaving its antigen binding site free for interaction. The results (Figure 6-36) clearly indicate antigen-binding activity of plant-produced scFv LKJ2. Strong binding of recombinant antibody to dsRNA argues in favor for correct scFv folding and its stable

soluble state in the ER. Undiluted tobacco extracts might have had too high concentration of interfering compounds inhibiting dsRNA binding. Nevertheless, diluting tobacco sap three times with BSA in PBS let scFv show up. The same effect was not observed when bacterially expressed scFv-J2 was analyzed.

6.5. Electron microscope investigation of scFv localization

Plants from three high expressing genotypes were subjected to microscopy studies. This examination was carried out by László Mustárdy at the Biological Research Centre. These were genotypes LKJ2 and LKP6, that presumably accumulated scFv J2 and P6, respectively in the endoplasmic reticulum, and genotype P6, where scFv P6 was targeted to the cytosol. The negative control, non-transformed tobacco, was always included.

Despite several attempts to localize scFv in transgenic plants *in situ* no convincing results were obtained. Antibody detection in transgenic plants is difficult in general, because antibodies are highly soluble in water and are easily lost at mild fixation conditions. In earlier experiments in N. Lukács's group the in situ localization of J2-IgG in the apoplast also failed by immunoelectron microscopy, although very high expression levels were reached and J2 could be easily detected by tissue printing (Lukács et al., 1994). In the present series of EM experiments an additional difficulty was: at all antibody concentrations tested unusually high background labeling was observed in all transgenic genotypes as well as in non-transgenic controls. Most gold grains were localized in chloroplasts and in the cytoplasm.

6.6. Summary on scFv expression and targeting in plants

The extensive results on different expression strategies of scFvs in transgenic tobacco are summarized in Table 6-10. Whenever scFv protein was not reliably recognized, the presence of scFv-encoding DNA and RNA sequences was strictly investigated to prove transformation and/or mRNA-expression. scFv P6 and KP6 were successfully expressed in the cytosol in contrast to scFv J2 and KJ2. None of the scFvs was in the apoplastic targeting, although the antibody fragments targeted to the endoplasmic reticulum accumulated to very high levels. Targeting to the inner side of plasma membrane with the aid of Fyn fatty acylation signal proved to be successful in case of GFP, but did not lead to detectable accumulation of scFv J2 at the plasma membrane. This is probably due to the intrinsic instability of scFv in the reducing environment of the cytoplasm.

Table 6-10: Summary of single-chain antibody fragment expression in transgenic Nicotiana tabacum *cv. Xanthi plants (n.d. stands for not determined)*

Localization	Signal	mRNA	scFv Protein
Cytoplasm	None	+ +	+ P6 - J2
Cytoplasm	KDEL	n.d. +	+ KP6 - KJ2
Apoplast	Leader peptide	n.d. ++	- LP6 - LJ2
Endoplasmic reticulum	Leader peptide + KDEL	n.d. n.d.	+ LKP6 + LKJ2
Plasma-membrane	Fyn fatty acylation signal	+ n.d.	- Fyn-J2 + GFP

6.7. Infecting transgenic plants with RNA viruses

Single-chain antibody fragments expressed in tobacco accumulated in the cells to different levels. In case of ER targeting, scFv LKJ2 exhibited detectable dsRNA-binding activity *in vitro*. The next intriguing question was whether single-chain antibody fragments could bind dsRNA molecules *in vivo*, and if yes, do they protect plants from virus multiplication or interfere rather with dsRNA-based virus silencing. To answer this question, transgenic tobaccos were infected with two plant RNA viruses. The Hungarian isolate of NTN strain of *Potato Virus Y* and *Cucumber Mosaic Virus* were chosen for the testes. Five experiments were carried out to evaluate difference in symptom development between transgenic and non-transgenic control plants. Figure 6-38 shows the variability of symptom intensity in PVY infected plants.

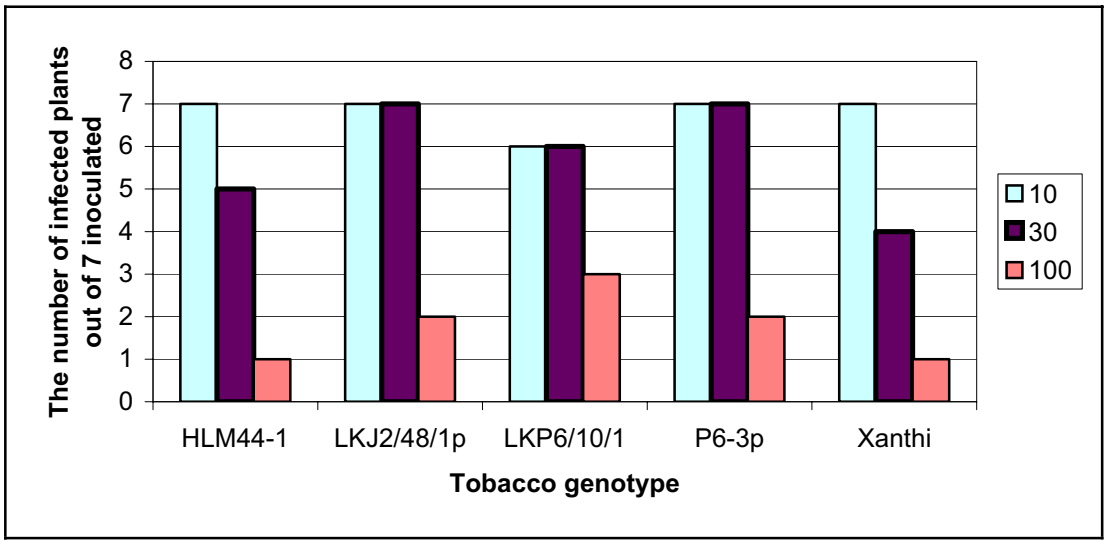

Figure 6-37: Number of plants showing disease symptoms at different PVY dilutions on the 9th day post inoculation

*Figure 6-38: Intensity of PVY symptoms in non-transgenic non-infected control tobaccos (**left**) comparing to transgenic LKP6 (**centre**) and LKJ2 (**right**) on the 22nd day post inoculation*

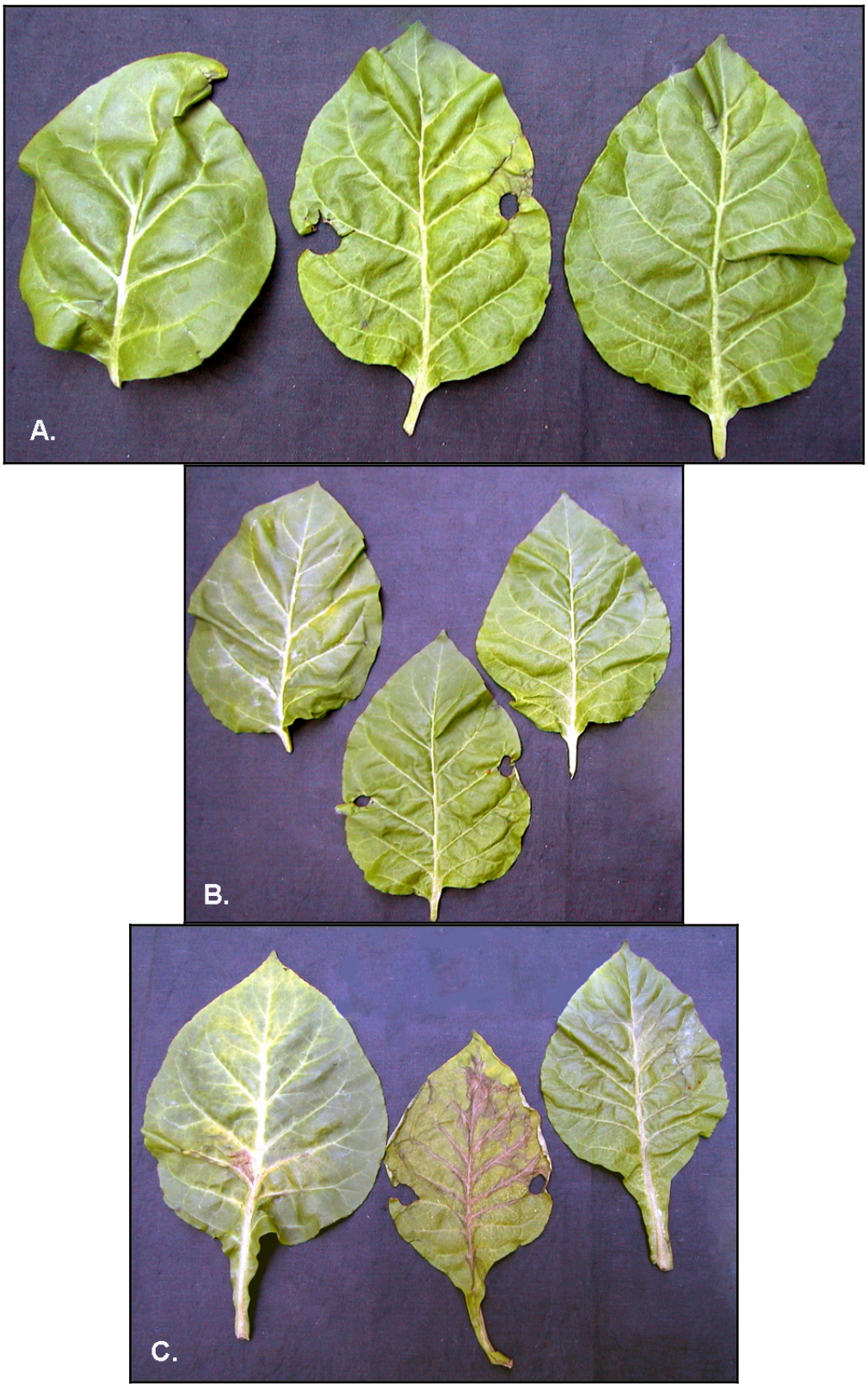

*Figure 6-39: Leaves zero (leaf inoculated with virus), three and five detached from PVY infected tobaccos non-transgenic control Xanthi (**A.**), transgenic LKP6 (**B.**) and LKJ2/48/1 (**C.**) on 23d day post inoculation. The third leaf is punctured due to collected tissue samples*

Virus stock in undiluted or 2 – 16 times diluted sap caused damage in all plants irrespective to genotype. At higher dilutions of PVY extract (30 or 100 times) there was also no significant difference in resistance among the plants (Figure 6-37) or any delay

in symptom development. This fact indicated that none of the differently localized scFvs could prevent PVY replication.

However, it was surprising to find that plants accumulating the ER targeted scFv J2 were more susceptible to symptom development than the control or the other transgenic plants. Leaves with viral symptoms towards the plant top dried out and hanged much faster than those in other genotypes (Figure 6-39). Usually these were the leaves where symptoms appeared the earliest and the strongest, leaf zero (inoculation leaf) and the third and fifth leaves. Data of this finding are presented in Figure 6-40.

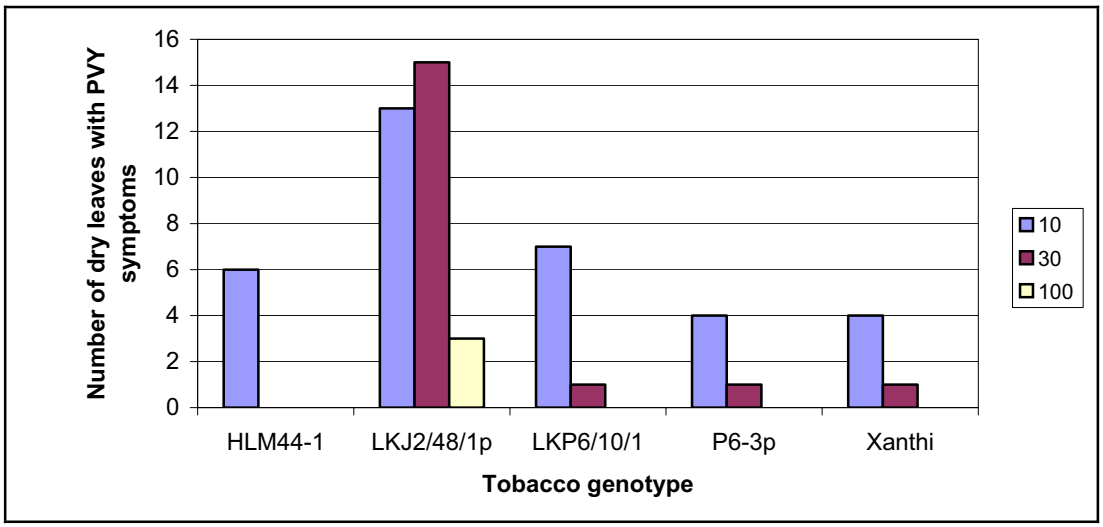

Figure 6-40: The total number of dry hanging infected leaves at different PVY dilutions on the 21st day post inoculation

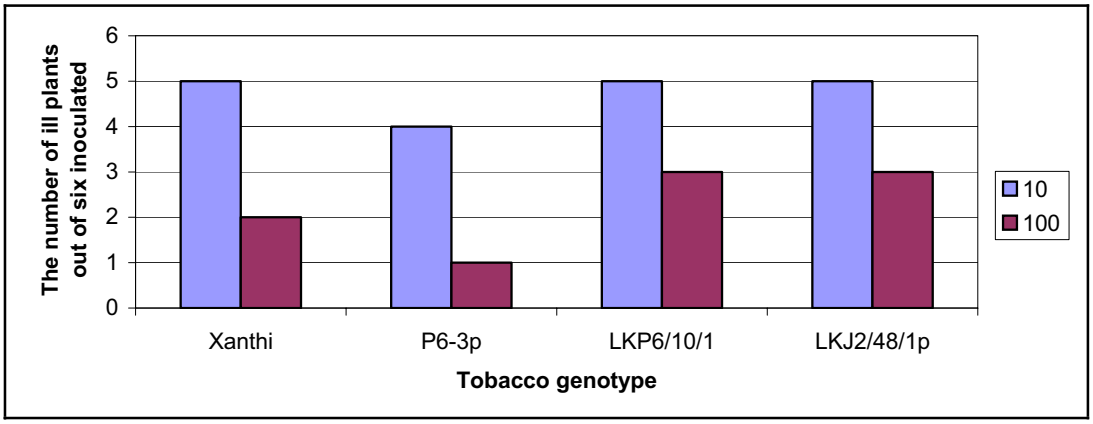

Figure 6-41: The total number of tobaccos with symptoms at different CMV dilutions on the 21 day post inoculation

To infect tobaccos with *Cucumber Mosaic Virus*, a frozen leaf of infected plant was homogenized under liquid nitrogen in water. After clearing by centrifugation, virus suspension was diluted in water 10 or 100 times. 0.2 ml of virus suspension with abrasive celite was applied to three young fully differentiated leaves. The intensity of CMV symptoms was different on individual plants, and symptoms appeared later comparing to PVY infected tobaccos. On the twenty first-day post inoculation mostly apical young leaves had light green mosaic spots or even areas reminding chlorophyll mutations. Sometimes there were spotty nets or leaf curling.

At 10 times diluted inoculum scFv P6 genotype was slightly less damaged then the others. In case of 100 times diluted inoculum again scFv P6 was slightly less infected while LKJ2 and LKP6 slightly more infected (Figure 6-41). Nevertheless, differences were not so much remarkable and most probably there was no resistance or tolerance in transgenic tobaccos to CMV. Furthermore, there was no such clear hypersensitivity to CMV infection like in case of PVY or the experiment was terminated to early to clearly distinguish it.

7. Discussion

7.1. Construction of active dsRNA-specific scFvs

Double-stranded RNA-specific scFvs were constructed in *E. coli*. scFv K1, J2 and P6 retained antigen-binding activity when expressed in the *E. coli* periplasma using the phage display technique. Since stability of heterologously expressed antibodies depends on their intrinsic properties and cannot be predicted, we decided to transform plants with scFv-fragments derived from two antibodies, J2 and P6.

The general layout of the different construct is shown in Figure 6-24. All constructs contain at or near to their C-terminus a c-myc tag for immunological detection of the expressed protein. Constructs A and B were intended for expression in the cytoplasm, therefore they do not contain N-terminal signal sequences but may contain an added KDEL-sequence at the C-terminus (construct B). It is well known that in the reducing environment of the cytoplasm conditions are very unfavorable for the formation of intradomain disulfide bridges, which, however, are necessary for correct folding and activity of scFv. Misfolded antibodies are inherently unstable and prone to degradation. It has been shown in the literature that the C-terminal KDEL sequence may increase the expression level of single-chain antibodies (Schouten et al., 1996). This was the reason for making construct B which, if the findings cited above are generally applicable, should give higher scFv levels in the cytoplasm than construct A.

Constructs C and D both contained the sequence of a mouse leader peptide, which directs scFv to the ER and thereafter for secretion into the apoplast. The difference between C and D is that because of the presence of the ER-retention signal KDEL in construct D the scFv from this construct escape secretion into the apoplast and become ER-resident. To attach antibody fragments to the cytoplasmic site of plasma membrane (construct E) we made use of the naturally occurring mechanism for the posttranslational modification of proteins with lipids (Dunphy et al., 1998). Two types of fatty acylated sequences were appended to the amino terminus of green fluorescent protein (GFP) and of scFv J2. The unmodified signal sequence from fyn kinase may lead to myristoylation and palmitoylation. The experimentally enhanced signal of src kinase encodes an additional polybasic sequence for electrostatic interaction to achieve stronger attachment to the membrane. The expected localization of the heterologous proteins was on the plasma membrane as well as on intracellular membranes (McCabe et al., 1999).

The different constructs were cloned into a plant expression vector and were then used for *Agrobacterium*-mediated transformation of *N. tabacum* cv. Xanthi leaf discs or of BY-2 cells.

7.2. Folding advantages of scFvs compared to intact antibody

Transgenic tobacco plants expressed the dsRNA-specific monoclonal antibody IgG J2 at a very high level, if antibody chains were directed to the endoplasmic reticulum by the original murine signal peptides (Lukacs et al., 1994). On the other hand the expression of antibody chains without a signal peptide was very low in transgenic tobacco and the ratio of H:L chains also differed from the 1:1 ratio of correctly assembled antibody. The results indicated that the antibody may spontaneously assemble in the cytoplasm of plants (see ELISA results in Figure 6-5 - Figure 6-6 on page 42) but if so the yield is very low and unlikely to cause any noticeable physiological effect on the plant. Ultimately the antibody polypeptides must have been removed from the cells, probably by some degradative process.

We reasoned that if the need for correct assembly was eliminated this would result in an increase in the intracellular antibody concentration. To achieve this goal we considered expressing antibodies in an altered form. Although the highly evolved IgG molecule has an optimized structure with respect to protein stability, affinity and avidity, it needs to adopt the quaternary structure for correct functioning.

It is known that the constant domains of both IgG chains are not crucial for antigen recognition and can therefore be dispensed with. There is a continually increasing number of reports in the literature that bare witness to the suitability of single-chain antibody fragments for providing an optimized minimal functional form of mAbs. Although scFvs possess only one antigen binding site and are sometimes unstable the probability that a single peptide will be able to fold correctly are higher. In addition the fragment is some five times smaller than the IgG, so that simply because of its physical properties it would be expected to diffuse faster and reach higher equilibrium concentration values. We therefore decided to make the expression of dsRNA-specific single-chain Fv fragments in the cytoplasm of tobacco our goal. The scFv constructions were made from several mAbs to increase the chances of obtaining significant results in expression studies.

7.3. Targeting and expression of scFv in *N. tabacum*

scFv expression in the cytoplasm differs greatly between P6 and J2 derived constructs

Transgenic plants were regenerated from transformations with constructs targeting scFv to the cytoplasm. scFv-mRNA reached high steady-state concentration in transformed plants in the case of both antibodies. However, scFv protein was only detected when A- or B-type constructs of the P6 antibody were used for transformation. We did not observe any difference between the expression levels of the unmodified scFv and the C-terminal KDEL containing scFv derived from P6. The results may mean that as a result of its intrinsic features P6-scFv can adopt a stable structure even under the reducing conditions in the cytoplasm, while for correct folding of J2-scFv passage through the ER, (i.e. strongly oxidizing conditions) is essential.

Both scFvs accumulate at high concentration in endoplasmic reticulum

scFv J2 as well as scFv P6 were targeted to the endoplasmic reticulum (ER) with an N-terminal heterologous mouse leader peptide and were made ER-resident by adding the C-terminal ER-retention signal KDEL. Very high levels of protein expression were observed for both scFvs. Although we have not yet proved the localization of the scFv in the ER by immunohistochemical analysis, indirect evidence is consistent with their localization in this compartment: We found that the specific activity of the ER-resident scFv P6 is several times higher than that of the scFv expressed in the cytoplasm (Morgun et al*.,* 2005).

scFv J2 expressed in endoplasmic reticulum binds double-stranded RNA

scFv J2 expressed in the endoplasmic reticulum was tested by AN-ELISA (Schönborn et al*.,* 1991) for its ability to bind double-stranded RNA. scFv J2 produced in the ER of tobacco plants (LKJ2, lines 48/1.3 and 48/1.4) turned out to be as active as the scFv expressed in the periplasma of recombinant *E. coli* clones (J2, lines 104.27 and 104.28).

scFv protein was not detected in the apoplast or on the plasma membrane

11 transgenic plants transcribing J2-scFv mRNAs were screened by Western blotting for protein expression, but no scFv protein was found. In earlier experiments high expression levels of intact J2-IgG had been obtained in the apoplast (Lukács et al*.,* 1994). Thus, J2-scFv seems to be much less stable in the apoplast than the original IgG.

P6-scFv was not detected in the intercellular space either. Our observations well agree with the literature (Figure 7-1).

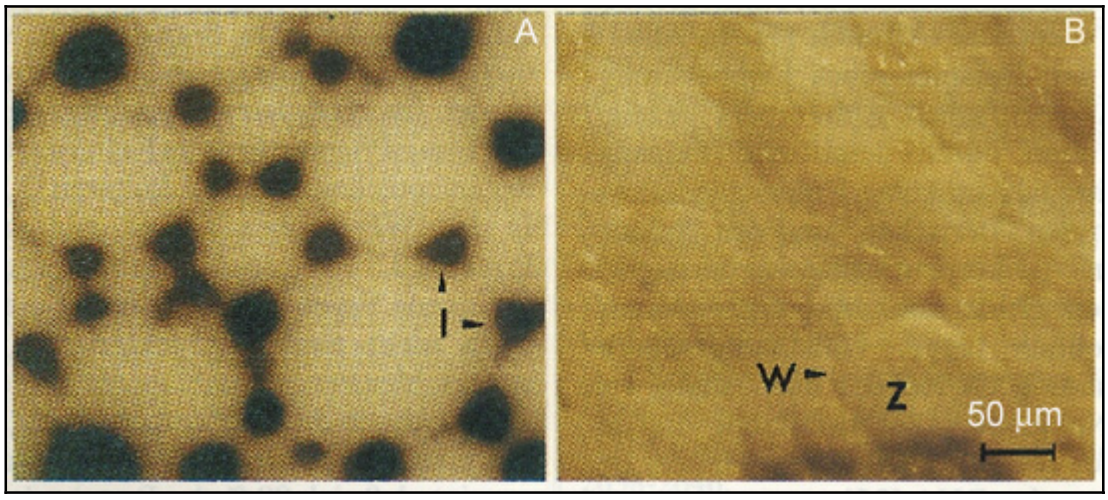

*Figure 7-1: Detection of plantibody IgG J2 in transgenic HLM tobacco. The Intercellular space is filled with plantibody (arrow I) in the investigated stem sample (**A.**) comparing to the non-transgenic control (arrow W) seen on image **B**. The detection was done by tissue immunoprinting. The "Z" sign marks a cell (Lukacs et al., 1994)*

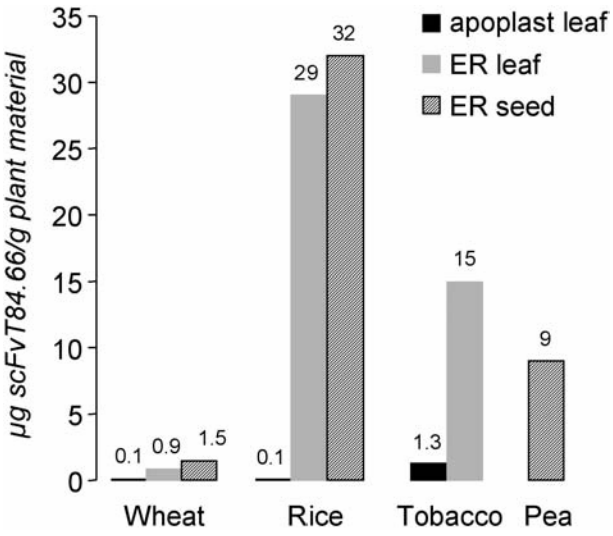

Figure 7-2: Across all the species tested, accumulation of the scFv in the endoplasmic reticulum by means of the KDEL retrieval signal significantly improved protein levels compared to antibodies secreted into the apoplast (Stoger et al., 2002b; Schillberg et al., 2003)

To target scFv to the cytoplasmic side of the plasma membrane by using anchoring signals E-type constructs were used. Here again we observed that high mRNA-levels did not lead to detectable protein expression. The targeting signals themselves were acting as expected in BY-2 cells, because by using the same sequences GFP fluorescence was confined to the plasma membrane (Deshmukh, personal communication). The signals changed the *in situ* localization of Green Fluorescent Protein in *N. tabacum* and *A. thaliana* plants too. While GFP localized in the cytoplasm and/or in the nucleus (A-type construct) was readily extracted by aqueous buffers, GFP

targeted to the plasma membrane (E-type construct) was only detectable when detergents were included in the extraction buffer.

The results of the expression studies are summarized in Table 6-10. In accordance with the literature our data show that the highest antibody levels can be obtained when scFvs are targeted to the ER and are made ER-resident. Although scFv-expression has been successfully achieved in the apoplast by other investigators, our scFv was not expressed in this compartment at detectable levels. Only P6-scFv was stable in the cytoplasm regardless of the presence or absence of KDEL protection signal. This indicates that both intrinsic properties and the primary sequence of heterologously expressed scFv are important for its stability. Since scFv P6 seems to provide a cytoplasmically stable scaffold, in future it could be used as a framework on which to graft and express new antigen binding sites from other specific antibodies (”CDR grafting”).

The experiment with targeting to the plasma membrane using fatty acylation signals showed that correct folding and cytoplasmic stability of an expressed protein appears to be the most important factor governing its fate in the cell irrespective of any targeting signal it may carry.

7.4. Physiological effects on virus infection

The expression studies were carried out to find out whether the expression of dsRNA-specific scFv in any of the cellular compartments might be at a sufficiently high level to influence replication of single-stranded RNA (ssRNA) viruses and/or dsRNA-dependent regulatory processes. From a practical point of view the most desirable effect that dsRNA-specific antibodies might have would be the inhibition of virus replication. During replication of ssRNA viruses double stranded regions consisting of the template and the nascent complementary strand are formed. In earlier experiments it was shown that the dsRNA-specific J2 antibody inhibits the *in vitro* replication of alfalfa mosaic virus on partially double-stranded template (de Graaff et al., 1995). We presume that the inhibition is a consequence of antibody binding, whereby the IgG stabilizes the replication intermediate and as a result inhibits replication. If this activity could be achieved *in vivo* at the site of virus replication then a broad resistance against ssRNA viruses might be obtained by expressing dsRNA-specific antibodies in plants.

However, the inhibition of virus replication is not the only effect that dsRNA-specific antibodies might have, because they recognize and bind to all long dsRNA species irrespective of their sequence and origin. Although long dsRNAs usually do not occur in plants, they play an important role in dsRNA-mediated gene silencing, which is one of the protective immune responses used by plants to defend themselves against viruses.

To analyze the effect of RNA-specific antibodies we infected transgenic plants with potato virus Y (PVY). PVY is a relatively slow virus, which induces well defined symptoms and which is known to be subject of dsRNA-mediated gene silencing (Metzlaff, 2002). Although we could not detect scFv-protein in all types of transformants, we inoculated all of them at three different virus concentrations. The rationale behind this decision was that in several cases in the literature the biological effect of a transgene was observed even when the protein concentration was below the level of detection. In our system, however, we only saw one significant difference between the transgenic lines. The non-infected plants developed normally and did not show any symptoms. After infection with PVY all transgenic lines behaved like the control, except the line expressing ER-resident J2-scFv. The symptoms in this line were seen earlier than in the others, but the most obvious difference to the other transgenic lines was that beginning with the third leaf above the infected leaf very severe necrosis was observed. Not only did the veins become so necrotic that they eventually

completely dried out, but 1-3 leaves also became limp, wilted and then died (Figure 6-38 - Figure 6-39). Such symptoms occasionally occurred in other, control or transgenic lines, but they appeared later and were less severe. The uppermost leaves of all transformants recovered and showed much weaker symptoms.

To evaluate the data statistically we infected 5 or 7 plants in each group at 10-, 30- and 100-fold dilutions of the PVY stock and counted the hanging dry leaves 21 days p.i. The experiment was repeated twice. The results of one experiment are shown in Figure 6-40. The ER-resident J2-scFv clearly led to more dramatic symptoms and to more serious damage than in the other lines. ER-resident P6 did not induce this kind of pathogenesis. It should be emphasized that although P6 binds to dsRNA, it was originally produced against viroid RNA and reacts with ssRNA as well. J2 is strictly specific for long, completely base-paired dsRNA species.

The observed effect indicates that the ER-resident J2-scFv influences symptom development. The effect could only be observed when the scFv was targeted to the ER lumen, because J2 IgG expressed in the apoplast did not give rise to such necrotic symptoms. The most probable explanation of the results is that J2-scFv confined to the ER is unable to protect the plant against PVY multiplication. It may, however, influence the dsRNA-mediated silencing of virus replication by protecting viral dsRNA against fragmentation by the dicer enzyme. It is still difficult to understand how an ER-resident protein may influence a process thought to take place in the cytoplasm. Potyviruses are known to replicate in association with large vesicular structures derived from endoplasmic reticulum. For tobacco etch virus (TEV) it has been shown that on infection with TEV the ER-network collapses into aggregated structures and this process probably begins with the binding of the 6 kDa virus protein to the ER-membrane (Hull, 2002). We believe that when PVY is replicating in association with the ER, the chances are relatively high that the highly concentrated scFv comes in contact with the replication complex. It is also possible that during the fragmentation of the ER, scFv is being set free and may act also outside the ER. Whichever explanation is correct; our results demonstrate that not only hapten- or protein-specific antibodies but also nucleic acid specific scFv are able to efficiently influence *in vivo* equilibria.

8. Conclusions

1. The assembly and activity of intact IgG J2 H- and L-chains expressed in the cytoplasm of *N. tabacum* cv. Xanthi was investigated. We found that although some association between H- and L-chains could be detected in ELISA by using γ- or κ-specific antibodies, after ammonium sulphate precipitation and affinity chromatography on Protein A only the heavy chains could be seen by gel electrophoresis and silver staining. We estimate that the H:L ratio of cytoplasmically expressed J2 in *N. tabacum* was at most 10:1 in contrast to the 1:1 ratio of hybridoma-produced antibodies. Since a similar difference was not observed in the ratio of H- and L-chain mRNAs we conclude, that the spontaneous assembly of the two chains is very inefficient and that the isolated H-chains are probably more stable in the cytoplasm than the L-chains. Virus challenge of transgenic plants with CMV or PVY viruses did not reveal any influence of J2 gene expression on virus replication or symptom development.

2. Four single-chain Fv fragments specific to dsRNA were constructed from hybridoma-derived cDNAs using the phage display technique. scFvs J2 and K1 originated from cloned IgG cDNAs, while scFv P6 and K2 from the polyA$^+$ mRNA-pool of hybridoma cell lines. All four scFvs were successfully expressed in *E. coli* and their RNA-binding activity was demonstrated by AN-ELISA. Some scFv-preparations, especially K1- and K2-scFv showed large variation in the extent of antigen binding, which could not be eliminated by rigorous standardization of cultivation, extraction and ELISA conditions. We suggest that this variability is due to the inherent instability of the structure of bacterially expressed scFvs, which may be inactivated by immobilization on ELISA plates. In the case of scFv P6 a change in fine specificity was observed: the rRNA-binding activity remained high, but dsRNA-binding was much lower than that of the original IgG P6. This change may also be caused by subtle structural changes at the antigen binding site of bacterially expressed scFv P6, because ER-resident scFvs expressed in tobacco did not show this effect. Clones J2.104, K1.21 and P6.84, which showed the highest activities and the lowest number of mutations in the given genotype, were selected for expression in tobacco.

3. Five types of gene constructs were designed to target scFv to the cytosol, to the endoplasmic reticulum or to the apoplast. Murine N-terminal leader peptide, C-terminal KDEL ER-retention signal and fatty acylation signals derived from Fyn and Src kinase were fused in different combinations to scFv or GFP sequences (Figure 6-

24). The genes were cloned into the co-integrative plant expression vector pGEJAE1 and transgenic *N. tabacum* cv. Xanthi plants were generated by *Agrobacterium*-mediated transformation of leaf discs.

4. The expression of scFv J2 and P6 in transgenic tobacco and its physiological effect on the plant were examined. As shown by Western blotting scFv P6 was stably expressed in the cytoplasm, while none of the two constructs led to any detectable accumulation of scFv J2 in the cytoplasmic compartment. Because of the stable expression of P6 in the cytoplasm, the framework of this antibody may be used in future experiments to express scFvs with different specificities in the cytoplasm by CDR-grafting. The highest levels of both scFvs were observed when the proteins were made resident in the ER. In contrast to the intact J2 IgG molecule neither J2- nor P6-scFvs were detected in the apoplast. Modification of GFP by the fatty acylation signal of Fyn kinase resulted in association of GFP in cellular membranes, but the same modification did not led to detectable accumulation of scFv J2 in the membrane fraction.

5. The expression of dsRNA-specific scFvs does not cause any agriculturally relevant tolerance or resistance to PVY infection in transgenic tobacco. However, it was observed that in plants expressing ER-resident scFv J2 the virus symptoms appeared somewhat earlier and more leaves developed necrotic lesions and died than in any other genotypes. The enhanced necrotic symptoms may indicate the interference of dsRNA-specific scFv J2 with the RNA silencing pathway. Recent results in our laboratory show that 14-20 % of non-infected transgenic plants usually develop characteristic morphological changes that may also be the result of an interaction with natural silencing mechanisms.

9. Resume

Antibodies have attracted researchers' attention for many years and much data on antibody sequences, folding, affinities, and transgenic expression have been obtained. However, the problem of how to express active antibody molecules in various subcellular compartments of heterologous systems has not yet been solved. The most critical location for antibody expression remains the cytoplasm, the replication site of many plant RNA-viruses.

A number of mouse monoclonal antibodies, named J2, K1, K2, P6, were produced by Noémi Lukács's group in earlier work (Schönborn et al., 1991). Antibodies J2, K1 and K2 specifically recognize structural features of double-stranded RNA (dsRNA) independent of the nucleotide sequence of the antigen, and their binding to single-stranded RNA, double-stranded DNA or RNA-DNA hybrids is below detection levels. Although the P6 antibody also reacts with dsRNAs independently of their sequence, it also interacts with structured single-stranded RNAs such as viroid- or ribosomal RNA as well. Double-stranded RNAs are known to play an important role in several basic biological processes, among other things in replication of ssRNA-viruses and in dsRNA-based gene silencing. Since more than 90 % of all plant viruses are RNA-viruses, dsRNA, which necessarily arises during replication, offers a potential target through which multiplication of most plant viruses might be influenced. Viral dsRNAs are also the targets of dsRNA-mediated gene silencing, which helps to protect plants against virus multiplication. We hypothesized, that when expressed in the right intercellular compartment in transgenic plants, dsRNA-specific antibodies may interfere with the replication process and/or with the dsRNA-mediated silencing mechanism of the host. It was shown in earlier experiments that the antibody J2 indeed prevents replication on partially double-stranded alfalfa mosaic virus template *in vitro* (de Graaff et al., 1995).

Considering the scientific and possible practical potential of dsRNA-specific antibodies to interfere with dsRNA-mediated viral and/or host processes within the plant cell, we set the following aims for the current doctoral studies:

(i) to establish strategies for the expression of correctly folded dsRNA-specific antibodies or antibody fragments inside the cytoplasm as well as in other cellular compartments in *N. tabacum*;

(ii) to identify antibody frameworks that can be stably expressed in the cytoplasm;

(iii) to construct single-chain antibody Fv fragments from full-length monoclonal antibodies using the phage display technique;

(iv) to investigate the physiological effects of dsRNA-specific antibodies and antibody fragments on the transgenic plants in which they are expressed.

First we investigated the stability and the assembly of intact J2 (IgG2a) antibodies expressed in the cytosol. We found that while the H-chain of J2 could be easily detected by ELISA, the L-chain concentration remained at background levels. The H:L ratio was estimated to be ≤10:1, in contrast to the 1:1 ratio of hybridoma-produced J2. After affinity purification on Protein A the eluted fraction weakly bound dsRNA. Infection of transgenic tobaccos with CMV or PVY viruses did not reveal any significant protection or tolerance against these viruses.

The low degree of assembly prompted us to express single-chain antibody fragments, which may fold more efficiently and which do not need the assembly of independent polypeptide chains to form the antigen binding site. Using the phage display technique

four scFv fragments specific to dsRNA were constructed and expressed in *E. coli*. scFvs J2 and K1 originated from cloned IgG cDNAs, while scFv P6 and K2 were directly amplified from mRNA purified from the corresponding hybridoma lines. All four scFvs were stably expressed in *E. coli* periplasma. Their antigen binding activity was demonstrated by ELISA: scFvs J2, K1 and K2 bound dsRNA, whilst scFv P6 preferentially bound rRNA. We found that the ELISA-activity of some scFvs varied widely from batch to batch, possibly because of the instability of the scFv proteins.

The most active clones, namely J2.104, K1.21 and P6.84, were selected for expression in higher plants. Expression studies were completed for J2 and P6 scFVs, which possess highly homologous VH domains and dissimilar VL domains. For both scFv J2 and P6 five types of constructs were made to express them in the cytosol (2 constructs), inside the endoplasmic reticulum, in the apoplast or to anchor them at the cytoplasmic side of the plasma membrane. The constructs were transferred from *E. coli* into the co-integrative plant expression vector pGEJAE1 and transgenic *Nicotiana tabacum* L. cv. Xanthi plants were generated by *Agrobacterium tumefaciens*-mediated transformation of leaf disks.

mRNA-expression was observed in all types of transformants. Investigating the expression of scFv proteins in the cytoplasm we found, that scFv P6 was stably expressed, but scFv J2 could not be detected in any of the transformants. To increase the stability of antibody fragments in the cytoplasm, and to protect them against proteolytic degradation we fused the ER retention signal KDEL to the C-terminus, because this sequence was shown to stabilize some scFvs in the cytoplasm (Figure 6-24 B). Close proximity to the plasma membrane has also been thought to stabilize antibody fragments and to elevate their concentration at the site of virus replication. To anchor scFv to the plasma membrane and possibly to other intracellular membranes we used the N-terminally linked fatty acylation signal of the Fyn kinase (Figure 6-24 E). Neither of these strategies resulted in the accumulation of detectable amounts of scFv J2. Nonetheless, the stably expressed P6 might provide the antibody framework in which antigen binding CDR-loops of other antibodies could be grafted for cytoplasmic expression in future experiments. In a control experiment using Green Fluorescent Protein (GFP) to evaluate the performance of Fyn signal, which is responsible for polypeptide myristoylation and palmitoylation and such for membrane targeting, membrane association of the reporter protein in tobacco plants could be clearly detected.

The oxidative environment inside the endoplasmic reticulum is optimal for formation and accumulation of correctly folded scFvs. Therefore we directed J2 and P6 scFvs into the ER lumen with the murine N-terminal IgG J2 leader peptide and made them ER-resident by adding the C-terminal KDEL retention signal. For both antibodies, the highest expression levels and the highest dsRNA-binding activity was achieved by using this strategy. When, however, the ER-retention signal was removed to achive scFv-secretion into the apoplast, none of the scFvs could be detected by Western-blotting. This finding indicates that although the scFvs seem to fold correctly in the apoplast, they are probably unstable in the intercellular space and quickly become degraded

To analyze the effect of RNA-specific antibodies on virus replication we infected transgenic plants with potato virus Y (PVY). PVY is a relatively slow virus, which induces well-defined symptoms and which is known to be subject of dsRNA-mediated gene silencing. Although we could not detect scFv-protein in all types of transformants, we inoculated all of them at three different virus concentrations. The rationale behind this decision was that in several cases in the literature the biological effect of a

transgene has been observed even when the protein concentration was below detection level. No significant tolerance or resistance to PVY was found among the transgenic tobacco plants. However, one significant difference between transgenic lines was observed: After infection with PVY all transgenic lines behaved like the control, except the line expressing ER-resident J2-scFv. Not only were pathogenic symptoms in this line seen earlier than in the others, but there was also an obvious increase in the severity of the disease caused by the virus. Beginning with the third leaf above the infected leaf very severe necrosis was observed and some leaves wilted and died. Such symptoms occasionally occurred in other, control or transgenic lines, but they appeared later and were less severe. ER-resident P6 did not induce this kind of pathogenesis. It should be emphasized that although P6 binds to dsRNA, it was originally produced against viroid RNA and reacts with ssRNA as well. J2 is strictly specific for long, completely base-paired dsRNA species. The effect could only be observed when the scFv was targeted to the ER lumen, because J2 IgG expressed in the apoplast did not give rise to such necrotic symptoms.

The most plausible conclusion to be drawn from these results is that J2-scFv confined to the ER cannot protect the plant against PVY multiplication but may influence the dsRNA-mediated silencing of virus replication by protecting viral dsRNA against fragmentation by the dicer enzyme. It is still difficult to understand how an ER-resident protein may influence a process thought to take place in the cytoplasm. Potyviruses are known to replicate in association with large vesicular structures derived from endoplasmic reticulum. For tobacco etch virus (TEV) it was shown that on infection with TEV the ER-network collapses into aggregated structures and this process probably begins with the binding of the 6 kDa virus protein to the ER-membrane (Hull, 2002). We believe that when PVY is replicating in association with the ER, the chances are relatively good that the highly concentrated scFv comes in contact with the replication complex. It is also possible that during the fragmentation of the ER, scFv is being set free and may act also outside the ER. Whichever explanation is correct; our results demonstrate that not only hapten- or protein-specific antibodies but also nucleic acid specific scFv may efficiently influence *in vivo* equilibria.

Thus, the strategies we developed for expression of correctly assembled antibodies or antibody fragments inside the cytoplasm of higher plants have been successfully executed. Single-chain antibody fragments were developed in *E. coli* and then expressed as plantibodies. The accumulation of scFvs was observed in the cytoplasm and in the ER. In addition, the physiological effects of the ER-localized expression of two scFv fragments as well as of the dsRNA-specific IgG J2 were investigated in *N. tabacum in vivo* upon PVY infection and in the case of J2-scFv a clear effect on viral pathogenesis was found.

10. Összefoglalás

Biológiai jelentőségük és sokoldalú alkalmazhatóságuk okán az ellenanyagok már hosszú ideje intenzív tudományos vizsgálatok tárgyát képezik, így napjainkig gazdag ismeretanyag gyűlt össze szekvenciájukról, szerkezetükről, affinitásukról és transzgénikus kifejeződésükről. Mindmáig nem létezik azonban egy általánosan alkalmazható stratégia, melynek követésével a növényi illetve állati szervezetek tetszőleges sejtalkotóiban aktív antitesteket lehetne kifejezni. Biológiai szempontból különösen érdekes, ellenanyag expresszió szempontjából viszont különösen problematikus a citoplazmában történő kifejezés.

Lukács Noémi csoportjában végzett korábbi kutatások során számos RNS-specifikus egér monoklonális antitestet állítottak elő, melyek közül a benyújtott disszertáció keretében négy ellenanyag, a J2, K1, K2 és P6 génsebészetével ill. növényi expressziójával foglalkoztam. A J2, K1 és K2 antitestek szigorúan duplaszálú RNS-(dsRNS)-specifikusak, azaz a dsRNS-ekhez azok nukleotid-összetételétől és szekvenciájától függetlenül kötődnek, de nem reagálnak DNS-sel, RNS-DNS hibridekkel és egyszálú RNS-ekkel (Schönborn et al., 1991). A P6 antitest az előzőektől abban különbözik, hogy nemcsak dsRNS-ekhez, hanem viroid-, riboszómális- és más ssRNS-ekhez is kötődik. Köztudott, hogy a dsRNS-ek számos alapvető biológiai folyamatban fontos szerepet játszanak, így az egyszálú-RNS vírusok replikációjában ill. a dsRNS-közvetített géncsendesítés folyamatában is. Mivel a növényi vírusok több mint 90 %-a RNS-vírus, a vírusreplikáció során szükségképp keletkező dsRNS-ek olyan potenciális célmolekuláknak tekinthetők, amelyeken keresztül a legtöbb növényi vírus multiplikációja befolyásolható. A növények antivirális védekezési mechanizmusában fontos szerepet játszó géncsendesítés is ezen célszerkezeten keresztül történik. Feltételeztük, hogy ha sikerül aktív dsRNS-specifikus ellenanyagokat kifejeznünk a megfelelő sejtkompartimentumban, akkor az antitestek befolyásolhatják a vírusreplikációt és/vagy a gazdanövény géncsendesítési reakcióit. Csoportunk korábbi kísérleteiben kimutatták, hogy a lucerna mozaikvírus *in vitro* replikációs rendszerben a J2 ellenanyag gátolja a parciálisan duplaszálú RNS-templáton történő replikációt (de Graaf et al., 1995).

Mivel a dsRNS-specifikus ellenanyagok potenciálisan minden dsRNS-re alapuló virális vagy növényi folyamatot befolyásolhatnak, növényi kifejezésük mind tudományos, mind gyakorlati szempontból ígéretesnek tűnik. Ezen megfontolásokból a jelen doktori munka keretében az alábbi célokat tűztük ki:

(i) Olyan expressziós stratégiák kidolgozása, amelyek segítségével dohányban (*N. tabacum* cv. Xanthi) mind a citoplazmában, mind más sejtalkotókban célzottan natív szerkezetű dsRNS-specifikus antitesteket és antitest fragmentumokat lehet kifejezni;

(ii) Olyan ellenanyag vázszerkezet azonosítása, amely a növényi citoszolban stabil kifejeződést biztosít;

(iii) Egyszálú Fv (scFv) antitest-fragmentumok előállítása monoklonális ellenanyagokból a "fág-display" módszer segítségével;

(iv) dsRNS-specifikus IgG és scFv-k fiziológiai hatásának vizsgálata transzgenikus dohány növényekben.

Először a citoszolban kifejezett intakt J2 (IgG2a) cDNS-ek fehérjetermékeinek stabilitását és az alegységek összerendeződését vizsgáltuk korábban előállított transzgenikus növényekben. Megállapítottuk, hogy míg a citoplazmában termelődött nehézlánc (H) könnyen kimutatható ELISÁ-val, addig a könnyűlánc (L) koncentrációja alig éri el a kimutatási határt. A láncok igen eltérő stabilitását mutatja, hogy a vizsgált növényekben a H:L arány nem vagy alig éri el a 10:1 értéket, míg a hibridóma sejtekben előállított J2 antitestekben az antitestekre jellemző 1:1 arány figyelhető meg. Protein A affinitáskromatográfiával tisztított frakciókban kisfokú dsRNS-kötést mutattunk ki, de CMV és PVY vírussal fertőzött transzgénikus dohányokban *in vivo* nem alakult ki sem védettség, sem tolerancia.

Ezek az eredmények arra késztettek bennünket, hogy egyszerűbb szerkezetű egyszálú antitest fragmentumokat (scFv) fejeztessünk ki a növényben. A fág-display technika felhasználásával négy dsRNS-specifikus scFv fragmentet állítottunk elő és expresszáltattunk *E. coli* periplazmában. A J2- és K1-scFv-ket klónozott IgG cDNS-ből, míg a P6- és K2-scFv-ket a megfelelő hibridóma vonalak polyA$^+$ mRNS-éből kiindulva szintetizáltuk. Mind a négy scFv protein stabilan expresszálódott. Az scFv-k aktivitását ELISA tesztekkel mutattuk ki: A J2-, K1- és K2-scFv-k specifikus dsRNS-kötést mutattak, a P6-scFv azonban preferenciálisan a riboszomális RNS-hez kötődött. Ugyanazon klónból származó különböző izolátumokat összehasonlítva nagy eltéréseket figyeltünk meg a fehérjék aktivitásában, aminek okát feltételezésünk szerint az scFv-szerkezet instabilitásában kell keresni.

További kísérleteinkben a legaktívabbnak talált klónokat (J2.104, K1.21, P6.84) használtuk fel. A növényi expressziót a J2- és P6-scFv-knél analizáltuk teljes körűen. E két scFv V_H doménje csak néhány aminosavban tér el egymástól, a V_L domének azonban különbözők. Mindkét scFv-vel öt konstrukciót készítettünk el, hogy a citoszolba (két konstrukció), az endoplazmatikus retikulum (ER) belsejébe vagy az apoplasztba irányítsuk a fehérjéket, ill. hogy lehorgonyozzuk őket a plazmamembrán citoplazma felőli oldalán. A konstrukciókat *E. coli*-ban állítottuk elő, majd a kointegratív pGEJAE vektor és *Agrobacterium tumefaciens* felhasználásával *Nicotiana tabacum* cv. Xanthi levélkorongokat transzformálva transzgenikus növényeket állítottunk elő.

Mind a 10-féle transzformánsban sikerrel kimutattuk a megfelelő scFv-t kódoló mRNS-ek szintézisét. A citoszolban a P6-scFv viszonylag magas koncentrációban expresszálódott, de a J2-scFv-t egyetlen transzformánsban sem tudtuk detektálni. Hogy az scFv-k stabilitását a citoplazmában fokozzuk, és védjük őket a fehérjebontó folyamatoktól, a fehérje C-terminális végét a KDEL ER-retenciós szignál hozzáadásával módosítottuk. Ez a szekvencia irodalmi adatok szerint egyes növényben expresszált scFv-knél stabilizáló hatást mutat (Schouten et al., 1996), kísérleteink során azonban egyik scFv-nél sem figyeltünk meg változást az expresszált fehérje mennyiségében. Azt a lehetőséget is megvizsgáltuk, hogy a J2-scFv-ket a Fyn kináz zsírsav-acilálási szignáljával N-terminálisan módosítva a plazmamemránhoz (és más intracelluláris membránokhoz) horgonyozva stabilizáljuk, s egyben a vírusreplikáció helyén feldúsítsuk. Ez a megközelítés sem eredményezett kimutatható J2-scFv felhalmozódást. Mindazonáltal kísérleteink a citoszolban történő scFv-kifejezés szempontjából azzal a pozitív eredménnyel jártak, hogy azonosítottuk a stabilan kifejeződő P6 scFv-szekvenciát, amely további kísérletekben fehérjevázként szolgálhat más

ellenanyagokból származó antigénkötő hurkok átültetésére ("CDR-grafting"). Ezt a technológiát állati eredetű ellenanyagok "humanizálásánál" széles körben alkalmazzák. Megemlítendő még, hogy ha a Fyn kináz mirisztilát és palmitilálát horgonyszekvenciák kapcsolódását lehetővé tevő szignálját nem J2-scFv-hez, hanem a "Green Florescent Protein"-hez (GFP) kapcsoltuk, akkor a riporter protein membránokkal való asszociációja kimutatható volt, tehát maga a szignál alkalmas arra, hogy dohányban *in vivo* a fehérje várt modifikációjához és redisztribúciójához vezessen.

Az endoplazmatikus retikulumban uralkodó oxidatív miliő kedvez a diszulfid-hidak kialakulásának, ezért optimális az scFv-k korrekt összerendeződése és felhalmozódása szempontjából. A J2- és P6-scFv-ket a J2 antitest eredeti egér N-terminális szignálpeptidjének segítségével irányítottuk az ER-be, ahol a fenn leírt C-terminális ER-retenciós szignál (KDEL) hatására váltak rezidenssé. Mindkét scFv-nél ezzel a stratégiával értük el a legmagasabb expressziós szinteket és a legnagyobb fajlagos dsRNS-kötő képességet. Ha a retenciós szignált eltávolítottuk, hogy a fehérje apoplasztba történő szekrécióját lehetővé tegyük, mindkét ellenanyag koncentrációja a kimutathatósági határ alá csökkent. Az eredmény arra utal, hogy bár az scFv-k minden jel szerint natív szerkezetet vesznek fel az ER-ben, az intercellulárisokban nem stabilak és hamar degradálódnak.

A J2- és P6-scFv-k fiziológiai hatását üvegházba kiültetett transzgenikus dohány növényeken vizsgáltuk. A növényeket burgonya Y vírussal (*Potato Virus Y,* PVY) fertőztük. A PVY egy viszonylag lassú vírus, amely jól meghatározott szimptómákat okoz. A vírus multiplikációját a gazdanövény a fertőzés során indukálódó dsRNS-közvetített géncsendesítéssel gátolja. Bár nem minden transzformánsunknál detektáltunk fehérjekifejeződést, minden transzformáns típust inokuláltunk három különböző PVY-koncentrációnál. Az irodalomban ugyanis több példát is találunk arra, hogy a transzgén biológiai hatása olyan esetekben is fellép, amelyekben a fehérjekoncentráció a kimutathatósági határ alatt marad. A transzgenikus dohány genotípusok egyike sem mutatott rezisztenciát vagy toleranciát a PVY vírussal szemben. Egy szignifikáns különbséget azonban megfigyeltünk: A fertőzés a J2-scFv-t az ER-ben kifejező növények kivételével minden genotípusnál a kontrollal azonos szimptómák kialakulásához vezetett. Az ER-rezidens J2-t expresszáló növényeken azonban súlyos nekrotikus szimptómák jelentek meg, amelyek egyes levelek lankadásához, majd pusztulásához vezettek. Hasonló szimptómák felléptek ugyan esetenként a többi genotípusnál is, de mindig sokkal kevésbé súlyosan (lsd. a 6-40. ábrát). Az apoplasztban magas koncentrációban kifejezett intakt J2-IgG nem vezetett a szimptómák megváltozásához, s a súlyos nekrotikus tüneteket az ER-rezidens P6-scFv-t expresszáló növényeknél sem figyeltük meg. Emlékeztetünk arra, hogy míg a J2 antitest dsRNS-specifikus, a P6 antitestek a dsRNS-eken kívül ssRNS-ekhez is nagy affinitással kötődik.

A leírt megfigyelések legkézenfekvőbb magyarázatának az tűnik, hogy bár az ER lumenjében felhalmozódó dsRNS-specifikus scFv-k nem képesek a vírusmultiplikációt meggátolni, a növény dsRNS-függő géncsendesítését befolyásolni tudják pl. a virális dsRNS-ek lefedésével. A dsRNS-hez kötött scFv-k védelmet nyújthatnak a dicer enzimmel történő feldarabolás ellen, azaz gátolhatják a géncsendesítési folyamat megindulását. Felvetődik a kérdés, hogy hogyan kerül az ER lumenjében lokalizált fehérje kapcsolatba a virális dsRNS-ekkel. A potyvírusok az endoplazmatikus

retikulumból származó kiterjedt vezikuláris struktúrákhoz kapcsolódva replikálódnak. A potyvírusokhoz tartozó dohány csíkoltság vírusról (*Tobacco Etch Virus,* TEV) tudjuk, hogy a TEV-fertőzés, valószínűleg a 6 kDa vírusfehérjének az ER-hez történő kötődése következtében, az ER-hálózat kollapszát és aggregált struktúrák kialakulást okozza (Hull, 2002). Feltételezzük, hogy az ER-rel asszociáltan replikálódó PVY esetében fennáll a lehetőség, hogy a virális RNS az ott magas koncentrációban jelen lévő scFv-kkel kapcsolatba kerül, ill. hogy az ER fragmentálódása során scFv-k válnak szabaddá s kötődnek az ER-en kívül előforduló dsRNS-ekhez. Bármelyik magyarázat is bizonyul helyesnek, eredményeink mindenesetre azt mutatják, hogy nemcsak haptén- és fehérje-specifikus antitestek, hanem nukleinsav-specifikus ellenanyagok is képesek biológiai egyensúlyok megváltoztatására *in vivo.*

A benyújtott disszertáció legfontosabb eredményei tehát a következők: Monoklonális ellenanyagokból kiindulva sikerrel expresszáltunk aktív dsRNS-kötő scFv-ket *E. coli* periplazmában. Két eltérő szekvenciájú és specifitású scFv-fragmentumot (J2 és P6) ötféle konstrukció segítségével kíséreltünk meg transzgenikus dohánynövények különböző sejtkompartimentumaiban kifejezni, s minden esetben sikerrel bizonyítottuk a megfelelő mRNS-ek szintézisét. scFv-proteinek felhalmozódását a citoplazmában kifejezett P6- ill. az ER-rezidens J2- és P6-scFv-k esetében mutattuk ki. A dsRNS-specifikus ellenanyagok vírusreplikációra gyakorolt *in vivo* hatását analizálva megállapítottuk, hogy az scFv-k nem gátolják a PVY vírus multiplikációját, sőt, az ER-rezidens J2-scFv súlyosbítja a vírusszimptómákat.

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12. Appendixes

12.1. Primers

Table 12-1: Oligonucleotides employed along the studies and their application

Name	Sequence, 5' to 3'	Length, nt	Application
For01	GTG CAA GTA ACT ACT ACT TTG AC	23	Forward primer, to screen K1 plants with IgG J2 background, annealed to K1VH CDR3
For02	CGA GCT CAC TCA GTC TCC AGC AAT CA	26	Forward primer, to screen K1 plants with IgG J2 background, annealed to scFvK1 VL start
FORSFIL	CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGA ATG GAG CTG GAT ATT TCT C	52	Forward primer used to construct scFv LJ2, annealed to IgG J2 leader and added SfiI site for cloning into pGEJAE1 vector.
J002	ATC CGA CGG TGG CGG TTC TAA CA	23	Forward primer, annealed to scFv J2 linker and VL.
J2-LT	ACT GTG CAA GGG GTG GTA AC	20	Forward primer, annealed at 3' end of scFv J2.104 VH, worked well with reverse pG-rev03; designed for screening scFv J2 constructs.
JFOR	CGT GAA AAA ATT ATT ATT CGC AAT TC	26	Forward primer to amplify scFv J2 from pCJ2 and clone into pQE-TriSystem vector; annealed at g3 leader, worked with JREV primer.
JK2NOT10	GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC	42	Reverse primer to attach SfiI site and amplify assembled scFv.
JREV	ACG GCT CGA GAC GCG GTT CCA	21	Reverse primer to amplify scFv J2 from pCJ2 and clone into pQE-TriSystem vector; annealed at E-tag, worked with JFOR primer; added XhoI site.

LF1	CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGG ATG T	37	Forward primer to construct Fyn-GFP and Fyn-scFv J2; contained SfiI site, ATG start codon; annealed to FF2 primer (coding Fyn signal) and pGEJAE1-Fyn-scFvJ2/GFP constructs; could be used with pG-rev03 reverse primer for screening transgenic plants by PCR.
M13FOR01	GTT TTC CCA GTC ACG AC	17	Standard forward M13 primer.
M13REV	CAG GAA ACA GCT ATG AC	17	Standard reverse M13 primer.
MJK1FONX	CCG TTT GAT TTC CAG CTT GGT GCC	24	Reverse primer for amplification of DNA encoding VL region.
MJK2FONX	CCG TTT TAT TTC CAG CTT GGT CCC	24	Reverse primer for amplification of DNA encoding VL region.
MJK4FONX	CCG TTT TAT TTC CAA CTT TGT CCC	24	Reverse primer for amplification of DNA encoding VL region.
MO-LINK-BACK	GGC ACC ACG GTC ACC GTC TCC TCA	24	Forward primer to amplify scFv linker.
MO-LINK-FOR	TGG AGA CTG AGT GAG CTC GAT GTC	24	Reverse primer to amplify scFv linker.
Oligo 11	GGC CGC AGA ACA AAA ACT CAT CTC AGA AGA GGA TCT GAA TGA TAT TAA GGA TGA ACT TTA AG	62	Forward oligonucleotide, annealed to NotI site and c-myc of pGEJAE1 vector, used with Oligo 12 to attach KDEL signal.
Oligo 12	TCG ACT TAA AGT TCA TCC TTA ATA TCA TTC AGA TCC TCT TCT GAG ATG AGT TTT TGT TCT GC	62	Reverse oligonucleotide, annealed to NotI site and c-myc of pGEJAE1 vector, used with Oligo 11 to attach KDEL signal.
P001	GTG CAA GTA ACT ACT ACT TTG AC	23	Primer to screen scFv K1
P03	AGG TSM ARC TGC AGC AGT CWG G	22	Forward primer of VH; homolog to VH1BACK, differing just in 1 nucleotide
P04	GGA ATG GAG CTG GAT ATT TCT CTT CCT CCT GTC AGG AAC TGC AGG TGT CCA CTC CAA GGT CAA GCT GCA GCA GTC	75	Forward primer to construct scFv LP6; encodes the antibody leader; anneals to scFv P6 VH 5' end and to FORSFIL primer.

pCANTAB5 R1	CCA TGA TTA CGC CAA GCT TTG GAG CC	26	Forward rescue and sequencing primer, for analysis and genetic manipulation of scFv fragments produced using the Recombinant Phage Antibody System of Amersham Biosciences (formerly Amersham Pharmacia Biotech, Inc.) Works well with pCANTAB5 R2.
pCANTAB5 R2	CGA TCT AAA GTT TTG TCG TCT TTC C	25	Reverse rescue and sequencing primer, for analysis and genetic manipulation of scFv fragments produced using the Recombinant Phage Antibody System of Amersham Biosciences (formerly Amersham Pharmacia Biotech, Inc.) Works well with pCANTAB5 R1.
pG-for02	TCC TTC GCA AGA CCC TTC CTC	21	Forward primer for screening scFv constructs in pGEJAE1 vector by PCR.
pG-rev02	AAC CGG CGG TAA GGA TCT GA	20	Reverse primer for screening scFv constructs in pGEJAE1 vector by PCR.
pG-for03	CTC AGG TGG AGG CGG TTC	18	Forward primer to pair with pG-rev03 for screening plants with PCR.
pG-rev03	CTT CTG AGA TGA GTT TTT GTT CTG C	25	Reverse primer, annealed to NotI and c-myc tag; worked well with J2-LT for screening scFv constructs in pGEJAE1 vector by PCR.
pG-rev04	CAG CCT AGC ATG CCC GAG GTG CAA CTG CAG CAG TCA	36	Forward primer, annealed to 5' region of scFv J2 VH in pGEJAE1, added PaeI site, worked with pG-rev02, used in cloning scFv J2 to pQE-3x expression vectors.
Primer D	AAA AAG GAT CCA CCG CCA CCG CTC CCG CCA CCG CCT GCA GAG ACA GTG ACC AGA G	55	Reverse primer, annealed to the scFv J2 3' region of VH and linker; together with Primer E needed for assembly of scFv J2 from VH and VL.

Primer E	AAA AAG GAT CCG GCG GTG GCG GTT CTA ACA TTA TGA TGA CAC AGT C	46	Forward primer, annealed to the scFv J2 linker and 5' region of VL; together with Primer D needed for assembly of scFv J2 from VH and VL.
SFOR	GTT ATT GTG CTG TCT CAT C	19	Forward primer of pQE-TriSystem vector used for scFv J2 sequencing in this expression vector.
SSF1	ACA AAA CTG ACT GAG GAG AGA GAG GTG CAA CTG CAG CAG TC	41	Forward primer to attach the Src signal sequence to scFv J2 in pGEJAE1.
VH1BACK	AGG TSM ARC TGC AGS AGT CWG G	22	Forward primer to amplify VH sequence.
VH1BACKSFI	CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC CSA GGT SMA RCT GCA GSA GTC WGG	57	Forward primer to incorporate SfiI restriction endonuclease site when constructing scFv.
VH1FOR-2	TGA GGA GAC GGT GAC CGT GGT GCC TTG GCC CC	32	Reverse primer to amplify VH sequence.
VK2BACK	GAC ATC GAG CTC ACT CAG TCT CCA	24	Forward primer to amplify VL sequence.
VLJ2FOR	TGT CGG CAG GAG AAA AGG TCA CTA	24	Forward sequencing primers located in VL scFv J2.
JK2NOT10	GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC	42	Reverse primer to incorporate NotI restriction endonuclease site when constructing scFv.

12.2. Sequences

12.2.1. Sequence of scFv J2 gene expressed in *E. coli*

DNA molecule name is pCJ2.104. scFv J2 sequence of clone 104 is in pCANTAB 5E phagemid. The entire molecule length is 5 222 bp.

Feature	Start	End
scFv J2 coding sequence of clone 104	1	861
GTG start codon	1	3
Signal peptide of gene 3 leading to periplasma	1	45
SfiI endonuclease site	48	60
Coding sequence of heavy chain variable domain	61	420
15-amino acid linker	421	465
Coding sequence of light chain variable domain	466	804
NotI endonuclease site	805	812
E-tag	814	852
Amber stop codon	859	861

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   1    GTGAAAAAAT TATTATTCGC AATTCCTTTA GTTGTTCCTT TCTATGCGGC CCAGCCGGCC
  61    ATGGCCGAGG TGCAACTGCA GCAGTCAGGA CCTGAGCTGG TAAAGCCTGG GGCTTCAGTG
 121    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
 181    CAGAAGCCAG GGCAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
 241    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 301    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGG
 361    GGTGGTAACC CCGCCTGGTT TGCTTACTGG GGCCAAGGGA CTCTGGTCAC TGTCTCTGCA
 421    GGCGGTGGCG GGAGCGGTGG CGGTGGATCC GGCGGTGGCG GTTCTAACAT TATGATGACA
 481    CAGTCGCCAT CATCTCTGGC TGTGTCGGCA GGAGAAAAGG TCACTATGAG CTGTAAGTCC
 541    AGTCAAAGTG TTTTATACAG TTCAAATCAG AAGAACTACT TGGCCTGGTA CCAGCAGAAA
 601    CCAGGGCAGT CTCCTAAACT GCTGATCTAC TGGGCATCCA CTAGGGAATC TGGTGTCCCT
 661    GATCGCTTCA CAGGCAGTGG ATCTGGGACA GATTTTACTC TTACCATCAG CAGTGTACAA
 721    GCTGAAGACC TGGCAGTTTA TTACTGTCAT CAATACCTCT CCTCGTACAC GTTCGGAGGG
 781    GGGACCAAGC TGGAAATAAA ACGGGCGGCC GCAGGTGCGC CGGTGCCGTA TCCGGATCCG
 841    CTGGAACCGC GTGCCGCATA G
```

12.2.2. Sequence of scFv K1 gene expressed in *E. coli*

DNA molecule name is pCK1.21. scFv K1 sequence of clone 21 is in pCANTAB 5E phagemid. The entire molecule length is 5 195 bp.

Feature	Start	End
scFv K1 coding sequence of clone 21	1	834
GTG start codon	1	3
Signal peptide of gene 3 leading to periplasma	1	45
SfiI endonuclease site	48	60
Coding sequence of heavy chain variable domain	61	411
15-amino acid linker	412	456
Coding sequence of light chain variable domain	457	777
NotI endonuclease site	778	785

Feature	Start	End
E-tag sequence	787	825
Amber stop codon	832	834

```
   1    GTGAAAAAAT TATTATTCGC AATTCCTTTA GTTGTTCCTT TCTATGCGGC CCAGCCGGCC
  61    ATGGCCCAGG TGAAGCTGCA GCAGTCAGGA CCTGAGCTGG TAAAGCCTGG GGCTTCAGTG
 121    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
 181    CAGAAGCCAG GGCAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
 241    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 301    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGT
 361    AACTACTACT TTGACTACTG GGGCCAAGGC ACCACGGTCA CCGTCTCCTC AGGTGGAGGC
 421    GGTTCAGGCG GCGGTGGCTC TGGCGGTGGC GGATCGGACA TCGAGCTCAC TCAGTCTCCA
 481    GCAATCATGT CTGCATCTCC AGGGGAGAAG GTCACCATGA CCTGCAGTGC CAGCTCAAGT
 541    GTAAGTTACA TGCACTGGTA CCAGCAGAAG TCAGGCACCT CCCCCAAAAG ATGGATTTAT
 601    GACACATCCA AACTGGCTTC TGGAGTCCCT GCTCGCTTCA GTGGCAGTGG GTCTGGGACC
 661    TCTTACTCTC TCACAATCAG CAGCATGGAG GCTGAAGATG CTGCCACTTA TTACTGCCAG
 721    CAGTGGAGTA GTAACCCATA CACGTTCGGA GGGGGGACCA AGCTGGAAAT AAAACGGGCG
 781    GCCGCAGGTG CGCCGGTGCC GTATCCGGAT CCGCTGGAAC CGCGTGCCGC ATAG
```

12.2.3. Sequence of scFv K2 gene expressed in *E. coli*

DNA molecule name is pCK2.32. scFv K2 sequence of clone 32 is in pCANTAB 5E phagemid. The entire molecule length is 5 195 bp.

Feature	Start	End
scFv K2 coding sequence of clone …	1	834
GTG start codon	1	3
Signal peptide of gene 3 leading to periplasma	1	45
SfiI endonuclease site	48	60
Coding sequence of heavy chain variable domain	61	411
15-amino acid linker	412	456
Coding sequence of light chain variable domain	457	777
NotI endonuclease site	778	785
E-tag sequence	787	825
Amber stop codon	832	834

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   1    GTGAAAAAAT TATTATTCGC AATTCCTTTA GTTGTTCCTT TCTATGCGGC CCAGCCGGCC
  61    ATGGCCCAGG TGAAGCTGCA GGAGTCAGGA CCTGAGCTGA TAAAGCCTGG GGCTTCAGTG
 121    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
 181    CAGAAGCCAG GACAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
 241    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 301    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGT
 361    AACTACTACT TTGACTACTG GGGCCAAGGC ACCACGGTCA CCGTCTCCTC AGGTGGAGGC
 421    GGTTCAGGCG GAGGTGGCTC TGGCGGTGGC GGATCGGACA TCGAGCTCAC TCAGTCTCCA
 481    GCAATCATGT CTGCATCTCC AGGGGAGAAG GTCACCATGA CCTGCAGTGC CAGCTCAAGT
 541    GTAAGTTACA TGCACTGGTA CCAGCAGAAG TCAGGCACCT CCCCCAAAAG ATGGATTTAT
 601    GACACATCCA AACTGGCTTC TGGAGTCCCT GCTCGCTTCA GTGGCAGTGG GTCTGGGACC
 661    TCTTACTCTC TCACAATCAG CAGCATGGAG GCTGAAGATG CTGCCACTTA TTACTGCCAG
 721    CAGTGGAGTA GTAACCCATA CACGTTCGGA GGGGGGACAA AGTTGGAAAT AAAACGGGCG
 781    GCCGCAGGTG CGCCGGTGCC GTATCCGGAT CCGCTGGAAC CGCGTGCCGC ATAG
```

12.2.4. Sequence of scFv P6 gene expressed in *E. coli*

DNA molecule name is pCP6.84. scFv P6 sequence of clone 84 is in pCANTAB 5E phagemid. The entire molecule length is 5 201 bp.

Feature	Start	End
scFv P6 coding sequence of clone 84	1	840
GTG start codon	1	3
Signal peptide of gene 3 leading to periplasma	1	45
SfiI endonuclease site	48	60
Coding sequence of heavy chain variable domain	61	417
15-amino acid linker	418	462
Coding sequence of light chain variable domain	463	783
NotI endonuclease site	784	791
E-tag sequence	793	831
Amber stop codon	838	840

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  1    GTGAAAAAAT TATTATTCGC AATTCCTTTA GTTGTTCCTT TCTATGCGGC CCAGCCGGCC
 61    ATGGCCAAGG TCAAGCTGCA GCAGTCTGGA CCTGAGCTGG TCAAGCCTGG GGCTTCAGTG
121    CAGATGTCCT GCAAGGCTTC TGGATACACA TTCACTAGCT ATGTTATGCA CTGGGTGAAG
181    CAGAAGCCTG GGCAGGGCCT TGAGTGGATT GGATATATTA ATCCTTACAC TGATACTACT
241    AAATACAATG AGAAGTTCAA AGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
301    GCCTACATGG AGCTCAGCGG CCTGACCTCT GAGGACTCTG CGGTCTATTA CTGTGCCCTT
361    CGTAGTCGCT ACTACTTTAA CTACTGGGGC CAAGGCACCA CGGTCACCGT CTCCTCAGGT
421    GGAGGCGGTT CAGGCGGAGG TGGCTCTGGC GGTGGCGCAT CGGACATTGA GCTCACCCAG
481    TCTCCAGCAA TCATGTTTGT ATCTCCAGGG GAGAAGGTCA CCATATCCTG CAGTGCCAAC
541    TCAACTGTAA GTTACATGTA CTGGTACCAG CAGAAGCCAG GATCCTCCCC CAAACCCTGG
601    ATTTATCGCA CATCCAACCT GGCTTCTGGA GTCCCTGTTC GCTTCAGTGG CAGTGGATCT
661    GGGACCTCTT ATTCTCTCAC AATCATCAGT ATGGAGGCTG AAGATGCTGC CACTTATTAC
721    TGTCAACAGT GGAGTAGTTA CCCGCTCACG TTCGGTGCTG GGACCAAGCT GGAGCTGAAA
781    CGGGCGGCCG CAGGTGCGCC GGTGCCGTAT CCGGATCCGC TGGAACCGCG TGCCGCATAG
```

12.2.5. Sequence of scFv J2 gene expressed in *N. tabacum*

scFv J2 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 2

```
  1    ATGGCCGAGG TGCAACTGCA GCAGTCAGGA CCTGAGCTGG TAAAGCCTGG GGCTTCAGTG
 61    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
121    CAGAAGCCAG GGCAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
181    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
241    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGG
301    GGTGGTAACC CCGCCTGGTT TGCTTACTGG GGCCAAGGGA CTCTGGTCAC TGTCTCTGCA
361    GGCGGTGGCG GGAGCGGTGG CGGTGGATCC GGCGGTGGCG GTTCTAACAT TATGATGACA
421    CAGTCGCCAT CATCTCTGGC TGTGTCTGCA GGAGAAAAGG TCACTATGAG CTGTAAGTCC
481    AGTCAAAGTG TTTTATACAG TTCAAATCAG AAGAACTACT TGGCCTGGTA CCAGCAGAAA
541    CCAGGGCAGT CTCCTAAACT GCTGATCTAC TGGGCATCCA CTAGGGAATC TGGTGTCCCT
601    GATCGCTTCA CAGGCAGTGG ATCTGGGACA GATTTTACTC TTACCATCAG CAGTGTACAA
661    GCTGAAGACC TGGCAGTTTA TTACTGTCAT CAATACCTCT CCTCGTACAC GTTCGGAGGG
721    GGGACCAAGC TGGAAATAAA ACGGGCGGCC GCAGAACAAA AACTCATCTC AGAAGAGGAT
781    CTGAATGGGG CCGCA
```

12.2.6. Sequence of scFv LJ2 gene expressed in *N. tabacum*

scFv LJ2 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 5

```
  1    ATGGAATGGA GCTGGATATT TCTCTTCCTC CTGTCAGGAA CTGCAGGTGT CCACTCCCAG
```

```
  61    GTCCAGCTGC AGCAGTCTGG ACCTGAGCTG GTAAAGCCTG GGGCTTCAGT GAAGATGTCC
 121    TGCAAGGCTT CTGGATACAC CTTCGCTAAC CATGTTATGC ACTGGGTGAA GCAGAAGCCA
 181    GGGCAGGGCC TTGAGTGGAT TGGATATATT TATCCTTACA ATGATGGTAC TAAGTACAAT
 241    GAGAAGTTCA AGGGCAAGGC CACACTGACT TCAGACAAAT CCTCCAGCAC AGCCTACATG
 301    GAGCTCAGCA GCCTGGCCTC TGAGGACTCT GCGGTCTATT ACTGTGCAAG GGGTGGTAAC
 361    CCCGCCTGGT TTGCTTACTG GGGCCAAGGG ACTCTGGTCA CTGTCTCTGC AGGCGGTGGC
 421    GGGAGCGGTG GCGGTGGATC CGGCGGTGGC GGTTCTAACA TTATGATGAC ACAGTCGCCA
 481    TCATCTCTGG CTGTGTCTGC AGGAGAAAAG GTCACTATGA GCTGTAAGTC CAGTCAAAGT
 541    GTTTTATACA GTTCAAATCA GAAGAACTAC TTGGCCTGGT ACCAGCAGAA ACCAGGGCAG
 601    TCTCCTAAAC TGCTGATCTA CTGGGCATCC ACTAGGGAAT CTGGTGTCCC TGATCGCTTC
 661    ACAGGCAGTG GATCTGGGAC AGATTTTACT CTTACCATCA GCAGTGTACA AGCTGAAGAC
 721    CTGGCAGTTT ATTACTGTCA TCAATACCTC TCCTCGTACA CGTTCGGAGG GGGGACCAAG
 781    CTGGAAATAA AACGGGCGGC CGCAGAACAA AAACTCATCT CAGAAGAGGA TCTGAATGGG
 841    GCCGCA
```

12.2.7. Sequence of scFv KJ2 gene expressed in *N. tabacum*

scFv KJ2 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 3

```
   1    ATGGCCGAGG TGCAACTGCA GCAGTCAGGA CCTGAGCTGG TAAAGCCTGG GGCTTCAGTG
  61    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
 121    CAGAAGCCAG GGCAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
 181    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 241    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGG
 301    GGTGGTAACC CCGCCTGGTT TGCTTACTGG GGCCAAGGGA CTCTGGTCAC TGTCTCTGCA
 361    GGCGGTGGCG GGAGCGGTGG CGGTGGATCC GGCGGTGGCG GTTCTAACAT TATGATGACA
 421    CAGTCGCCAT CATCTCTGGC TGTGTCTGCA GGAGAAAAGG TCACTATGAG CTGTAAGTCC
 481    AGTCAAAGTG TTTTATACAG TTCAAATCAG AAGAACTACT TGGCCTGGTA CCAGCAGAAA
 541    CCAGGGCAGT CTCCTAAACT GCTGATCTAC TGGGCATCCA CTAGGGAATC TGGTGTCCCT
 601    GATCGCTTCA CAGGCAGTGG ATCTGGGACA GATTTTACTC TTACCATCAG CAGTGTACAA
 661    GCTGAAGACC TGGCAGTTTA TTACTGTCAT CAATACCTCT CCTCGTACAC GTTCGGAGGG
 721    GGGACCAAGC TGGAAATAAA ACGGGCGGCC GCAGAACAAA AACTCATCTC AGAAGAGGAT
 781    CTGAATGATA TTAAGGATGA ACTT
```

12.2.8. Sequence of scFv LKJ2 gene expressed in *N. tabacum*

scFv LKJ2 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 7

```
   1    ATGGAATGGA GCTGGATATT TCTCTTCCTC CTGTCAGGAA CTGCAGGTGT CCACTCCCAG
  61    GTCCAGCTGC AGCAGTCTGG ACCTGAGCTG GTAAAGCCTG GGGCTTCAGT GAAGATGTCC
 121    TGCAAGGCTT CTGGATACAC CTTCGCTAAC CATGTTATGC ACTGGGTGAA GCAGAAGCCA
 181    GGGCAGGGCC TTGAGTGGAT TGGATATATT TATCCTTACA ATGATGGTAC TAAGTACAAT
 241    GAGAAGTTCA AGGGCAAGGC CACACTGACT TCAGACAAAT CCTCCAGCAC AGCCTACATG
 301    GAGCTCAGCA GCCTGGCCTC TGAGGACTCT GCGGTCTATT ACTGTGCAAG GGGTGGTAAC
 361    CCCGCCTGGT TTGCTTACTG GGGCCAAGGG ACTCTGGTCA CTGTCTCTGC AGGCGGTGGC
 421    GGGAGCGGTG GCGGTGGATC CGGCGGTGGC GGTTCTAACA TTATGATGAC ACAGTCGCCA
 481    TCATCTCTGG CTGTGTCTGC AGGAGAAAAG GTCACTATGA GCTGTAAGTC CAGTCAAAGT
 541    GTTTTATACA GTTCAAATCA GAAGAACTAC TTGGCCTGGT ACCAGCAGAA ACCAGGGCAG
 601    TCTCCTAAAC TGCTGATCTA CTGGGCATCC ACTAGGGAAT CTGGTGTCCC TGATCGCTTC
 661    ACAGGCAGTG GATCTGGGAC AGATTTTACT CTTACCATCA GCAGTGTACA AGCTGAAGAC
 721    CTGGCAGTTT ATTACTGTCA TCAATACCTC TCCTCGTACA CGTTCGGAGG GGGGACCAAG
 781    CTGGAAATAA AACGGGCGGC CGCAGAACAA AAACTCATCT CAGAAGAGGA TCTGAATGAT
 841    ATTAAGGATG AACTT
```

12.2.9. Sequence of scFv K1 gene expressed in *N. tabacum*

scFv K1 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 2

```
   1    ATGGCCCAGG TGAAGCTGCA GCAGTCAGGA CCTGAGCTGG TAAAGCCTGG GGCTTCAGTG
  61    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
 121    CAGAAGCCAG GGCAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
 181    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 241    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGT
 301    AACTACTACT TTGACTACTG GGGCCAAGGC ACCACGGTCA CCGTCTCCTC AGGTGGAGGC
```

```
361    GGTTCAGGCG GCGGTGGCTC TGGCGGTGGC GGATCGGACA TCGAGCTCAC TCAGTCTCCA
421    GCAATCATGT CTGCATCTCC AGGGGAGAAG GTCACCATGA CCTGCAGTGC CAGCTCAAGT
481    GTAAGTTACA TGCACTGGTA CCAGCAGAAG TCAGGCACCT CCCCCAAAAG ATGGATTTAT
541    GACACATCCA AACTGGCTTC TGGAGTCCCT GCTCGCTTCA GTGGCAGTGG GTCTGGGACC
601    TCTTACTCTC TCACAATCAG CAGCATGGAG GCTGAAGATG CTGCCACTTA TTACTGCCAG
661    CAGTGGAGTA GTAACCCATA CACGTTCGGA GGGGGGACCA AGCTGGAAAT AAAACGGGCG
721    GCCGCAGAAC AAAAACTCAT CTCAGAAGAG GATCTGAATG GGGCCGCA
```

12.2.10. Sequence of scFv LK1 gene expressed in *N. tabacum*

scFv LK1 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 4

```
  1    ATGGAATGGA GCTGGATATT TCTCTTCCTC CTGTCAGGAA CTGCAGGTGT CCACTCCCAG
 61    GTCCAGCTGC AGCAGTCTGG ACCTGAGCTG GTAAAGCCTG GGGCTTCAGT GAAGATGTCC
121    TGCAAGGCTT CTGGATACAC CTTCGCTAAC CATGTTATGC ACTGGGTGAA GCAGAAGCCA
181    GGGCAGGGCC TTGAGTGGAT TGGATATATT TATCCTTACA ATGATGGTAC TAAGTACAAT
241    GAGAAGTTCA AGGGCAAGGC CACACTGACT TCAGACAAAT CCTCCAGCAC AGCCTACATG
301    GAGCTCAGCA GCCTGGCCTC TGAGGACTCT GCGGTCTATT ACTGTGCAAG TAACTACTAC
361    TTTGACTACT GGGGCCAAGG CACCACGGTC ACCGTCTCCT CAGGTGGAGG CGGTTCAGGC
421    GGCGGTGGCT CTGGCGGTGG CGGATCAGAC ATCGAGCTCA CTCAGTCTCC AGCAATCATG
481    TCTGCATCTC CAGGGGAGAA GGTCACCATG ACCTGCAGTG CCAGCTCAAG TGTAAGTTAC
541    ATGCACTGGT ACCAGCAGAA GTCAGGCACC TCCCCCAAAA GATGGATTTA TGACACATCC
601    AAACTGGCTT CTGGAGTCCC TGCTCGCTTC AGTGGCAGTG GGTCTGGGAC CTCTTACTCT
661    CTCACAATCA GCAGCATGGA GGCTGAAGAT GCTGCCACTT ATTACTGCCA GCAGTGGAGT
721    AGTAACCCAT ACACGTTCGG AGGGGGGACC AAGCTGGAAA TAAAACGGGC GGCCGCAGAA
781    CAAAAACTCA TCTCAGAAGA GGATCTGAAT GGGGCCGCA
```

12.2.11. Sequence of scFv KK1 gene expressed in *N. tabacum*

scFv KK1 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 2

```
  1    ATGGCCCAGG TGAAGCTGCA GCAGTCAGGA CCTGAGCTGG TAAAGCCTGG GGCTTCAGTG
 61    AAGATGTCCT GCAAGGCTTC TGGATACACC TTCGCTAACC ATGTTATGCA CTGGGTGAAG
121    CAGAAGCCAG GGCAGGGCCT TGAGTGGATT GGATATATTT ATCCTTACAA TGATGGTACT
181    AAGTACAATG AGAAGTTCAA GGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
241    GCCTACATGG AGCTCAGCAG CCTGGCCTCT GAGGACTCTG CGGTCTATTA CTGTGCAAGT
301    AACTACTACT TTGACTACTG GGGCCAAGGC ACCACGGTCA CCGTCTCCTC AGGTGGAGGC
361    GGTTCAGGCG GCGGTGGCTC TGGCGGTGGC GGATCGGACA TCGAGCTCAC TCAGTCTCCA
421    GCAATCATGT CTGCATCTCC AGGGGAGAAG GTCACCATGA CCTGCAGTGC CAGCTCAAGT
481    GTAAGTTACA TGCACTGGTA CCAGCAGAAG TCAGGCACCT CCCCCAAAAG ATGGATTTAT
541    GACACATCCA AACTGGCTTC TGGAGTCCCT GCTCGCTTCA GTGGCAGTGG GTCTGGGACC
601    TCTTACTCTC TCACAATCAG CAGCATGGAG GCTGAAGATG CTGCCACTTA TTACTGCCAG
661    CAGTGGAGTA GTAACCCATA CACGTTCGGA GGGGGGACCA AGCTGGAAAT AAAACGGGCG
721    GCCGCAGAAC AAAAACTCAT CTCAGAAGAG GATCTGAATG ATATTAAGGA TGAACTT
```

12.2.12. Sequence of scFv LKK1 gene expressed in *N. tabacum*

scFv LKK1 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 8

```
  1    ATGGAATGGA GCTGGATATT TCTCTTCCTC CTGTCAGGAA CTGCAGGTGT CCACTCCCAG
 61    GTCCAGCTGC AGCAGTCTGG ACCTGAGCTG GTAAAGCCTG GGGCTTCAGT GAAGATGTCC
121    TGCAAGGCTT CTGGATACAC CTTCGCTAAC CATGTTATGC ACTGGGTGAA GCAGAAGCCA
181    GGGCAGGGCC TTGAGTGGAT TGGATATATT TATCCTTACA ATGATGGTAC TAAGTACAAT
241    GAGAAGTTCA AGGGCAAGGC CACACTGACT TCAGACAAAT CCTCCAGCAC AGCCTACATG
301    GAGCTCAGCA GCCTGGCCTC TGAGGACTCT GCGGTCTATT ACTGTGCAAG TAACTACTAC
361    TTTGACTACT GGGGCCAAGG CACCACGGTC ACCGTCTCCT CAGGTGGAGG CGGTTCAGGC
421    GGCGGTGGCT CTGGCGGTGG CGGATCGGAC ATCGAGCTCA CTCAGTCTCC AGCAATCATG
481    TCTGCATCTC CAGGGGAGAA GGTCACCATG ACCTGCAGTG CCAGCTCAAG TGTAAGTTAC
541    ATGCACTGGT ACCAGCAGAA GTCAGGCACC TCCCCCAAAA GATGGATTTA TGACACATCC
601    AAACTGGCTT CTGGAGTCCC TGCTCGCTTC AGTGGCAGTG GGTCTGGGAC CTCTTACTCT
661    CTCACAATCA GCAGCATGGA GGCTGAAGAT GCTGCCACTT ATTACTGCCA GCAGTGGAGT
721    AGTAACCCAT ACACGTTCGG AGGGGGGACC AAGCTGGAAA TAAAACGGGC GGCCGCAGAA
781    CAAAAACTCA TCTCAGAAGA GGATCTGAAT GATATTAAGG ATGAACTT
```

12.2.13. Sequence of scFv P6 gene expressed in *N. tabacum*

scFv P6 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 4

```
   1    ATGGCCAAGG TCAAGCTGCA GCAGTCTGGA CCTGAGCTGG TCAAGCCTGG GGCTTCAGTG
  61    CAGATGTCCT GCAAGGCTTC TGGATACACA TTCACTAGCT ATGTTATGCA CTGGGTGAAG
 121    CAGAAGCCTG GGCAGGGCCT TGAGTGGATT GGATATATTA ATCCTTACAC TGATACTACT
 181    AAATACAATG AGAAGTTCAA AGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 241    GCCTACATGG AGCTCAGCGG CCTGACCTCT GAGGACTCTG CGGTCTATTA CTGTGCCCTT
 301    CGTAGTCGCT ACTACTTTAA CTACTGGGGC CAAGGCACCA CGGTCACCGT CTCCTCAGGT
 361    GGAGGCGGTT CAGGCGGAGG TGGCTCTGGC GGTGGCGCAT CGGACATTGA GCTCACCCAG
 421    TCTCCAGCAA TCATGTTTGT ATCTCCAGGG GAGAAGGTCA CCATATCCTG CAGTGCCAAC
 481    TCAACTGTAA GTTACATGTA CTGGTACCAG CAGAAGCCAG GATCCTCCCC CAAACCCTGG
 541    ATTTATCGCA CATCCAACCT GGCTTCTGGA GTCCCTGTTC GCTTCAGTGG CAGTGGATCT
 601    GGGACCTCTT ATTCTCTCAC AATCATCAGT ATGGAGGCTG AAGATGCTGC CACTTATTAC
 661    TGTCAACAGT GGAGTAGTTA CCCGCTCACG TTCGGTGCTG GGACCAAGCT GGAGCTGAAA
 721    CGGGCGGCCG CAGAACAAAA ACTCATCTCA GAAGAGGATC TGAATGGGGC CGCA
```

12.2.14. Sequence of scFv LP6 gene expressed in *N. tabacum*

scFv LP6 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 2

```
   1    ATGGAATGGA GCTGGATATT TCTCTTCCTC CTGTCAGGAA CTGCAGGTGT CCACTCCAAG
  61    GTCAAGCTGC AGCAGTCTGG ACCTGAGCTG GTCAAGCCTG GGGCTTCAGT GCAGATGTCC
 121    TGCAAGGCTT CTGGATACAC ATTCACTAGC TATGTTATGC ACTGGGTGAA GCAGAAGCCT
 181    GGGCAGGGCC TTGAGTGGAT TGGATATATT AATCCTTACA CTGATACTAC TAAATACAAT
 241    GAGAAGTTCA AAGGCAAGGC CACACTGACT TCAGACAAAT CCTCCAGCAC AGCCTACATG
 301    GAGCTCAGCG GCCTGACCTC TGAGGACTCT GCGGTCTATT ACTGTGCCCT TCGTAGTCGC
 361    TACTACTTTA ACTACTGGGG CCAAGGCACC ACGGTCACCG TCTCCTCAGG TGGAGGCGGT
 421    TCAGGCGGAG GTGGCTCTGG CGGTGGCGCA TCGGACATTG AGCTCACCCA GTCTCCAGCA
 481    ATCATGTTTG TATCTCCAGG GGAGAAGGTC ACCATATCCT GCAGTGCCAA CTCAACTGTA
 541    AGTTACATGT ACTGGTACCA GCAGAAGCCA GGATCCTCCC CCAAACCCTG GATTTATCGC
 601    ACATCCAACC TGGCTTCTGG AGTCCCTGTT CGCTTCAGTG GCAGTGGATC TGGGACCTCT
 661    TATTCTCTCA CAATCATCAG TATGGAGGCT GAAGATGCTG CCACTTATTA CTGTCAACAG
 721    TGGAGTAGTT ACCCGCTCAC GTTCGGTGCT GGGACCAAGC TGGAGCTGAA ACGGGCGGCC
 781    GCAGAACAAA AACTCATCTC AGAAGAGGAT CTGAATGGGG CCGCA
```

12.2.15. Sequence of scFv KP6 gene expressed in *N. tabacum*

scFv KP6 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 1

```
   1    ATGGCCAAGG TCAAGCTGCA GCAGTCTGGA CCTGAGCTGG TCAAGCCTGG GGCTTCAGTG
  61    CAGATGTCCT GCAAGGCTTC TGGATACACA TTCACTAGCT ATGTTATGCA CTGGGTGAAG
 121    CAGAAGCCTG GGCAGGGCCT TGAGTGGATT GGATATATTA ATCCTTACAC TGATACTACT
 181    AAATACAATG AGAAGTTCAA AGGCAAGGCC ACACTGACTT CAGACAAATC CTCCAGCACA
 241    GCCTACATGG AGCTCAGCGG CCTGACCTCT GAGGACTCTG CGGTCTATTA CTGTGCCCTT
 301    CGTAGTCGCT ACTACTTTAA CTACTGGGGC CAAGGCACCA CGGTCACCGT CTCCTCAGGT
 361    GGAGGCGGTT CAGGCGGAGG TGGCTCTGGC GGTGGCGCAT CGGACATTGA GCTCACCCAG
 421    TCTCCAGCAA TCATGTTTGT ATCTCCAGGG GAGAAGGTCA CCATATCCTG CAGTGCCAAC
 481    TCAACTGTAA GTTACATGTA CTGGTACCAG CAGAAGCCAG GATCCTCCCC CAAACCCTGG
 541    ATTTATCGCA CATCCAACCT GGCTTCTGGA GTCCCTGTTC GCTTCAGTGG CAGTGGATCT
 601    GGGACCTCTT ATTCTCTCAC AATCATCAGT ATGGAGGCTG AAGATGCTGC CACTTATTAC
 661    TGTCAACAGT GGAGTAGTTA CCCGCTCACG TTCGGTGCTG GGACCAAGCT GGAGCTGAAA
 721    CGGGCGGCCG CAGAACAAAA ACTCATCTCA GAAGAGGATC TGAATGATAT TAAGGATGAA
 781    CTT
```

12.2.16. Sequence of scFv LKP6 gene expressed in *N. tabacum*

scFv LKP6 nucleotide sequence in pGEJAE1 plant expression vector, sequenced clone 62

```
  1    ATGGAATGGA GCTGGATATT TCTCTTCCTC CTGTCAGGAA CTGCAGGTGT CCACTCCAAG
 61    GTCAAGCTGC AGCAGTCTGG ACCTGAGCTG GTCAAGCCTG GGGCTTCAGT GCAGATGTCC
121    TGCAAGGCTT CTGGATACAC ATTCACTAGC TATGTTATGC ACTGGGTGAA GCAGAAGCCT
181    GGGCAGGGCC TTGAGTGGAT TGGATATATT AATCCTTACA CTGATACTAC TAAATACAAT
241    GAGAAGTTCA AAGGCAAGGC CACACTGACT TCAGACAAAT CCTCCAGCAC AGCCTACATG
301    GAGCTCAGCG GCCTGACCTC TGAGGACTCT GCGGTCTATT ACTGTGCCCT TCGTAGTCGC
361    TACTACTTTA ACTACTGGGG CCAAGGCACC ACGGTCACCG TCTCCTCAGG TGGAGGCGGT
421    TCAGGCGGAG GTGGCTCTGG CGGTGGCGCA TCGGACATTG AGCTCACCCA GTCTCCAGCA
481    ATCATGTTTG TATCTCCAGG GGAGAAGGTC ACCATATCCT GCAGTGCCAA CTCAACTGTA
541    AGTTACATGT ACTGGTACCA GCAGAAGCCA GGATCCTCCC CCAAACCCTG GATTTATCGC
601    ACATCCAACC TGGCTTCTGG AGTCCCTGTT CGCTTCAGTG GCAGTGGATC TGGGACCTCT
661    TATTCTCTCA CAATCATCAG TATGGAGGCT GAAGATGCTG CCACTTATTA CTGTCAACAG
721    TGGAGTAGTT ACCCGCTCAC GTTCGGTGCT GGGACCAAGC TGGAGCTGAA ACGGGCGGCC
781    GCAGAACAAA AACTCATCTC AGAAGAGGAT CTGAATGATA TTAAGGATGA ACTT
```

13. Declaration

Hereby I declare that the work presented in this manuscript is my own and was carried out entirely with help of literature and aid cited in the manuscript.

Bogdan Morgun

Budapest, September 7, 2005