A single chain antibody against a viral RNA polymerase (TBSV-BS3-Statice)

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to
my parents

and
my teachers

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Abbreviation

BAP	6-Benzylaminopurin
BSA	Bovine serum albumin
EDTA	Ethylene diamine tetraacetic acid
ER	endoplasmic reticulum
EtOH	ethanol
h	hour
ddH2O	double distilled water
IPTG	Isopropyl-β-D-thiogalactopyranosid
KD	Kilo Dalton
Min	minute
NAA	1-Naphthaleneacetic acid
ORF	Open reading frame
PAGE	Polyacrylamine gel electrophoresis
PEG	Polyethyleneglycol
RdRp	RNA dependent RNA polymerase
rpm	round per minute
scFv	single variable fragment
SDS	sodiumdodecylsulfate
SDS-PAGE	sodiumdodecylsulfate polyacrylamine gel electrophoresis
sec	second
UV	ultraviolet
Wt	wild type

I. Summary

Antibody-mediated resistance in transgenic plants is an attractive alternative to the various forms of pathogen-derived resistances, because it circumvents the danger of unintended side effects such as heteroencapcidations and recombination of viral genomes. Engineered single-chain Fv antibodies (scFvs) are particularly suitable for the expression in transgenic plants to achieve virus resistance because of their small size and the lack of assembly requirements.

All positive-strand RNA viruses encode a RNA-dependent RNA polymerase (RdRp) which is essential for the replication of the viral genome. Currently there are eight conserved RdRp motifs known. Four of these eight motifs are present in all classes of polymerases and reside in the catalytic portion of the "palm domain". These functional domains emerge as a promising target for antiviral intervention and may enable to achieve broad range resistance. Moreover viral RdRps are present in only small amounts in infected cells and localised to membranous cytosolic structures therefore presenting a favourable target for antiviral strategies mediated by scFvs.

To get a high a amount of RdRp as antigen for scFvs selection from scFv phage libraries, it is almost impossible to obtain the soluble pure RdRp by purification from virus infected plants, since RdRp is a membranous protein and expressed only in very low amounts in infected plant cells. Therefore in this study the different fragments coding for the RdRp (p92) of Tomato Bushy Stunt Virus (TBSV) were cloned into different plasmids and were expressed in different E. coli expression host strains to obtain the different fragment of RdRp in soluble from. However, soluble proteins could not be obtained by using this system, even many attempts to optimise the soluble expression were done. Therefore the purified denatured proteins were used as antigens to select scFvs from scFv phage libraries.

The monoclonal phages expressing the scFvs in soluble form were selected and characterised. The scFvs which gave the highest binding activity both in ELISA and western blot were selected and used to study the scFv-mediated RdRp inhibition.

An epitope mapping was performed to define exactly the domain of the RdRp to which the selected scFvs bind. It could be shown that all of the selected scFvs bind to the motif E of RdRp. This domain acts as a thumb clamp while binding to the template and is therefore highly important for the RdRp activity.

Establishing transgenic plants expressing a foreign gene is time consuming. Therefore before transforming the selected scFvs into plants, their activity was studied in in vitro and in in vivo assays. Partially purified CNV RdRp was used to study the scFv-mediated RdRp inhibition in in vitro. It was shown that the selected scFvs inhibit the transcription activity of the RdRp to different degrees. The inhibition activity of scFv of other viral RdRps were also investigated in vitro. The motif E of TCV and HCV RdRps share amino acid homology with the TBSV RdRp, therefore the affinity of the selected scFvs for the RdRps of these viruses was studied in vitro. The results clearly showed that the selected scFvs show specific binding to the HCV RdRp and the scFvP55H9 can inhibit the activity of the E. coli expressed TCV in in vitro.
To study the inhibition activity of the selected scFv in planta two transient in vivo assays were developed. Agroinfiltration is a valuable tool to transiently express a foreign gene in plant cells. Applying this technique the scFvs can be expressed in planta and their inhibition activity can be studied when virus particles or RNA are inoculated on infiltrated leaves. Expressing a foreign gene via a virus based vector is another method by which the foreign gene can be transiently expressed during the viral replication cycle. In this assay the scFv can directly inhibit the RdRps while being expressed from the same viral genome. Both in vivo assays showed that the scFvs selected from denatured RdRp fragments can inhibit the viral RdRps in plant cells.

Furthermore transgenic N. benthamiana plants were established expressing the different scFvs to test for broad range viral resistance resulting from the scFv-mediated inhibition of the RdRp. Since it is not clear yet in which plant cell compartment the RdRps are translated, the scFv constructs were designed to be expressed in the cytoplasm or in the endoplasmic recticulum (ER). The self fertilised seeds from T0 plants were germinated and challenge inoculated with viruses from two different virus families (TBSV, Tombusvirus and RCNMV, Dianthovirus). Transgenic plant lines expressing the scFv in the cytosol and ER showed to be resistant to TBSV and RCNMV infection. The resistance against the homologous and a heterologous virus upon scFv expression in the cytosol or the ER indicates that the folding of the scFv upon expression in the plant cell is adequate to preserve an efficient binding to the viral RdRps. Further experiments have to be done to clarify the efficient mechanism of inhibition upon scFv expression in the ER.

Wide-range virus-resistance based on antibodies to RdRps is a successful new strategy and adds a new tool to the repertoire for combating plant viruses.

II. Introduction

Plant viruses cause serious losses of yield and quality in many crops. Viruses may cause mild or even symptomless infection, which nevertheless is, however, associated with an economically significant reduction in yield, but also cause catastrophic disease outbreaks resulting in complete loss of marketable crops.

In contrast to the control of fungal diseases no chemical pesticides are available for use as direct antiviral agent, thus alternative control strategies are required. Effective measures can include control of viral vectors, use of virus free seed material or production of pathogen free plant stocks and good agricultural practices to minimise transmission from infected plants. Traditional plant breeding such as crossing, mutant screening and backcrossing, has been successful applied to develop resistance to a number of agronomically important crop pathogens. However, this strategy is time-consuming and does not guarantee that the selected resistance will be durable. Production of stable resistant varieties is, however, the most important and efficient of the control measures.

Genetic engineering to develop virus resistance in plants has been studied more widely than for any other group of pathogens, because of the relatively simple nature of viruses. It is generally easier to identify and clone a viral gene rather than genes from other plant pathogens. Due to the shortage of natural resistance genes, genetically engineered viral resistance has been focused on " pathogen-derived resistance" using viral sequence to increase resistance by interfering with some particular aspects of the viral life cycle (Hamilnton 1980; Abel et al., 1986). A number of transgenic crops tolerant or resistant to particular viruses have been developed using this strategy (review by Lomonossoff 1995; Beachy, 1997). However, this approach may have undesirable consequences, such as the potential restoration of wild-type viruses or generation of new viruses by recombination events, and concern has been raised over its safety (Aaziz and Tepfer 1999; Borja et al., 1999; Rubino et al., 1999).

Antibodies are a major host defence against many viruses in vertebrates, and the mechanisms of interaction between the antibodies and viral antigens are now being understood. But the application in plant virology has been under-utilised yet. The mechanisms of virus neutralisation can include virus aggregation, masking of receptor attachment sites, steric

inhibition of receptor binding, stabilisation of the capsid or replicases (De Jaeger et al., 2000). These mechanisms offer an untapped potential to engineer virus resistance into plant and elucidation of structure-function relationships in vivo.

Therefore an alternative approach to create plants that are resistant to pathogens is the expression of antibodies or antibody fragments that bind to and inactivate pathogens in plants. This strategy progressed by a better understanding of the mechanism of plant diseases and the identification of many proteins critical to pathogen infection, development, replication and spread of the pathogen in question. This knowledge is fundamentally useful for developing antibody-based resistance, because it allows the tailoring of antibodies that bind to and inactivate key targets in pathogenesis. Building on these efforts, the first step towards the generation of pathogen-resistant plants requires antibody selection and cloning, efficient antibody expression, antibody stabilisation and targeting to appropriate cellular compartments to efficiently inhibit the pathogen in question.

Functional whole molecule antibodies (rAbs) were first expressed in transgenic plants in 1989 (Hiatt et al., 1989). Expression of the whole recombinant antibody molecule in plant cells is rather difficult because of the size of the protein and its need to assemble after expression. A single chain Fv (scFv) is a part of an antibody molecule consisting of variable heavy chain (V_H) and variable light chain (V_L) domains. It is the smallest fragment that maintains the binding specificity and affinity of the whole antibody (Glockshuber et al., 1990). Therefor the expression of scFvs is more promising than the expression of the whole antibody. Many authors were able to express scFv fragments in plant cells but most of them aimed to express scFvs to modulate plant hormones (Artsaenko et al., 1995; Fiedler and Conrad 1995; Owen et al., 1992), to detect viral infection (Fecker et al., 1996; Firel et al., 1993).

Tavladoraki et al. (1993) demonstrated for the first time the expression of functional scFv expressed against the coat protein of artichoke mottled crinkle virus (AMCV) can cause viral resistance. However, using the coat protein for selection of the scFv may not result in broad range resistance, since this protein is rather variable not only in different groups of virus, but also in the same group of viruses. Moreover viral coat proteins accumulate to high amounts in plant cells making it difficult to reach a sufficient scFv titre in the plant cells to achieve complete neutralisation of the antigen.

All positive-strand RNA viruses encode a RNA-dependent RNA polymerase (RdRp) which is essential for the replication of the viral genome. RdRp is the first protein which is translated from viral genome by the host translation machinery. Most of viral RdRps contain a conservative region named palm domain, which consists of different motifs. This domain functions as an active centre for RNA transcription (Koonin 1991). Therefore using this region as the target for scFvs to inhibit the activity of the viral polymerase might more easily results in broad range resistance.

In the present study it was tried to verify this theory by producing and expressing scFvs against different motifs of the replicase of Tomato Bushy Stunt Virus (TBSV)-BS3-Statice. This viral strain was characterised and cloned by Galetzka et al. (2000) before.

Symptomatology, Host Range and Geographic Distribution of Tombusviruses

Tombusviruses show a wide geographic distribution being reported in North and South America (Pontis et al., 1968) , several locations in Europe, Mediterranean countries and also in Algeria. Generally individual members of the tombusvirus group have, however, a restricted geographic distribution with the exception of tomato bushy stunt virus (TBSV) and pelargonium leaf curl tombusvirus (PLCV) which were probably spread internationally through infected geraniums. Most tombusviruses naturally infect only a single or a few hosts. This is in contrast to their experimental host range which is wide and diversified comprising as a whole over 120 plant species in 20 different monocotyledonous and dicotyledonous plant families (Hollings and Stone 1965; Hollings and Stone 1970). Tombusviruses systemically invade naturally infected plants but remain localised in the majority of experimentally infected plants; common exceptions to this rule are the experimental hosts *Nicotiana clevelandii* and *Nicotiana benthamiana.* Tombusvirus-infected crops generally show stunting and diffuse mottling and malformation of leaves. Characteristic lesions associated with the indicator plants basil, globe amaranth and *Chenopodium quinoa willd* can be used to identify tombusviruses at the group level. However, identification of individual tombusviruses using host responses alone can not be relied upon. The diseases, host ranges and geographical distribution of tombusviruses have recently been reviewed by Martelli et al. (1988).

Fig. 1 Statice (*Goniolimon statarienum*) field infected with the Statice strain of TBSV-BS3. Infected plants show malformation, do not develop flowers and often die.

Transmission

Tombusvirus particles are very stable and reach high concentrations in infected tissues. In addition, tombusviruses are efficiently spread and can become established in diverse environments. Different tombusviruses have been shown to be spread in nature by a number of means including seed and pollen transmission, transmission through propagation material and possibly mechanical transmission. There is little doubt that several tombusviruses are even spread through the soil. Attempts to transmit different tombusviruses using aphides, white flies or mites have been unsuccessful (Orlob 1968). Several reports have identified tombusviruses in association with rivers and lakes throughout the world, and there is evidence that tombusviruses can enter a field through irrigation water (Tomlinson and Faithful 1984; Koenig and Lesemann 1985). TBSV is still infectious after passage through the human alimentary tract, suggesting that man may be an important carrier as tombusviruses may possibly enter rivers through sewage treatment of infective faeces (Tomlinson et al., 1982). Experimentally, tombusviruses are readily transmitted via sap, and infected leaf extracts retain infectivity after freezing for several years.

Genome structure and molecular biology

Tombusviruses have noneveloped 30 nm isometric particles consisting of 180 copies of a single type of coat protein subunits of approximately 41kD (fig.2). The genome is a single copy of a linear monopartite positive polarity RNA approximately 4.7 kb in length (Russo et al., 1988; Rubino et al., 1995). The greatest sequence diversity among different tombusviruses is in the capsid protein with the protruding domain exhibiting the most extensive variation. The high degree of nucleic acid sequence similarity among members of the tombusvirus group suggests, however, that tombusviruses should be considered as related strains with distinctive biological properties rather than separative viruses.

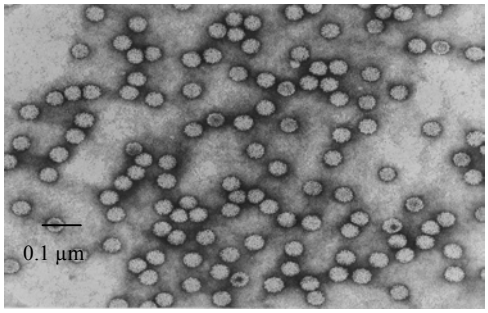

Fig. 2 Electromicroscopie picture of TBSV-BS3-Statice

The genomic organisation of tombusviruses is shown in fig.3. The tombusvirus genome consists of five long open reading frames (ORFs) encoding proteins with approximate molecular masses of 33, 92, 41, 22 and 19 kD. The 33 kD (ORF1) protein terminates with an amber codon. Readthrough of this amber terminator produces a protein of 92 kD (ORF2). ORF3 codes for 41 kD protein. The 19 kD (ORF5) is completely nested within a different reading frame of the 22 kD (ORF4) (Russo et al., 1994).

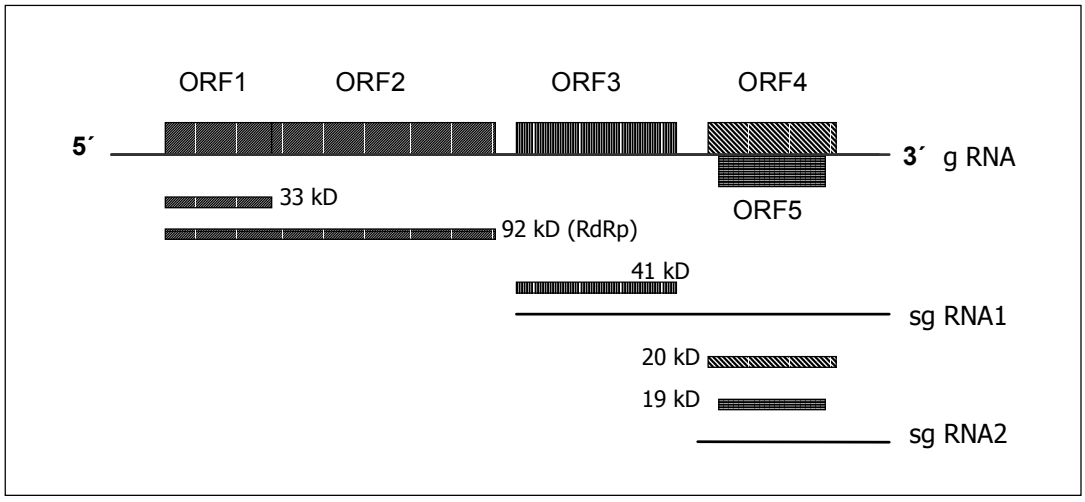

Fig. 3 Schematic representation of the genomic organisation of TBSV.

The genomic of TBSV consists of 5 open reading frames. The ORF1 and 2 are translated from the viral genome. The ORF3 is translated from subgenomic (sg) RNA1 and the ORF 4 and 5 are translated from sgRNA2.

The role of the different proteins encoded by the Tombusvirus genome in the viral replication cycle.

1. The 33K and 92K Proteins

The amino acid sequence of the 33 kD protein does not contain distinct motifs that may help us to understand its role. It lacks domains involved in virus replication like a methyltransferase domain usually near the N terminus, which is involved in the replication of the genome of viruses belonging to the "Sindbis-like" group of positive-stand viruses (Rozanov et al.,1992). However, it was clearly shown that expression of the 33 kD protein is necessary for replication of the Cymbidium ringspot virus (CyRSV, tombusvirus) RNA, because the introduction of a frame shift in ORF 1 with formation of a stop codon at position 650, resulting in the synthesis of a truncated protein of about 16 kD, or the substitution of the stop codon at position 1048 with a methionine codon, abolished infectivity (Dalmay et al., 1993). These results also showed that expression of the 92 kD alone is not sufficient to promote replication of CyRSV RNA. When antibodies raised against the 33 kD protein or the readthough region of the 92 kD protein were used for western blot analysis of tissues infected by TBSV-Ch (Scholthof et al., 1993) or CyRSV (Lupo et al., unpublished observation), it was shown that both types of protein are produced *in vivo*, the 33k in larger amounts than the 92 kD protein.

The 92 kD protein contains the canonical Gly-Asp-Asp tripeptide and surrounding hydrophobic amino acids which are characteristic for putative polymerase domains of most RNA viruses sequenced to date and thus likely forms at least part of the viral RNA replicase. The tombusvirus genome lacks coding information for the helicase domain found in most RNA viral genomes, leading to speculation that such a function may be acquired from a host protein during infection.

2. The 41 kD protein

The 41 kD protein has been demonstrated to be the coat protein (CP) based on *in vitro* translation and immunoprecipitation studies (Burgyan et al., 1986; Hayes et al., 1988, Johnston and Rochon 1990; Li et al., 1993) as well as amino acid sequence similarities with the coat protein of the BS-3 strain of TBSV, which was sequenced directly (Hopper et al.,

1984). Because the coat protein may have additional functions beside protecting the viral genome from the external environment, in different laboratories viral mutations were established that do not express this protein or express it in mutated form, and the effect of mutation on viral replication and movement was studied.

Taken altogether, the results obtained with CP mutants of tombusviruses permit the following conclusions to be drawn (Russo et al., 1996).

a) The CP gene is dispensable for replication and the presence of whole virus particles is not necessary for short-distance movement.

b) Depending on the host and the virus, long-distance movement is favoured by the presence of virions; this, however, does not constitute a strict requirement for systemic host invasion. Tombusvirus RNA, probably complex with a movement protein (the 22 kD protein), may pass through plasmodesmata from an infected cell to the next until it reaches the vascular tissues for long-distance spread. However, movement through the vascular system in this form may expose the putative RNA-protein complex to nuclease attacks, which may account for the relative inefficiency in systemic invasion of *N. clevelandii* by CyRSV mutant CP, and of both *N. clevelandii* and *N. benthamiana* by mutants lacking a long portion of the CP gene (Dalmay et al.,1992; McLean et al., 1993; Scholthof et al., 1993).

3. The 22 kD and 19 kD proteins

Two small nested genes, located near the 3´terminus of the tombusvirus genome, are translated from a second subgenomic RNA (Hillman et al., 1989; Rochon and Johnston 1991) to yield two proteins (Hayes et al., 1988; Johnston and Rochon 1990) of 22 kD (p22) and 19 kD (p19). Point substitutions introduced into the AUG codons for the 22 and 19 kD proteins have demonstrated that both proteins are likely to be produced *in vivo* since full-length or subgenomic-length transcripts which lack either of the AUG codons alter the infectivity, symptomatology and *in vitro* translation profiles of Cucumber necrosis virus (CNV) transcripts. The 22 kD protein seems to be essential for viral RNA accumulation since inoculation with full-length transcripts containing a mutated 22 kD AUG codon does not result in symptoms, and viral RNA cannot be detected by northern blot analysis. It is possible

that the 22 kD protein has a role in cell to cell movement but this has yet to be substantiated (Dalmay et al., 1993; Rochon and Johnston 1991; Russo et al., 1994; Scholthof et al., 1993). Plants inoculated with mutant transcripts which lack the AUG codon show symptoms of viral infection, but the symptoms are very mild in comparison to the symptoms produced by the wild-type transcripts. Examination of an infected leaf which was sub-inoculated from transcript-inoculated plants revealed that 19 kD mutant inoculated plant accumulates high levels of symptom-attenuating CNV defective interfering (DI) RNAs which are generated *de novo* from the mutant transcript. Since such a rapid accumulation of DI RNA is not observed in wild-type inoculated plants, it would appear that the CNV 19 kD protein plays a role in viral RNA replication preventing the formation or accumulation of aberrant replication products such as DI RNAs. It is also likely that the DI RNAs formed in 19 kD mutant infected plants are responsible for the observed symptom attenuation. However, even early in infection when DI RNAs are not detected, 19 kD mutant plants still show only mild symptoms (Rochon and Johnston 1991).

In vitro and *in vivo* studies have demonstrated that a single subgenomic mRNA acts as the template for the expression of both the 22 and 19 kD proteins. Expression of both proteins from this bifunctional subgenomic mRNA may be regulated by a leak scanning mechanism, as the upstream AUG codon (22 kD protein) has been found to be in a suboptimal context for efficient expression in animal or plant cells whereas the downstream AUG codon (19 kD protein) is in a more favourable context (Rochon and Johnston 1991).

Viral replication

Replication of plant viruses has been notoriously difficult to study directly because of the problems associated with obtaining synchronous infection of whole plants. Since plant cells that have had their cell walls enzymatically removed regenerate walls fairly quickly, stable cultures of plant cells without walls are not available as they are for many animal species. Therefor, experiments aimed at examining timing of events during synchronous replication of plant viruses must be performed in protoplasts isolated from whole plants, suspension cultures, or callus cultures (Hillman 1998).

A RNA virus replicase specifically replicates its own RNA or very closely related viral RNAs. This specificity is determined by recognition of and attachment of RNA viral

replicases to certain structures in the viral RNA. The whole step of viral replication can be concluded in 4 steps as in the schematic picture (fig.4).

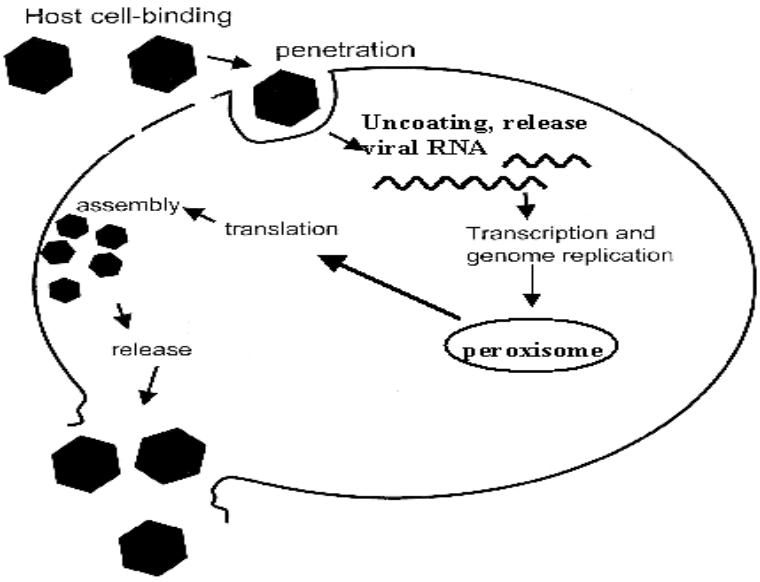

Fig. 4 Overview of TBSV replication

The replication cycle consists of several steps:

1. Virus reaches plant cells by a vector or mechanical inoculation
2. Viral polymerase is translated by the host translation machinery and transcribes the viral RNA at the peroxisome membrane
3. Viral proteins (coat protein and movement protein) are produced.
4. Generation of new virus particles (overview of viral replication see http://ag.arizona.edu /~zxiong/)

One of the major cytopathological features of tombusvirus infected plant tissue is the occurrence of membranous cytoplasmic inclusions called multivesicular bodies (MVB) in infected cells (Martelli et al., 1988). MVB consist of a main body surrounded by many spherical to ovoid vesicles measuring 80-150 nm in diameter. These vesicles contain fine fibril material consisting of double-strand RNA (Martelli et al., 1984; Di Franco et al., 1984). More recently the proteins of the replicase complex of TBSV (Scholthof et al., 1995) were found to be associated with membranous cell components sedimenting at 3000 g (Lupo et al., 1994). This provides evidence that MVB are the site of tombusvirus replication. Ultrastructural and cytochemical studies have shown that, in the cell of *N. bentamiana* and *N. clevelandii* systemically infected by TBSV, the MVB contained glycolate oxides and were derived from peroxisomes (Russso et al., 1983; Martelli et al., 1984).

Viral RdRp

RNA-dependent RNA polymerases (RdRps) function as the catalytic subunit in concert with host proteins and sometimes viral proteins in the replication of the viral genome. (Buck 1996; Lai 1998) and they are required for the replication of all positive strand RNA viruses. Currently, there are eight conserved RdRp motifs (Koonin 1991; Poch et al., 1989). Four of these eight conserved motifs are now known to be present in all classes of polymerases and reside in the catalytic portion of the " palm" domain (Hansen et al., 1997; Ollis et al., 1985). The motifs of the palm sub-domain are named A, B, C and D, plus a fifth motif, E , unique to RdRp and Reverse transcriptase (RTs) (Poch et al., 1989) (see fig.5).

Motif A

The motif A of TBSV p92 is composed of ß-strand, turn, α-helix followed by a longer α-helix. Motif A is involved in magnesium coordination and possible sugar selection. The aspartate near the end of the ß-strand is completely conserved (Argos 1988; Delarue et al., 1990; Koonin 1991; Poch et al., 1989). This aspartate is likely involved in coordination of divalent cations during nucleotidytransfer catalysis (Steitz 1998). The mutation of this residue or even subtle changes at this position cause the polymerase to be completely inactive or nearly inactive *in vitro*.

Motif B

The first half of motif B contains a highly conserved glycine in the loop. (Jacobo-Molina et al., 1993). The mutation of the conserved glycine to arginine, leucine or aspartate completely abolishes polymerase activity *in vitro* (Lohmann et al., 1997). The specific function of this residue remains to be elucidated. The extended α-helix of motif B contains the highly conserved asparagine residue. This residue is also thought to play a role in the discrimination of ribose versus deoxyribose during the replication.

Motif C

Motif C forms a ß-strand, turn, ß-strand structure that contains the highly conserved GDD motif found in RdRps (Kamer and Argos 1984; Koonin 1991). This structure is very similar in all classes of polymerases (Hansen et al., 1997). The first aspartate of the GDD motif is thought to be involved in coordination of a second divalent cation. There appears to be a strict requirement for the first aspartate as any changes to this position, including a change to a

negatively charged glutamate, are almost never tolerated for *in vivo* viral replication and/or *in vitro* RNA synthesis (Inokuchi and Hirashima 1987; Longstaff. et al., 1993). The second aspartate of the GDD motif is not absolutely conserved in all classes of polymerases, suggesting some flexibility at this position.

Motif D

The structure of this motif completes the core palm structure (Hansen et al., 1997). There is some variability in the structure of motif D in published polymerase crystal structures (Hansen et al., 1997). Up to now, it is unclear whether motif D in all RdRps will adopt the α-helix, turn, ß-strand structure observed in poliovirus polymerase. Additionally, the function of the residues in this motif are unclear.

Motif E

The hydrophobic residues in this motif are important for the interaction with the palm core structure. The hydrogen bond interactions with the ß-strand of the thumb are thought to be important in proper positioning of the thumb on substrate binding (Hansen et al., 1997; Jacobo-Motina et al., 1993). TBSV p92 is predicted to have an α-helix in this region, and it is unknown whether an α-helix in this position can also act as thumb clamp.

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METIKRMIWP KKEIFVGDFA IGVNRTAPVD IFQLVCRVVL RYMRTGKIEC   50
DSDSITKFVI ELLKTDCAAK WEWFMKRRQR GDYIIPLSIA SLPIIPLLSY  100
TTRVRAVSVK AFGNELSFNI RVPRPSVPKK GLLLRLAAGL ALAPICALAM  150
YATLPREKLS VFRLRTEART HMEDEREATD CLVVEPAREL KGKDGEDLLT  200
GSRMTKVTAS TGRPRRRPYA AKIAQVARAK VGYLRNTPEN RLIYQRVMIE  250
IMDKDCVRYV DRDVILPLAI GCCFVYPDGV EESAALWGSQ ESLGVK.GGL  300
VRLPGVVTQI NRDIPSDVLL PQEVLEVRTG PPNAKDRNIF MVAGCPSQAR  350
FLVHNHCLKN LKRGLVERVF CVERNGKLTR TPQPTKGAFG RLSPFRKAVC  400
EKVGVAHRYG YDGFLSYYSG AKLRTYTRAV ESLHITPVSE RDSHLTTFVK  450
AEKISTAKSD PAPRVIQPRN PRYNVELGRY LRHMESKLMK AVDGVFGETT  500
CIKGYTADEV GAIFREKWDR FDKPVAIGLD ASRFDQHCSM EALQYEHSFY  550
RAMYPGNKLL SKLLEWQLHN KGKGYVPDGT ITYRKEGCRM SGDINTSLGN  600
YLLMCAMVHG YMRHLGINEF SLANCGDDCV LIVERRNLKQ VQRTLPEYFL  650
NLGYTMKVEA PVFQMEEVEF CQAHPVQFQG GWKMVRNVRT AMSKDVHCVN  700
NIRDLATRKA WSNAQHHGGL ALSAGIPVVE RFYSRFTLYD TPRKHQRIDT  750
VTNVHKWRGS GGSYVVTPEA RASFWAAFGL TGDEQLALED RLDRWEMDLF  800
GIEGVDAHEP SILDSAVA  818
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Fig. 5 TBSV replicase protein sequence; **blue alphabet** = motif A, **pink alphabet** = motif B, **light green alphabet** = motif C, **green alphabet** = motif D and **dark red alphabet** = motif E

In this study several parts of the TBSV-BS3-Statice RdRp, carrying 5 motifs were cloned and expressed in *E. coli*. The purified proteins were used for scFv selection from scFv libraries and the selected scFvs were tested for their RdRp inhibition activity in *in vitro* and *in vivo* assays. Finally the resistance behaviours of transgenic plants expressing selected scFv were examined by challenge inoculation with different viruses.

III. Materials and methods

Chemical substances and reagents which were not specially mentioned in the text were purchased from Boehringer (Mannheim), Merck (Darmstadt), Sigma (Deisenhofen), Roth (Karlsruhe), Difco Laboratories (Detroit, MI, USA). Restriction enzymes and other DNA modified enzymes or DNA standards were purchased from GibcoBRL (Eggenstein). The media and hormones for establishment the transgenic plants *in vitro* culture were purchased from Fischer Lab world (Nidderau) and Neolab company (Heidelberg). For the media and buffers preparation see appendix I and for the plasmid maps see appendix II

Molecular cloning

1. Cloning of RdRp fragments

1.1 Amplification of the DNA fragments

To express the target proteins for using to select scFvs from the single chain library, different parts of replicase protein were cloned in expression plasmid. The ORF1 gene which code for the p33 protein, a part of ORF2 starting after amber codon till 3´end and the ORF2 gene which code for RNA dependent RNA polymerase (RdRp, p92) and the gene which codes for only five motifs were amplified and *BamHI* and *SalI* restriction sites were introduced by PCR. The oligonuecleotides which were used as the primer are listed in table 1. The full clone of TBSV (DM1) (Galetzka *et al*., 2000) was used as the template. The PCR products were electrophoresed to check the amount and size and were then cloned into plasmids (e.g. pET23a^{+} and pET26b^{+})which were digested with the same enzymes.

The PCR cycles were:

Cycle 1	94°C, 5 min
Cycle 2-41	Denaturation at 94°C, 30 sec
	Annealing at X°C, 30 sec
	Extension at 72°C, Y sec

The annealing temperature was derived from the lowest melting temperature of the two primers minus 5 degree. The extension time was calculated from the amplified size with approximately 50 bases per second. The PCR products were checked by electrophoresis.

Table 1. The primers which were used for cloning of the replicase fragments in expression vectors.

Segment	Forward primer	Reverse primer
P33	**p33BamHI5´** 5´-ACG ATG GCG GCC GCG GCC CAG CCG GAT CCA TGG AGA CCA TTA AGA GGA TGA- 3´	**2.Rep3´prt33** 5´-TGG TCA GTC GAC TTT GAC ACC TAG GGA TTC CTG TGA ACC-3´
P55	**p55BamHI5´** 5´-ACG ATC GCG GCC GCG GCC CAG CCG GAT CCA TGG GAG GCC TAG TAC GTC TAC-3´	**2.Rep3´prt55** 5´-TGG TCA GTC GAC TGC TAC GGC GGA ATC AAG GAT GCT-3´
P92	**p33BamHI5´** 5´-ACG ATG GCG GCC GCG GCC CAG CCG GAT CCA TGG AGA CCA TTA AGA GGA TGA- 3´	**2.Rep3´prt55** 5´-TGG TCA GTC GAC TGC TAC GGC GGA ATC AAG GAT GCT-3´
5 motif	**5motifsBamHI5´** 5´-GGC CCA GCC GGA TCC GGC TGC CGC ATG AGT GGG-3´	**2.Rep3´5motifs** 5 -TGG TCA GTC GAC TGG AAT TCA ACC TCT TCC ATT TG-3´

1.2 Low melting agarose Extraction

The digested DNA or plasmid was loaded in a low melting gel and electrophoresis was done at 80 volt. The expected bands were cut out under a UV light detector. The piece of the gel was put in a eppendorft cup and 100 µl of TE buffer were added. The mixture was incubated at 75°C for 5 min, then 300 µl of phenol were added. The mixture was vortexed for 1 min and centrifuged for 10 min at 4°C. The upper part of the mixture was transferred to another cup. 200 µl of phenol/chloroform (1:1) mixture were added and the mixture was centrifuged again for 2 min at 13000 rpm. The upper part was transferred to a new cap, and 200 µl of chloroform were added, then the mixture was centrifuged for 1 min. The upper part was transferred and the amount was measured. 1/10 volume of 2 M NaCl and 2.5 volume of 100% EtOH and 1 µl of tRNA (5 µg/µl) were added. The mixture was stored at –20°C for 30 min, then it was centrifuged at 13500 rpm for 10 min. The supernatant was discarded and 100 µl of 70% EtOH were added. The mixture was centrifuged for 2 min at 13000 rpm and the supernatant was discarded. The DNA pellet was dried in a speedvac or at room temperature.

10 □l of H_2O DEPC were added. 2 □l of the solution were electrophoreses to detect the amount of DNA.

1.3 Ligation

An expression vector (table 2) and DNA-fragments with compatible cohesive termini were ligated in 10 µl reaction mixture containing DNA (molar ratio vector : DNA-fragment, 1:2),1 µl 20 mM $HOCOCL_2$, 1□l PEG 8000 40%, 1□l 10x ligation Buffer and 0.5 µl ligase enzyme. The mixture was incubated at room temperature for 4 h or overnight.

Table 2. Plasmids which were used for cloning of the replicase fragments; the map of these plasmids can be seen in Appendix I.

Vector	Resistant gene	Promoter	Tag at C-terminus	Tag at N-terminus	Signal Tag
pET 23	Amp^R	T7	His-Tag	T7-tag	-
pET 26	Kan^R	T7lac	His-Tag	-	Periplasmic localization
pET 43	Amp^R	T7lac	HSV-tag and His-Tag	S-tag, Nus tag	-

*All plasmids listed in table 2 were purchased from NOVAGEN.

1.4 Transformation

1.4.1 Competent bacteria

The bacteria (table 3) were cultivated overnight at 37°C 180 rpm. The next morning 20 µl of inoculated overnight bacteria were cultured in 10 ml of LB media at 37°C for 3.5 h. Then they were centrifuged for 5 min at 6000 rpm. The supernatant was discarded and the pellet was resuspended slightly with 4 ml of KZB solution and put on ice for 20 min. The mixture was then centrifuged at 6000 rpm for 5 min, the supernatant was discarded. The pellet was

resuspened again with 600 µl of KZB solution. 50-100 µl of competent cells were used for transformation.

Table 3. The bacterial strains which were used for transformation and expression of the replicase fragments.

Bacteria	Function	Characteristics	Source
DH5α	Cloning	Φ80d*lac*ZΔ M15	GIBCO BRL
BL21(DE3)	Expression of the Replicase parts	F^-*omp*ThsdS$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Udo Conrad, IPK Gatersleben
BL21(DE3)pLysS	”	F^-*omp*ThsdS$_B$($r_B^-m_B^-$)*gal dcm* (DE3)pLysS(Cm^R)	NOVAGEN
JM109(DE3)	”	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^- m_k^+), *rel*A1, *sup*E44,λ-,Δ(lac-*pro*A^+B^+),[F^-, *tra*D36, *pro*AB, *lac*I^qZΔM15], λDE	Promega
Novablue(DE3)	”	*end*A1*hsd*R17(r_{k12}^- m_{k12}^+)*sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F-[*pro*A^+B^+ *lac*I^q ZΔM15 ::Tn 10(Tc^R)] (DE3)	NOVAGEN

1.4.2 Transformation

The cloned plasmids were transformed in competent *E. coli* (DH5α, Gibco BKL). Two micro-litre of each ligation mixture were added in 25 µl of competent DH5α and incubated on ice for 30 min. After that a heat chock was performed by incubating the mixture at 42°C (water bath) for 45 seconds. Then it was put on ice, 175 µl of SOC solution were added to the mixture and then incubated at 37°C for 1 h. The mixture was plated on an antibiotic supplemented LB Agar plate and the plate was incubated overnight at 37°C.

1.5 Mini-preparation of plasmid DNA

2 ml of LB medium were inoculated with a single colony in the presence of the appropriate antibiotic. The medium was inoculated overnight with vigorous shaking at 37°C. The overnight culture was spined down for 1 min at 13000 rpm. The pellet was resuspended in 100 µl lysis buffer (GTE solution) and incubated for 5 min at room temperature. 200 µl NaOH/SDS solution were added and incubated on ice for 5 min. Then 150 µl Na-acetate (5M pH 4.8) was added and mixed by vortexing 2 seconds and incubated on ice again for 5 min, then it was centrifuged for 3 min at 13000 rpm. 400 µl supernatant were transferred to a new eppendorf tube. 800 µl of 100 % EtOH were added to precipitate the plasmid DNA and incubated at room temperature for 2 min. Then the mixture was centrifuged at full speed for 3 min. The supernatant was removed and the pellet was once washed with 1 ml 70% ethanol by centrifugation for 2 min at full speed. The supernatant was removed and the pellet was dried under vacuum. The pellet was dissolved in 15 µl H_2O. Three microlitre of the pellet solution were digested with suitable restriction enzyme.

1.6 Digestion of cloned plasmids

For analytical digests 1 µg of DNA was digested with 10 U of the restriction enzyme in question and 2 µl 10x enzyme buffer in a total volume of 20 µl. One microlitre aliquot was taken after 1 h of digestion and 1 µl loading dye and H_2O were added to 10 µl end volume. The solution was loaded in the electrophoresis gel to check for complete digestion. Completely digested plasmids were precipitated by adding 137 µl of 100% ice cold EtOH and 5 µl 2 M NaCl. The solution was incubated at room temperature for 2 min and then centrifuged by 13000 rpm for 10 min. The supernatant was discarded and 100 µl 70% ice cold EtOH were added. The solution was incubated at room temperature for 2 min and then centrifuged again for 5 min at 13000 rpm. The supernatant was discarded and the pellet was dried under vacuum. 10 µl of DEPC treated water were added to resuspened the digested plasmid.

1.7 Purification of plasmid

A positive colony was inoculated in 10 ml antibiotic supplemented LB at 37°C by shaking over night. The next morning the bacteria cells were harvested by centrifugation at maximum

speed (13000 rpm) for 2 min and the supernatant was discarded. The pellet of bacteria cells was purified by Nucleo Spin Plus kit (Macherey-Nagel company) as follows. The pellets were resuspended by vigorous vortexing in 250 µl buffer A1,which added RNase. 250 µl buffer A2 were added. The suspension was mixed gently and incubated at room temperature for maximum 5 min. 300 µl buffer A3 were added and the suspension was mixed gently by inverting the tube 6-8 time. The mixture was centrifuged at 13000 rpm for 12 min. The supernatant was added in the spin tube and centrifuged at maximum speed for 1 min. The flow through was discarded. 700 µl buffer A4 was added in the spin tube and the mixture was centrifuged at maximum speed for 1 min. The flow through was discarded and 50µl AE buffer was added and centrifuged at full speed to elute the DNA. The concentration of DNA was measured by mixing 5 µl of the DNA solution and 95 µl of water. The mixture was measured with a photometer (UV-160 1PC Schimadzu) at 260 and 280 nm. ΔE_{260} = 1, DNA[concentration] = 50 µg/ml (Sambrook et al., 1989).

1.8 Agarose gel electrophoresis

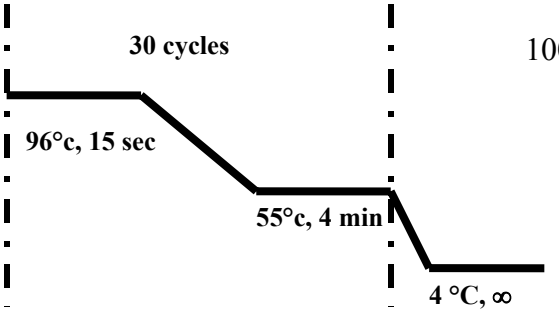
To 2 µl of digested DNA 7 µl of water and 1 µl of 10x loading buffer were added. The DNA mixture was loaded in 1 % TBE agarose gel to separate DNA fragments according to the size. To stain the DNA 0.5 µl Ethidium bromide (1%, 10 mg/ml) was added before pouring the gel. The electrophoresis was performed in TBE buffer for 30 min at 100 V. The DNA fragments were visualised by illuminating with UV light.

1.9 Sequencing

The recombinant plasmid was confirmed to be in frame by sequencing. 700 ng of purified DNA were added in the reaction mix. The reaction mixture was amplified by PCR by using the program as shown below.

Reaction mix:

100-500 ng	DNA
4 µl	Bigdye (Gentreprise, Mainz)
1 µl	10 pM Primer
ddH_2O	to 20 µl



PCR cycle for DNA sequencing

20 µl of PCR products were precipitated with 2 µl of 3 M pH 5.2 sodiumacetate pH. 5.2 and 50 µl 100% EtOH. The mixture was incubated on ice for 10 min, then centrifuged by 13000 rpm for 30 min. The supernatant was discarded and 200 µl 70% EtOH were added. The mixture was centrifuged again at 13000 rpm for 10 min. The supernatant was discarded and the pellet was dried by speed vacuum. The dry DNA was sent to analyse to Genterprise GmbH in Mainz.

2. Bacterial expression of RdRp fragments

2.1 Induction

The target genes were cloned under the T7 promoter which can be induced to express the proteins by adding IPTG. A single colony was inoculated in 5 ml LB media supplemented with antibiotics and incubated by shaking overnight at 37°C. The overnight culture was diluted 1:100 with fresh media supplemented with antibiotics, then incubated at 37°C with shaking till OD_{600} 0.4-0.6. 100 mM IPTG were added to the culture till the final concentration was 1 mM. A time course experiment was performed to determine the time point of maximal protein concentration in the bacterial cell after induction with IPTG. One millilitre ml of the culture was taken every h after adding IPTG. The aliquots were centrifuged for 30 seconds at 13000 rpm and the supernatant was discarded. The pellet was resuspended in 75 µl lysis buffer. The mixture was boiled for 5 min and 25 µl 4x SDS sample buffer and 10 µl □-mercaptoethanol were added. The mixture was boiled again for 5 min and 10 µl of each aliquot was applied in a 12% SDS-PAGE gel.

2.2 SDS-PAGE gel

Proteins can be separated according to their molecular weight by SDS polyacrylaminde gel electrophoresis (SDS-PAGE). For the experiments the Biorad mini-protein I dual slab gel was used. For a mixture of 10 ml of 12.5% separating gel composed of 4.2 ml of 30% / 0.8% acrylamind / bisacrylamind (Roth company), 4.6ml distilled water, 0.1 (10%) ml SDS, 1.25 ml 3 M Tris/HCl (pH 8.8), 120 µl 10% APS and 12 µl TEMED (N,N,N,N,tetramethylethy-leneamine) was poured between the glass plates. It was overlaid with 1-2 ml H_2O saturated Butanol. After polymerisation of the gel, the overlay was removed and the surface of the gel was cleaned with running buffer. A 4% collecting gel composed of 0.67 ml 30% / 0.8%

acrylaminde / bisacrylamind, 3.63 ml distilled water, 0.05 ml SDS (10%), 0.625 ml 1M Tris/HCl (pH 6.8), 120 □l APS (10%) and 12 µl TEMED was poured onto the separating gel, then the combs were placed. Electrophoresis was carried out in a Laemmli buffer system. After filling the upper and lower reservoir with running buffer, the combs were removed and protein samples were applied to the slots using a Hamilton syringe. The protein samples contained about 0.25 volumes of 4 x sample buffer and 0.1 volumes of □-mercaptoethanol. For protein stacking the gel was run at 75 volt and for the protein separation the gel was run at 140 volt, 30 mA (maximum voltage).The gels were stained for 30 min by shaking in coomasineblue staining solution and were destained for a few min with destaining solution.

2.3 Soluble and Insoluble protein determination

2.3.1 Shearing with syringe method

The bacterial expressed protein can be found in the soluble and/or insoluble (inclusion bodies) cell fraction. To determine the cell fraction which contained the target proteins a crude soluble and insoluble fraction was prepared and analysed by using SDS-PAGE. For this 2 h after induction with IPTG a 1.5 ml aliquot from the culture was taken and centrifuged for 1 min. The cell pellet was resuspended in 100 µl 50 mM Tris/HCl (pH 8.0), 2 mM EDTA. 1/10 volume of Triton X-100 and lysozyme was added to the suspension to a final concentration of 100 µg/ml. The mixture was incubated at 30°C for 15 min and the DNA was sheared with an injection syringe afterwards. The mixture was centrifuged for 15 min at 13000 rpm at 4°C. To the supernatant, which contained the soluble proteins, 25 µl 4xSDS sample buffer and 25 µl □-mercaptoethanol were added. The pellet, which contained the insoluble proteins, was resuspended in 125 µl 4x sample buffer and 25□l □-mercaptoethanol. 10 µl of each fraction were applied to a 12% SDS-PAGE gel.

2.3.2 By using BugBuster® reagent

The bacteria were induced as described above. Two millilitre of liquid culture bacteria were harvested and centrifuged for 3 min at 13000 rpm using a preweighed centrifuge tube. The supernatant was removed and the wet weight of pellet was determined. The pellet was completely resuspended at room temperature with BugBuster® (NOVAGEN) reagent by gentle vortexing, using 5 ml reagent per gram of wet pellet. The Benzonase (NOVAGEN)

was added, using 1 □l (25 units) of Benzonase per ml of BugBuster reagent for resuspension. The mixture was incubated on a shaking platform at slow setting for 10-20 min at room temperature. The supernatant was collected and loaded directly in a 12% SDS- PAGE to check the soluble protein after the mixture was centrifuged at 13000 rpm for 20 min at 4°C.

2.3.3 Osmotic Shock

The bacteria were grown and induced as described above. The cell pellet was resuspended thoroughly in 30 ml of 30 mM Tris-HCl pH 8, 20% sucrose. Then 600 µl 0.5 M EDTA, pH 8 (final concentration of 1 mM) was added. The resuspended cells were stirred slowly at room temperature for 10 min. The cells were collected by centrifugation at 13000 rpm at 4°C for 10 min. The supernatant was removed. The pellet was thoroughly resuspended in 30 ml of ice cold 5 mM $MgSO_4$ and stirred slowly for 10 min. During this step, the periplamic proteins are released into the buffer. The mixture was centrifuged for 10 min at 13000 rpm at 4°C. The supernatant was transferred to a new tube. An aliquot of this fraction was loaded in a 12% SDS-PAGE to check the soluble proteins.

2.4 Analysis of expressed proteins

2.4.1 Western blot analysis

A protein membrane (Hybond-P, Amersham Pharmacia Biotech) was cut at the size of the gel and washed with methanol for 20 sec. Then it was washed 4 times with water. This membrane was incubated in transfer buffer until use. To blot the protein on the membrane, 5 pieces of whatman paper were laid on the tray (Trans-blot SD, semi-Dry transfer cell, BIO-RAD), the gel was laid on the top, the protein membrane and the other 5 pieces of whatman paper were put on the top of the gel respectively. The electricity was applied by the ampere (mA) was calculated according the dimension of the gel (height x width x 0.8x Number of gel) and 25 volt were applied overnight. The successful protein transfer can be detected by incubation of the blotted protein membrane with Ponceau solution.

2.4.2 Immunodetection with Anti-His Antibodies (chemiluminescent method)

The protein membrane was washed twice for 10 min with TBS buffer at room temperature and incubated 1 h in blocking buffer (5% skim milk) at room temperature. The membrane was incubated in Anti-His Antibody (NOVAGEN) solution (1:1000) dilution of antibody stock solution in 5% skim milk in TBS at room temperature for 1 h. Then the membrane was washed 4 times with TBS-Tween buffer and once with TBS buffer, each for 5 min at room temperature. The membrane was incubated with 1:2000 dilution of second antibody conjugated with peroxides (Anti-mouse-POD, BM Chemiluminescence Western blotting Kit Mouse/Rabbit, Boehringer Mannheim) for 1 h at room temperature. After incubation with the secondary antibody, the membrane was washed 4 times for 10 min each time in TBS-Tween buffer. The membrane was incubated with substrate (supplied in the western blot kit) for 5 min and exposed to a film (BioMax®, Leight-1,13 x16, Sigma). The film was developed according to the manufacturer's recommendation

3. Purification of *E. coli* expressed RdRp fragments

Since most of the expressed proteins were in the from of inclusion bodies, the purification of target proteins was done under denaturing condition. The inducted cells were harvested by centrifugation at 13000 rpm, the pellet were resolubilized in 8 M Urea pH 8.0. The solution was centrifuged at 1000 rpm, 4 °C for 10 min. The supernatant was mixed with Ni-NTA resin (NOVAGEN) in a column, shaken slowly for 20 min, then the solution was flowed slowly through the column. The column was washed twice with washing buffer (8 M Urea pH 6) and the target proteins were eluted by 8 M Urea pH 4.5. The purified proteins were electrophoresed in 12% SDS-PAGE and analysed by straining with coomasineblue and western blot. The concentration was determined by using the Bradford reagent (Bio-Rad).

4. Phage Display for scFvs selection

4.1 Tritation of Phage Library

E. coli strain TG1 cells (Stratagene) were streaked on minimum media and incubated at 37°C overnight. A single colony was picked and cultured in 10 ml 2X TY broth, 37°C overnight. 100 µl of overnight culture were sub cultured in 10 ml fresh 2XTY media, at 37°C till OD_{600}

= 0.4. 100 µl of 10X dilution of the phage library (kindly provided by MRC, Cambridge University) were added to 1ml of TG1 culture. 50 µl of the mixture were plated on triple plate of TYE supplement with 100 µg/ml Ampicillin and 1% glucose, then incubated at 37°C overnight. The colonies were counted, then the titre was calculated.

4.2 Selection of scFvs

100 µl of 100 µg/ml of each target protein in 8 M Urea pH 7 were coated in immunoplate (Nunc-Immuno™ plate), then incubated overnight at 4°C. The plates were washed with PBS 3 times and 100 µl of 2% BSA in PBS were added to block unspecific binding. The plates were incubated at room temperature for 2 h, then were washed 3 times with PBS. 90 µl of diluted phages (titre about 10^{12} to 10^{13}) of the library were added and incubated at room temperature for 30 min with shaking, then the plates were incubated further without shaking for 90 min. Non binding phages were removed by washing 20 times with 1% Tween-PBS, then once with PBS. Bound phages were eluted by adding 90 µl of 100 mM Triethylamine, then incubated with shaking for 10 min. Eluted phages were transferred in a new tube and neutralised with 1 M Tris pH 7.2. Nine millilitre of TG1 cells OD_{600} = 0.4 were added to the eluted phages, then incubated in a water bath at temperature 37°C for 30 min. To titrate the eluted phages, the infected cells were serially diluted 1:10², 1:10^4 and 1: 10^6 with 2XTY media and 50 µl of each dilutions were plated on triple plates of TYE media supplement with 100 µg/ml Ampicillin and 1% glucose and the plates were incubated at 37°C overnight. The colonies were counted and the titre of eluted phages was calculated. The remaining cells were spanned down at 3300 rpm for 10 min. The pellets were resuspend in 1 ml of 2XTY media, then plated on large square Bio-assay dish TYE supplement with 100 µg/ml Ampicillin and 1% glucose, incubated at 37°C overnight. The cells on large square Bio-assay dish were washed off with 7 ml of 2XTY media supplement with 15 % glycerol. 50 µl of these cells were cultured in 50 ml of 2XTY supplement with 100 µg/ml Ampicillin and 1% glucose, incubated at 37°C till OD_{600} = 0.4. To rescue the phages, 5X10^{10} Helper phages were added into 10 ml of the culture. The mixture was incubated at 37°C for 30 min, the mixture was spanned down at 3700 rpm for 10 min. The pellet were resuspended in 50 ml 2XTY media supplemented with 100 µg/ml Ampicillin and 50 µg/ml Kanamycin, incubated overnight at 30°C 160 rpm. The overnight culture was spanned down at 3700 rpm 15 min. Ten millilitre of PEG/NaCl were added into 40 ml of supernatant, then the mixture was incubated on ice for 1 h. The mixture was spanned down at 3700 rpm for 30 min. The pellet was resuspended in 1.6

ml of TE and 0.4 ml PEG/NaCl, then incubated on ice for 20 min and spanned down at 9000 rpm for 10 min. The pellet was resuspended in 1.2 ml PBS and spanned down at 11600 g for 10 min. 600 µl of supernatant was used for the next round of panning.

4.3 Monoclonal phage ELISA

Single colonies from the 3rd round of panning were picked and cultured in 96 microtitre plate in 200 µl of 2XTY media supplemented with 100 µg/ml Ampicillin and 1% glucose and shaken at 300 rpm at 37°C overnight. 2 µl from each well were transferred into a new microtiter plate with 200 µl of 2XTY media supplemented with 100 µg/ml Ampicillin and 1% glucose, incubated at 37°C for 30 min then shaken at 300 rpm overnight. The culture plates were centrifuged at 1800 rpm at 4°C for 10 min. The pellets were resuspended in 200 µl of 2XTY supplement with 100 µg/ml Ampicillin and 50 µg/ml Kanamycin, then incubated at 30°C 300 rpm overnight. The overnight culture was spanned down at 1800 rpm for 10 min. Forty microlitre 6% BSA-PBS were added to 40 µl of the supernatant. The mixture was added to microtitre plates coated with 100 µl of each antigens (100 µg/ml) and the unspecific binding was blocked with 3% BSA-PBS. The plates were incubated at room temperature for 90 min, then washed 5 times with PBS-1% Tween 20. A 1:2000 dilution of Anti M13 antibody conjugated with Horse reddish peroxidase (HRP-Anti M13, Sigma) in 3% BSA-PBS was added and incubated at room temperature for 90 min. The plates were washed 5 times with PBS-0.1% Tween 20, then 80 µl of a freshly prepared substrate solution were added. Forty microlitre of 1M sulphuric acid were added to stop the reaction when the colour of reaction turned blue. The reaction was detected in a ELISA reader at 650 and 450 nm.

4.4 Soluble expression of scFvs

To expressed soluble scFv, the positive monoclonal phages have to infected HB2151 *E. coli* strain (Stratagene). Therefore 10µl of eluted phage (10^4-10^7) were added in 200 µl exponentially growing HB2151 bacteria (OD_{600} = 0.4) for 30 min at 37°C. 50 µl of infected bacteria were plated on TYE agar plate supplement with 100 µg/ml Ampicillin and 1% glucose. The plates were incubated overnight at 37°C. The positive clones were cultured overnight in 2xTY media supplemented with Ampicillin 100µg/ml and 1% glucose. The overnight cultures were diluted 1:100 in 2xTY media supplement with Ampicillin 100µg/ml and 0.1% glucose and cultured by shaking at 200 rpm at 37°C till OD_{600} = 0.9, then IPTG was

added to a final concentration of 1 mM. The mixture was further cultured by shaking at 200 rpm at 30°C overnight. The overnight induced cultures were pelleted by centrifugation at 5000 rpm and the supernatant was saved for the detection of expressed scFvs by western blot or ELISA.

4.5 Purification of soluble expressed scFvs

The expressed scFvs clones were cultured and inducted as described above. Two hundred millilitre of the supernatant were concentrated by centrifugation through a vivaspin column (Sartorius™) which has cut off 10 KD till the total amount of supernatant was about 60 ml. Then the concentrated supernatant was loaded in a 1 ml Protein-L pack column which was pre- incubated with PBS buffer. The column was washed with washing buffer (PBS). The scFvs were eluted with 0.1 M glycine pH 2.0. 2 ml fractions were collected. 30 µl of each fraction were used to determine the amount and purity of eluted scFv by western blot. The positive fractions were pooled and dialysed against PBS pH 8.0. The dialysis bag which contained scFv solution was submerged in PEG 20000 at 4°C for concentration. The concentration of scFv was measured by the Bradford method (Bradford reagent, Bio-Rad).

5. Antibody-mediated RNA dependent RNA polymerase inhibition assay

5.1 *In vitro* assay

The RdRp of cucumber necrosis virus (CNV), a tombusvirus , was successfully purified from infected plant material and it showed the activity to amplify a given RNA template (Nagy et al., 1999). Therefore the inhibition activity of selected scFvs can be investigated by incubation of these scFvs with the RdRp prior to adding the reaction mix and the formation of *de novo* products can be measured via the incorporation of radio-labelled nucleotide.

5.1.1 Preparation of RNA templates

DI-72 plasmid (kindly provided by Nagy) was linearlised by digestion with *SmaI* for at 25 °C for 3 h. The digested plasmid was electrophoresesed to detect the result of digestion. The digested plasmids were purified by adding 70 µl of sterile water and 100 µl saturated phenol : chloroform (RNA quality). The mixture was vortexed for 30 sec and centrifuged at 13000 rpm

for 4 min. The aqueous phase was transferred into a new tube and 300 µl of isopropanol-ammonium acetate (10:1) were added. The mixture was incubated on ice for 15-20 min, then centrifuged at 13000 rpm for 10 min. The supernatant was discarded carefully. The DNA pellet was resuspended in 700 µl of 70% ethanol. The mixture was centrifuged at 13000 rpm for 5 min and the ethanol was discarded. The pellet was dried at room temperature. The pellet was resuspended with 10 µl of sterile water and 5 µl were used as the template for *in vitro* transcription.

Reaction mix:

Y	µl	dH_2O (up to volume of 50 µl)
5	µl	10 mM rNTPs (A,G,C,U) (Roche, Mannheim)
5	µl	10X T7 RNA polymerase buffer with DTT
X	µl	linearized DNA (~ 1 µg)
0.1	µl	24 U/µl RNAse inhibitor from Pharmacia
1	µl	T7 RNA polymerase (50 U/µl) (Biolab)

The reaction mixture was incubated at 37°C for 2 h. The amplified RNA was detected by electrophoresis in a 0.8 % TBE gel. The transcribed RNA was purified by adding 70 µl of sterile water and 100 µl saturated phenol : chloroform (RNA quality). The mixture was vortexed for 30 sec and centrifuged at 13000 rpm for 4 min. The aqueous phase was transferred into a new tube and 300 µl of isopropanol-ammonium acetate (10:1) were added. The mixture was incubated on ice for 15-20 min, then centrifuged at 13000 rpm for 10 min. The supernatant was discarded carefully. The transcribed RNA pellet was resuspended in 700 µl of 70% ethanol. The mixture was centrifuged at 13000 rpm for 5 min and the ethanol was discarded. The pellet was dried at room temperature and resolved with 100 µl of sterile water. The transcribed RNA was purified again by isopropanol precipitation and washing step as described above. 50 µl sterile water were added to resolve the RNA pellet. One microlitre of transcribed RNA was electrophoresed in a 0.8% TBE agarose gel to determine the purity and amount.

5.1.2 ScFv-mediated inhibition of RdRp

The reaction mix (see below) was incubated with various amounts of a scFv (0.1 µg to 0.3 µg) on ice for 30 min. Then 2 µl of purified template (*in vitro* RNA transcribed DI-72 negative strand) were added to the reaction and the mixture was incubated for further 2 h at 25°C.

Reaction mix:

2.5µl 10X RdRp buffer (500 mM Tris pH 8.0, 100 mM $MgCl_2$, 100 mM DTT)
2.5 µl 2M potassium glutamate
5.0 µl 10 mM rACG + 0.1 mM rUTP mix
0.1 µl RNase inhibitor
10 µl RdRp fraction (Mix RdRp fraction by pipetting before removing it from the tube)
Add ddH_2O to 50 µl
1 µl α-P^{32} UTP (10 µci/µl, ICN)

The reaction was terminated by adding 50 µl SDS/EDTA stop solution and 100 µl phenol-chloroform was added. The mixture was shaken for 2-3 min in a circular eppendorf tube vortex adapter (3.5 "gear"), then was centrifuged for 4 min at 13000 rpm. The upper phase of the mixture was transferred into a new tube containing 300 µl isopropanol-ammonium acetate mix (10:1) and 3 µl glycogen was added. The mixture was mixed by turning the tube upside down a few times, then incubated on ice for 10-20 min. The mixture was centrifuged for 20 min at 13000 rpm at 4°C.The supernatant was discarded and the pellet was washed with 600 µl 70% ethanol by turning the tube up and down twice and centrifuged again for 5 min at 1300 rpm at 4°C. The supernatant was discarded and the pellet was air dried for about 5-10 min. The pellet was dissolved in 16 µl dH_2O. Eight microlitre of the samples were incubated for 5 min at 85 °C, then placed immediately on ice. The samples were loaded on a 5% pre-run acrylamide/urea gel. The gel was dried followed by analysis with a phosphorimager.

5. 2 *In vivo* assay

Two different methods were applied to investigate the inhibition efficiency of the different scFvs to RdRp in plant cells. First intact leaves of *Nicotiana bethamiana* were agroinfiltrated

with a solution of *Agrobacteria* carrying the different scFvs gene under the control of the 35S promoter in their binary vector. This leads to transient expression of scFvs in infiltrated leaves.

5.2.1 *In vivo* assay by Agroinfiltration method

5.2.1.1 Cloning

The scFvs genes from pIT1 vector were subcloned in pRTRA 7/3 (kindly provied by Moenke, IPK) between *NcoI* and *NotI* restriction sites, resulting in an expression cassette in which the scFv genes were flanked upstream by 35S promoter and downstream by a terminator. The whole cassette was subcloned into a binary vector named pPZP200 (kindly provide by Jach) at the *HindIII* restriction site of the right border. The orientation of the insert was checked by digesting the plasmid with *BamHI.* 50-100 ng of positive clones were electroporated into 50 µl of the competent *Agrobacterium tumufacien* ATHV strain (2.5 volt, 25 µF and 200 ohm, Bio-Rad). The bacterial suspension was immediately added with 1 ml SOC media and the mixture was incubated at 28°C for 1 h. The electroporated bacteria were plated onto LB agar supplemented with 100 µg/ml of spectinomycin and streptomycin. The plates were incubated at 28°C till the colonies grew.

5.2.1.2 Agro-infiltration, intact leaves method

5.2.1.2.1 Preparation of *Agrobacteria* suspension

Single transformed colonies were cultured at 28°C in 50 ml of YEB media containing antibiotics with shaking until reaching an OD_{600} of about 1.0. The culture was centrifuged at 5000 g for 10 min at 15°C to pellet the bacteria cells. The cells were resuspended in 2-5 ml induction media then the cell suspension was transferred into a new Erlenmeyer flask containing 200 ml induction media. The cells were cultured at 28°C with shaking overnight to log phase (OD_{600} about 0.8). The cell suspension was transferred into GS3 tubes and centrifuged at 5000 g for 10 min at 15°C. The cells were resuspended in 2-5 ml of MMA media and the density of the cell suspension was adjusted with MMA media till OD_{600} = 2.4. The suspension was incubated at room temperature(22°C) for 2 h.

5.2.1.2.2 Infiltration of intact leaves

N. benthamiana which was not watered for one day before infiltration was infiltrated with the bacteria suspension by using a syringe. One milliliter of the cell suspension was slowly pressed into an intact leave until the suspension spread throughout almost the whole leaf. The infiltrated plants were watered and kept in the dark at 22°C overnight, then were further cultured in the green house.

5.2.1.2.3 Challenging the infiltrated plants with virus particles

Purified virus particles of TBSV-BS3-statice were diluted with inoculation buffer to at about 0.1 µg/1 µl. Ten microlitre of virus mixture was rub inoculated onto the infiltrated leave 4 days after infiltration. The plants were cultured in the greenhouse and the symptoms (local lesions and systemic infection) were monitored 4, 7, 14 and 21 days after challenge inoculation.

5.2.2. *In vivo* assay by using a virus based vector

Schothof et al. (1993) demonstrated that the coat protein of TBSV is not necessary for viral infection. Removing the gene which codes for the coat protein and replacing it with a heterogeneous gene will only slightly affect the viral infection but the heterogeneous gene will be expressed during the virus infection cycle. FC8-T7 is an infectious clone from which the coat protein gene was partially removed and 2 cloning sites were introduced, *SphI and KpnI*, (Galetzka, unpublished data). Since the scFvs were selected against TBSV RdRp which is necessary for viral replication, cloning of these genes into the infectious clone (FC8-T7) will result in decreasing the infection due to the inhibition of the RdRp activity by different scFvs.

5.2.2.1 Cloning the scFvs gene into the infectious clone

The scFvs genes were amplified by a PCR which introduced *SphI* and *KpnI* restriction sites at their 5´and 3´end respectively. The genes were cloned into the FC8-T7 plasmid which was digested with the same enzyme. The positive clones were selected and purified as described above.

FcscFv*SphI* 5´-CTC GAG GCA TGC CGA GGT G-3´
FcscFv*KpnI* 5´GTC ACG GGT ACC ATT CAG ATC CTC TTC TG-3´

The primers which were used to amplify the scFvs gene and introduce *SphI* and *KpnI* restriction site at 5´end and 3´end of the genes

5.2.2.2 *In vitro* transcription

The purified recombinant plasmid (5.2.2.1) was linearlized by digestion with *SmaI* and was used as the template for the *in vitro* transcription. Since the infectious clone contains the T7 promoter, the viral RNA genome can be obtained by using the T7 polymerase in *in vitro* transcription.
Reaction mix:

Y µl	ddH_2O (to make up total volume to 50 µl)
5 µl	10 mM rNTPs (A,G,C,U) (Roche, Mannheim)
5 µl	10X T7 RNA polymerase buffer with DTT
X µl	linearized DNA (~ 1 µg)
0.1 µl	24 U/µl RNAse inhibitor from Pharmacia
1 µl	T7 RNA polymerase (50 U/µl) (Biolab)

The reaction mixture was incubated at 37°C for 2 h. The amplified RNA was detected by electrophoresis in 0.8 % TBE gel. 10 µl of transcribed RNA were mixed with 90 µl inoculation buffer. 2-3 leaves of a *N. benthamiana* were rub-inoculated with 10 µl of the mixture each. The inoculated plants were cultured in the greenhouse and the symptoms (local and systemic) were monitored after 4, 7, 14 and 21 days after inoculation.

6. ELISA detection of the binding of scFvs to HCV NS5B RdRp

A microtiter plate was coated with 1 µg of HCV RdRp NS5B (kindly provided by Bartenschlager, Mainz university) at 4°C overnight. Unspecific binding was blocked with 3% BSA in PBS buffer for 1 h. The plate was washed 3 time with PBS buffer, then 0.5 µg of

scFvs diluted in PBS buffer were added and incubated at room temperature for 2 h. The unbound scFvs were washed out with 0.001% Tween-PBS buffer for 4 times and 1 time with PBS buffer. Binding of scFvs to HCV NS5B RdRp was detected by ELISA with 1:100 of anti-myc (provided by Conrad, IPK) and 1: 2000 of anti-mouse antibody conjugated with alkaline phosphatase (AP) (Sigma).

7. Total protein extraction from plant material

100 mg infiltrated or inoculated leaf was harvested and homogenized with 100 µl SDS-loading buffer. The homogenized material was boiled for 10 min and centrifuged at 13000 rpm for 15 min. 30 µl of supernatant was mixed with 10 µl Protein-loading buffer. The mixture was electrophoresed in 10% SDS-PAGE at 120 volt for 3 h. The proteins were blotted and detected by western blot as described in 2.4.2. Anti-myc (kindly provided by Conrad, IPK) and anti-mouse antibody conjugated with peroxidase (POD) (Sigma) were used as the first and second antibody respectively.

8. Establishment of *N. benthamiana* transgenic plants expressing scFvs

The recombinant binary plasmid pPZP200 carrying scFv gene was introduced into *Agrobacterium tumefaciens* (strain ATHV) by electropolation (as in 5.2.1 in Material and methods). *Agrobacterium* suspension was prepared as described in 5..2.1.2 in Material and Methods. *N. benthamiana* leaves were cut carefully with a razor blades and inoculated with the *Agrobacterium* suspension for 5 min. Leaves were blotted on filter paper and placed on MS media for 2 days in *in vitro* culture chamber. They were then transferred to a regeneration medium (MS media supplement with Kanamycin 200 mg/ml for selecting the transformed plants Cabinicillin 500 mg/ml for inhibiting the growth of *Agrobacterium*, NAA 0,01 mg/l and BAP 1 mg/l) then they were incubated for 4 days in *in vitro* culture chamber. Subsequent transfers were made every 10 days. Shoots regenerated 20-30 days post-inoculation were transferred into MS media supplement with Kanamycin (200 mg/ml) and Cabinicillin (500 mg/ml). The plantlets (T_0) were grown in a greenhouse.

9. Challenge inoculation of transgenic plants with viruses

Self fertilized transgenic seeds were harvested and geminated in a MS media supplement with Kanamycin 200 mg/ml. The plantlets (T_1) were transferred in pots and incubated in a greenhouse. The plants growing 4-6 leaves were challenge inoculated with virus particles or infected plant sap. The local and systemic symptoms were observed comparing with inoculated wt plants.

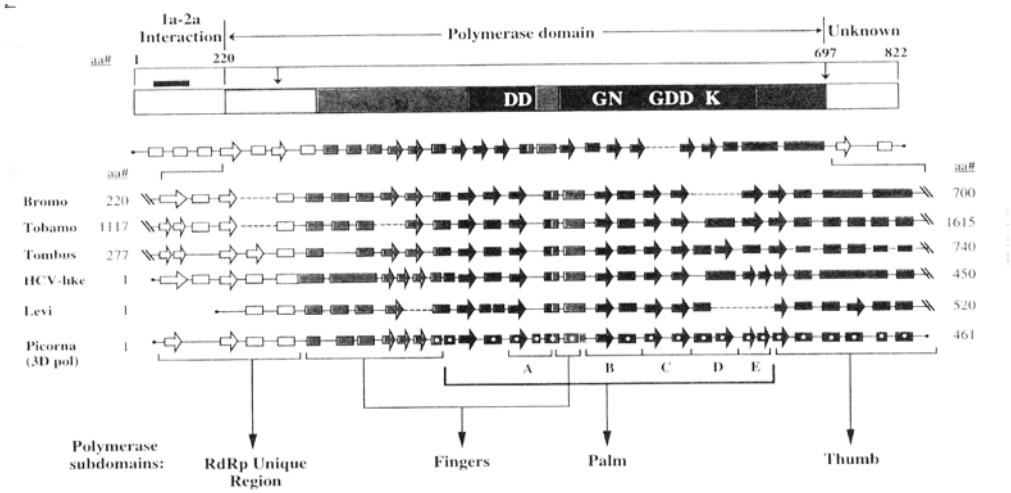
IV. Results

1. Target selection

As already mentioned, the success of an antibody-based resistant strategy depends very much on the binding site of the target antigen. The binding site of the target protein should be exposed on the surface of the protein molecule in question to be easily assessable for the antibody. Moreover the binding site should be essential for protein function.

To obtain broad rang resistance, the palm domain which contains 5 motifs was selected in this studies as the target protein to produce scFvs, since theses motifs are important for enzyme activity and are conserved among viral RdRps. Lack of information about the 3D structure of this RdRp makes it difficult to predict the conformation of the target binding site. However, the alignment results of RdRp of different viruses (O´Reilly et al., 1998) might be used as primary data for the prediction of the conformation of the TBSV RdRp target binding domain (see fig.6A).

A



B

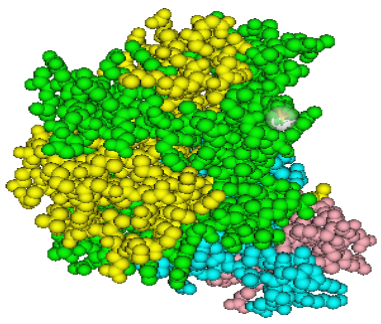


Fig.6 (A) Sequence alignments of motif A-E of RdRp including Brome mosaic virus (BMV 2a), Tomato mosaic virus (TMV p183), Tomato bushy stunt virus (TBSV p92), Hepatitis C virus (HCV NS5B), Phage Qβ replicase subunit II, poliovirus polymerase $3D^{pol}$, and the Human immunodifficient virus reverse transcriptase (HIV RT). Bold underlines indicate α-helices; thin underlines, β-strands. Arrow and rods represent the consensus secondary structures of palm motif. (Pic. A was obtained from O´Reilly et al., 1998). The most highly conserved residues in each motif are shown in bold letters. (B) shows 3D structure of $3D^{pol}$, the yellow area indicate the palm domain. (The 3D structure was obtained from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=&DB=structure).

Fig. 6A shows that the 2D structure of TBSV polymerase and 3D-polio polymerase are quite similar especially the polymerase domain. Therefor it might be postulated according to the 3D structure of 3D polio polymerase (fig. 6B) that the palm domain (contain motif A,B,C,D and E) of TBSV polymerase is also exposed on the surface of the RdRp molecule and accessible to antibodies.

Therefore, in this study several parts of the TBSV-BS3-Statice RdRp, carrying 5 motifs were cloned and expressed in *E. coli*. The purified proteins were used for scFv selection from scFv libraries and the selected scFvs were tested for their RdRp inhibition activity in *in vitro* and *in vivo* assays. Finally the resistance behaviours of transgenic plants expressing selected scFv were examined by challenge inoculation with different viruses.

2. Cloning of TBSV RdRp fragments

To get high amounts of target proteins for the scFv selection by Phage display, the best approach is expressing the target proteins in bacteria. The pET system is the most powerful system developed for the cloning and expression of recombinant proteins in *E. coli*. The target genes are cloned in the pET plasmids under control of strong bacteriophage T7 transcription and translation signals. The p33, p55, p92 protein and the five motifs of RNA polymerase were amplified with specific primers (see table 1) introducing restriction sites compatible with the vectors (*NcoI* and *SalI*). The amplification of target genes was carried out by using the full length clone of TBSB-BS3-statice strain, named DM1 (Galetzka et al., 2000) as the template (fig.7).

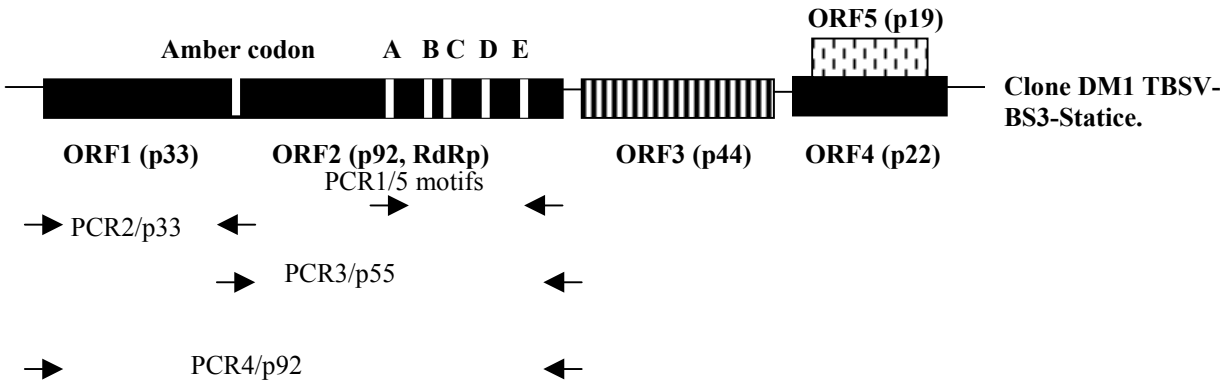


Fig. 7 Schematic drawing showing the amplification of the RdRp fragments from full clone DM1 TBSV-BS3-Statice. A, B, C, D, E indicate the motifs in the replicase gene; Arrows indicate the direction of primers.

After amplification, the PCR products were electrophoresed in a 1% TBE gel to check the size and the amount of PCR products. The results showed that the amplification was successful and gave specific products (data not shown). All PCR products were further purified to remove primer dimer and any impurity from the amplification reaction and were digested with restriction enzymes as described in Material and Method. The PCR products of p55 and p92 were partially digested, since they contained *NcoI* restriction sites in frame. Experiments were done to find the best combination of incubation time and amount of enzyme to yielded the highest amount of digested products. The results showed that 1 µl of restriction enzyme (10 U/µl) of *NcoI* and an incubation time for 10 min is the most efficient combination. Digestion with *NcoI* and *SalI* gave a overhang (sticky) site. The purified digested PCR products were ligated into expression vectors which were digested with the same enzymes. In this study a set of pET system expression vectors were used to optimise the expression profile such as solubility and yield. Table 2 (see Material and Method) describes the different vectors used in this study.

The ligated plasmids were transformed into competent cell *E. coli* (DH5□) to select recombinant plasmids by plating the transformed cells on LB agar media supplemented with antibiotics depending on the resistant gene of the vector. The positive colonies were selected by miniprep followed by digestion with specific enzymes. The positive recombinant plasmids were further confirmed by DNA sequencing.

After verification of the sequences, clones 38/6/26b+ and 38/8/ 26b+ were chosen for overexpression. Clone 38/8/26b+ contains the whole replicase (RdRp, p92) and 38/6/26b+ contains a part of the replicase, named " Hand of replicase ", starting at amino acid 540 of the RdRp. By digesting with *BamHI* and *SalI* which are also present in the primer, the clone 27/23a+, 115/26b+ and 111/23a+ were obtained. The clone 27/23a+ contains the p55 segment, starting after amber codon of the RdRp, the clone 115/26b+ contains a p33 segment and clone 111/23a+ contains the 5 motifs of replicase. All of these clones were sequenced and were confirmed to be in frame (data not shown).

3. Expression of the RdRp fragments in *E. coli*

After cloning, the positive clones were transformed in the expression hosts. *E. coli* strain BL21(DE3), BL21(DE3) pLysS were used as the expression host for the clones of p33, p55

and 5 motifs proteins. Since the p92 segment contains the amber stop codon in frame, it was necessary to chose an expression host, which can read though this stop codon. Therefore *E. coli* strain JM109 (DE3) and Novablue (DE3) were used to express the p92 protein in this experiment.

To search the suitable combination of vector/host for expression of the target proteins, the positive clones were transformed into different expression hosts (see table. 4). A single colony was cultured in 50 ml LB culture media, which contains appropriate antibiotics until the density of cells reached OD_{600} = 0.4-0.5. The cells were induced with 0.4 mM IPTG for the 23a+ plasmid and 1.0 mM IPTG for 26b+ plasmid. 1 ml of each sample was harvested and 10 µl of total proteins were loaded in a 12%SDS-PAGE gel to detect protein expression.

Protein segment	**Clone**		**Expression results**
	Clone No.	**plasmid**	
p33	115	26b+	-
p55	27	23a+	in BL21(DE3)LysS
p92	38/6	26b+	-
Hand	38/8	26b+	in BL21(DE3) and BL21(DE3) LysS
28H3 (5 motifs)	111	23a+	in BL21(DE3) and BL21(DE3) LysS

Table 4. The table shows the combination between plasmids and different expression hosts. The expression hosts which were used in this experiment were BL21(DE3), BL21(DE3)pLysS, JM109(DE3), Novablue(DE3), BL21(DE3), BL21(DE3)pLysS, BL21(DE3).

Fig.8 and 9 show that only the clones 27/23a+(p55) and 38/8/26b+ (Hand protein) can be overexpressed. The clone 38/8/26b+(Hand protein) can be overexpressed in Bl21(DE3) and BL21(DE3)pLysS but the clone 27/23a+ can be overexpressed only in BL21(DE3)pLysS. For the clone 111/23a+ (5 motifs) was also expressed in BL21(DE3) and BL21(DE3)LysS (data not shown). The yield of expressed proteins is quite high, when compared with total protein. A time course experiment was performed to determined the suitable induction time which target proteins were expressed in a high yield and not degraded. Fig.9 indicates that 2 h after induction was a good time point for protein expression of the Hand protein whereas the

overnight expression led to the degradation of target proteins (lane 8 and 10 in fig.9). The same results were also obtained from the expression of p55 and 28H3 (5 motifs) (data not shown).

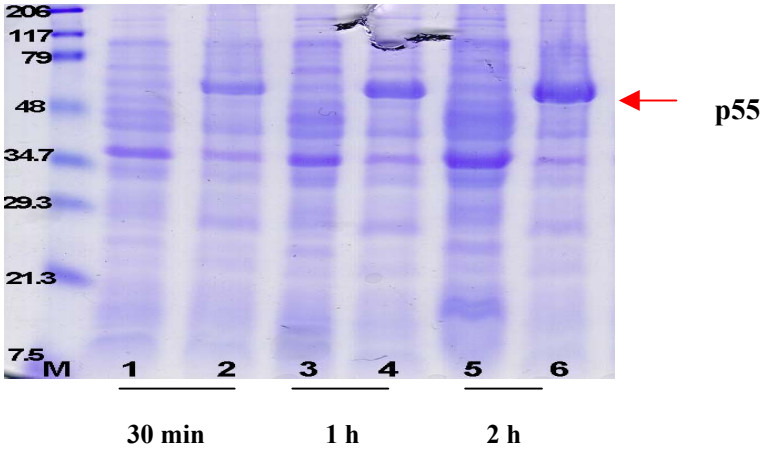


Fig. 8 The overexpression of p55 from 27/23a+ in Bl21(DE3) pLysS. The cell cultures were grown at 37°C to OD_{600} of approximately 0.5 and expression induced with 0.4 mM IPTG. At 30 min, 1 h and 2 h, the cells were harvested. The pellets were resuspended in lysis buffer. Total protein samples were run in 12%SDS-PAGE followed by staining with Coomassie blue. Lane M= marker, lane 1, 3, 5 are non induced cells and lane 2, 4, 6 are induced cells: arrow indicate the overexpression bands.

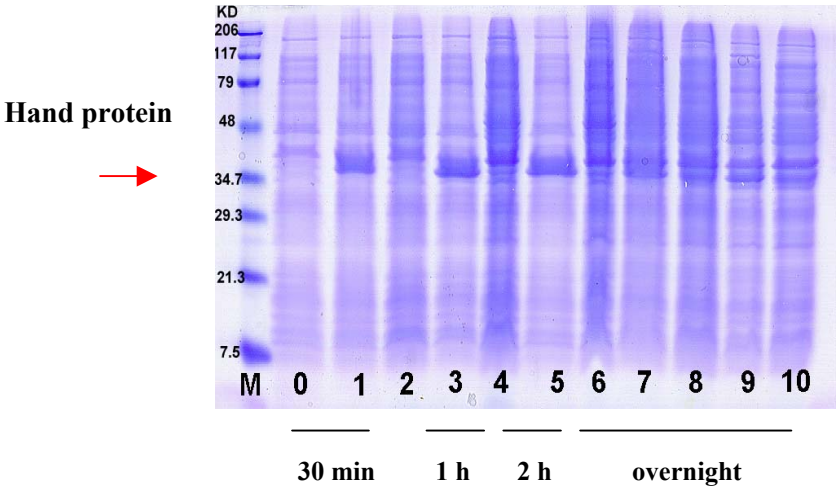


Fig. 9 The expression of Hand protein from colony 38/8/26b+ in BL21(DE3). The cell cultures were grown at 37°C to OD_{600} of approximately 0.5 and expression induced with 1.0 mM IPTG. At 30 min, 1 h, 2 h and overnight, the inducted cells were harvested. The pellets were resuspended in lysis buffer. Total protein samples were run along with 12%SDS-PAGE followed by staining with Coomassie blue. Lane M= marker, lane 0, 2, 4, 6, 7 and 9 are non induced cells and lane 1, 3, 5, 8 and 10 are induced cells: arrow indicate the overexpression bands. Expression is optimal after 2 h.

The clone 115/26b+ and 111/23a+ could not be expressed in any expression hosts even though the sequencing of both clones confirmed them to be in frame. The variation in the amount of IPTG, time and temperature were also performed to optimise the condition for expression, but these factors could not give any positive results (data not shown).

3. Determination of protein expression

Recombinant proteins may be expressed in soluble or insoluble (inclusion body) form *in E. coli*. Insoluble, inactive inclusion bodies are, however, frequently formed upon recombinant protein production in transformed microorganisms. The production of inclusion bodies may be the result of many different factors, such as high S-bond in recombinant proteins, the recombinant proteins might toxic to the cells or misfolding after expression *etc*. The binding site of insoluble proteins may, however, be changed after refolding and lead to unsuccessful antibody-antigen interaction.

To get the best antigen, the target proteins should therefore remain in soluble from. To determine whether proteins accumulate in the insoluble fraction (inclusion body) or in the soluble fraction, 50 ml of cell culture were induced with 0.4 mM IPTG for colony 27/23a+ in Bl21(DE3)pLysS and with 1.0 mM IPTG for colony 3878/26b+ in BL21(DE3) at 37°C. 2 ml of induced cells were harvested at 30 min,1 h, and 2 h after induction respectively. Crude soluble and insoluble fractions were prepared by two methods. First cells were washed with Tris/HCl buffer and resuspend with lysozyme and TritonX-100. After centrifugation the supernatant contains soluble, and the pellets contains insoluble proteins (fig 10).

Another method, which was used to prepare soluble and insoluble proteins is the extraction of proteins by using the BugBuster® reagent (Novagen). BugBuster® Protein Extraction is formulated for the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble proteins. The proprietary formulation utilise a mixture of non-ionic detergents that is capable of cell wall perforation without denaturing soluble protein. Cells were harvested by centrifugation, followed by resuspension in BugBuster® reagent. During a brief incubation soluble proteins were released. The extract was clarified by centrifugation, which removed cell debris and insoluble proteins. Aliquots of both fractions were applied to a 12% SDS-PAGE gel (fig.11).

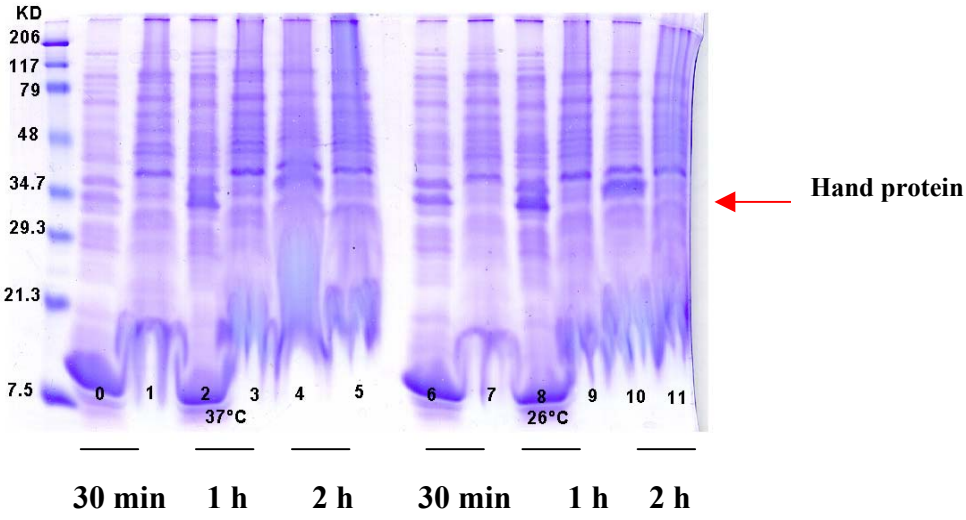


Fig.10 Soluble and insoluble protein detection of Hand protein 38/8/26b+ in BL21(DE3). Cells were induced with 1.0 mM IPTG at 37°C and 26°C. The induced cells were harvested at 30 min, 1h and 2h respectively. The pellet was treated with lysis buffer and 1µl of lysozyme and was sheared with a 10 ml syringe. The lysised cells were centrifuged. 10 µl of supernatant, which contains soluble proteins and 10 µl of resuspended pellets (insoluble proteins) in lysis buffer were loaded in a 12% SDS-PAGE followed by staining with Coomassieblue. Lane 0, 2, 4 and 6, 8, 10 are insoluble fractions which were inducted at 37°C and 26°C respectively. Lane 1, 3, 5 and 7, 9, 11 are the soluble fractions which were inducted at 37°C and 26°C receptively, arrow indicate the target proteins.

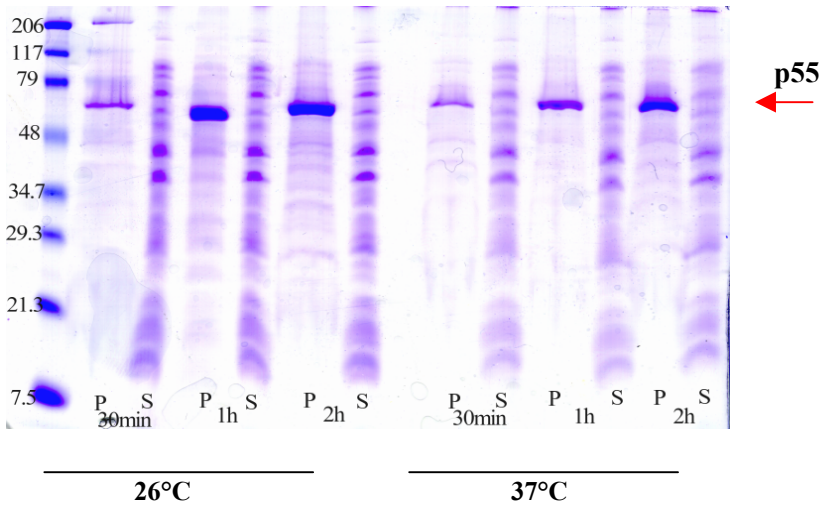


Fig.11 Soluble and insoluble protein detection of p55, 27/23a+ in Bl21(DE3)pLysS. Cells were grown and induced with 0.4 mM IPTG and harvested as in fig.10. The pellets were incubated with BugBuster® reagent 20 min. The cells were centrifuged and 10 µl of both fractions were loaded in a 12%SDS-PAGE followed by staining with Coomassieblue. P = insoluble fraction, S = soluble fraction.

The results show that most of the proteins accumulated in inclusion bodies. Extraction of the cells with Bugbuster® reagent is easier than using the syringe method and less time consuming, therefore it is the preferable method for the next experiments.

Culture conditions can also have a dramatic effect on solubility and localisation of a given target protein. In general, conditions that decrease the rate of protein synthesis, such as low induction temperature and/or low concentration of IPTG, trend to increase the percentage of target protein found in soluble form.

Fig.10 and 11 show a comparison of the expression pattern between the induction at 26°C and 37°C. The soluble fraction of target proteins was not dramatically different at different temperatures. Reducing the IPTG concentrations from 1:2 to 1:10 of recommended concentration of each clone was also done. The results (data not shown) indicated that these factors were not be able to increase the soluble fraction of any of the clones.

4. Western blot and Chemiluminescence Immuno detection

Since the target proteins were fused with a His-tag for purification, it was necessary to verify the presence of detection tags in the target proteins. Therefore western blot and chemilu - minescence immuno detection were performed. The cells were grown until the density reached OD_{600}=0.4-0.5. They were induced with 0.4 mM IPTG for 27/23a+/ BL21 (DE3) pLysS and 1.0 mM IPTG for 38/8/26b+/ BL21(DE3). 2 ml of induced cells were harvested after 1 h and 2 h. The induced cells were centrifuged and resuspended in Bugbuster® reagent. Ten microlitre of expressed target proteins were loaded in a 12% SDS-PAGE. After running the gel, the proteins were transferred to a nitro-cellulose membrane by semidry electroblotting as described in Material and Methods.

After the target proteins were transferred, the remaining protein-free sites on the membrane were blocked to prevent high background due to binding of the primary or secondary antibody. The membrane was incubated in 5% of non-fat dried milk (Marvel™) in TBS buffer then with anti-His antibodies (1:1000). The anti-His antibody bound to immobilised 6X His-tagged protein was visualised by using a secondary anti-mouse IgG antibody conjugated to horseradish peroxide (POD) together with enzyme substrate. The highest recommended (1:1000) dilution was used to avoid non-specific signals. The chemiluminescence substrate

gives rise to products that spontaneously emit light at the enzyme's location. This was recorded by using a photographic film.

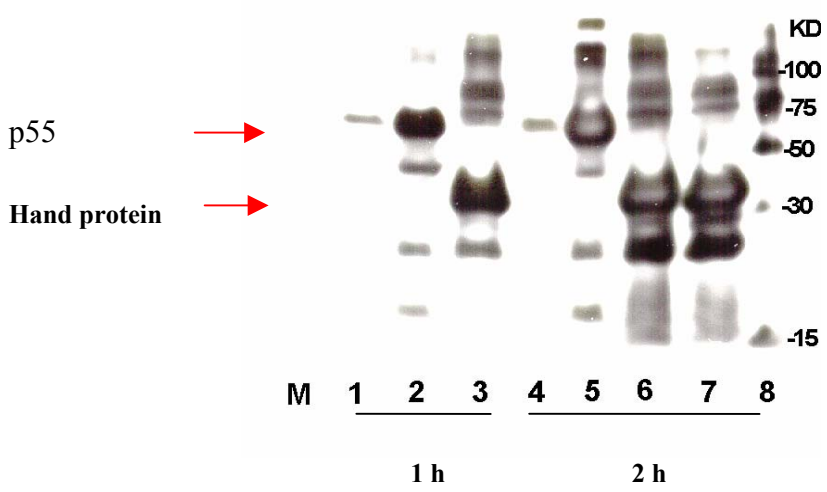


Fig.12 The detection of His-tagged protein by western blot and chemiluminescence method. The cell cultures were grown at 37°C to OD_{600} of approximately 0.5 and expression induced with 0.4 mM IPTG for clone 27/23a+in BL21(DE3)pLysS and 1.0 mM IPTG for clone 38/8/26b+/BL21(DE3). At 30 min, 1 h and 2 h the cells were harvested. The pellets were incubated with lysis buffer. Total protein samples were run in a 12%SDS-PAGE. The target protein were transferred to a nitrocellulose membrane (Hybond-P) by semidry electroblotting. The membrane was incubate with 1:1000 anti-His antibody. The position of anti-His antibody bound to immobilised His-tagged protein was visualised with a second anti-mouse IgG antibody (1:1000) conjugated to horseradish peroxide together with enzyme substrate. M = protein marker, lane 1, 4 = non induction, lane 2, 5 = overexpressed protein of 27/23a+in BL21(DE3)pLysS (p55), lane 3, 6 and 7 = overexpressed protein of 38/8/26b+ in Bl21(DE3) (Hand protein), lane 8 = His-tagged marker , arrow indicate the His-tagged target proteins.

Fig.12 shows that proteins expressed from both clones contain the His-tag (see arrow position). In non induced cells a band of the same size as the target proteins appeared (lane 1 and 4). This band is very likely the result of basal expression.

5. Purification of *E. coli* expressed RdRp fragments

5. 1. Soluble proteins

5.1.1 Column purification

The results from western blot analysis (fig.12) showed that the target proteins contain a His-tag, so they can be purified by using a chromatographic purification. Ni-NTA Spin column provide a simple method for rapid screening and purification of 6X His-tagged proteins from small scale bacterial expression cultures. The high affinity of the Ni-NTA resins for 6X His-tagged proteins is due to both specificity of the interaction between histidine residues and immobilised nickel ions and to the strength with which these ions are bound to the NTA (nitrilotriacetic acid) resin.

The 27/23a+/BL21(DE3)pLysS, 38/8/26b+/Bl21(DE3) and 111/23a+/BL21(DE3) clones were grown and induced at 37°C. 2.5 ml of induced cells were harvested 1 h after induction with IPTG. The cells were extracted with BugBuster® reagent and centrifuged respectively. A low concentration of imidazole (10 mM) was added to the supernatant to inhibit unspecific protein to bind with the column. The supernatant which contains soluble proteins was adjusted to pH 8.0, since in this pH range the His-tagged protein will become completely negative charged. The soluble fraction was loaded in the prewetted Ni-NTA Spin column. The washing and eluting step were performed as described in Material and Methods.

The attempts to purify the soluble His-tagged proteins by using Ni-NTA Spin column were, however, unsuccessful (data not shown). Even optimised conditions, such as increasing the amount of imidazole in the binding step, adding others additive like NaCl to prevent the ionic interactions and the addition of glycerol to prevent hydrophobic interaction between His-tagged proteins with contaminants were unsuccessful.

5.1.2 Batch purification

Since most proteins are in the inclusion from, a large scale cell culture was needed to get enough soluble protein for purification. Batch purification was used to purify the low expression soluble target proteins. Two hundred millilitre of cell culture were grown at 37°C

and induced with IPTG (0.4 mM for clone 27/23a+and 111/23a+ and 1.0 mM for clone 38/8/26b+) for 1 h. 200 ml inducted cells were harvested and extracted with BugBuster® reagent. The cell debris was removed by centrifugation for 20 min at 4°C. and the supernatant was adjusted to pH 8.0. 20 mM of imidazole were added in the soluble fraction and it was incubated with Ni-NTA Agarose at 4°C for 1 h. The washing and eluting steps were performed as described in Materials and Methods.

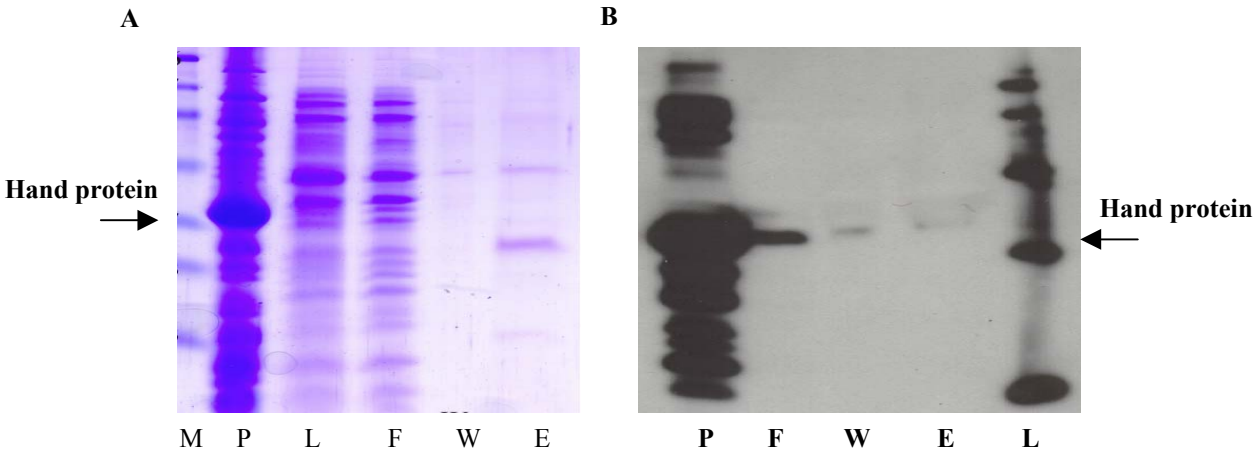


Fig. 13 Purification and detection of Hand protein (38/8/26b+/BL21(DE3) by using Batch Purification.
(A) Cells were grown at 37°C until OD_{600} =0.5 and induced with 1.0 mM IPTG. After 1 h, the induced cells were harvested and proteins were extracted with BugBuster® reagent. 20 mM imidazole were added to the supernatant and adjusted to pH 8.0. The clear lysate was incubated with Ni-NTA agarose at 4°C for 1 h. The mixture was loaded in a purified column. The column was washed and the His-tagged proteins were eluted with elution buffer. The aliquots of each purification steps were loaded in a 12% SDS-PAGE followed by staining with Coomassieblue. Lane M = protein marker, P = pellet, L = lysate, F = flowthrough, W = washing, E = elution after dialysis. (B) Western blot Detection of His-tagged Hand protein (38/8/26b+/BL21(DE3) pLysS. Aliquots of each purification steps were loaded in a 12% SDS-PAGE. The gel was blotted on a nitrocellulose membrane by semidry electroblotting. The membrane was incubated with 1:1000 anti-His antibody. The position of anti-His antibody bound to immobilised His-tagged protein was visualised by a second anti-mouse IgG antibody (1:1000) conjugated with horseradish peroxidase together with enzyme substrate. Lane P = pellet, F= flowthrough, W = washing, E = elution, L = His-tagged ladder.

Fig.13 showed that the target proteins could not bind to the Ni-NTA agarose (no target protein band in the elution, lane E), although the there are some soluble target proteins (lane F in B). The result from western blot and chemiluminescence confirmed that the target protein bands contain His-tag. An attempt to optimise the binding conditions was also performed but it was not possible to increase the binding affinity between the target proteins and Ni-ions. The same results were also obtained from 27/23a+ (p55) and 111/23a+ (5 motifs) (data not shown).

5.2 Insoluble proteins

Since the target proteins were expressed and formed inclusion bodies, the purification of theses protein was done under denaturing condition. The induced cells were harvested and resuspended in BugBuster® reagent to get rid of the non-target soluble proteins. The inclusion bodies were resuspended in extraction buffer (8M Urea pH 8). The supernatant was loaded into Ni-NTA column preincubated with extraction buffer. The column was washed 3 times with washing buffer and the bound proteins were eluted with elution buffer.

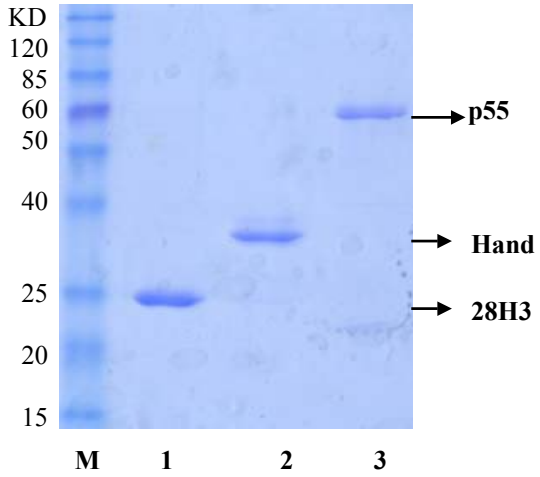


Fig.14 Purification of target proteins under denaturing condition. Cells were grown and induced with 0.4 mM IPTG and harvested 4 h after induction. The pellet was first extracted with Bugbuster® reagent for 30 min, then resuspended in 8 M Urea pH 8.0 for 2 hrs. The supernatant was loaded in Ni-NTA column and the column was washed with 8 M Urea pH 6.5. The target proteins were eluted with 8 M Urea pH 4.5. The purified proteins were pooled and concentrated by using a concentrator. The concentration of proteins was determined by using Bio-Rad reagent. 1 µg of each proteins was loaded in a 12%SDS-PAGE followed by staining with Coomassie blue. M = protein marker , Lane 1 = 28H3 (5 motifs) protein, lane 2 = Hand protein, lane 3 = p55.

The result shows that the purification of theses proteins under denaturing condition results in high amount of target proteins (fig.14). The purified proteins were also redenatured by dialysis against PBS or in a Urea buffer with reduced concentration, however, the proteins tend to precipitated under these conditions. Therefore the denatured purified proteins were used as antigens for selecting scFvs from the Phage library.

6. Phage display

Phage technology offers a valuable tool for selecting scFvs from a single chain phage library. Repertories of antibody fragments were first generated by polymerase chain reaction (PCR) from rearranged V-genes of populations of lymphocytes, spleen or bone marrow. Cloning of V-gene in phagmid results in expressing the gene as a fusion with a phage protein and display on the phage surface. By panning the phage library with target antigens, the specific scFvs can be selected.

6.1 Panning

In this study two naive human repertories (unimmunized scFv phage library) which were provided by the Cambridge University were used for the selection of scFvs against fragments of the purified denatured RdRp of TBSV-BS3 statice from expressed in *E. coli*. One microgram of each antigen in Urea buffer pH 8 was coated in immuno-microtiter plates and incubated at 4°C overnight. The phage libraries (A and B) were titered and 10^3 phages of each library were added to each coated well for the first round of selection. The selected phages were rescued by using the helper phage M13. The panning result showed the enrichment of selected phage after 3 rounds of panning (fig.15).

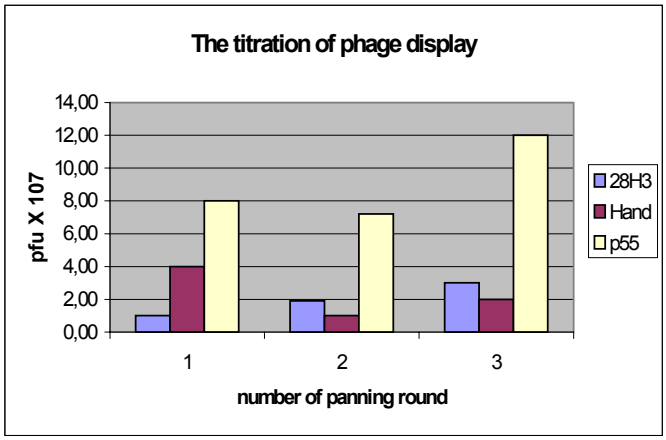


Fig.15 The bar graph demonstrates the enrichment of selected phages after each round of panning. The eluted phages were infected TG1 cells. The infected bacteria were serial diluted and plated on TYE plate as described in Material and Methods. The colonies were counted. The titration of eluted phages was done in triplicates.

6.2 Monoclonal phage selection

After the third round of panning, a monoclonal phage ELISA was performed to select single phages which can bind to the given antigen. Single colonies were picked and incubated in microtiterplates at 37°C. The phages were rescued by helper phage M13 and used to bind with the given antigens. The result showed that 58% of the monoclonal phage against the 28H3 protein, 73.4% and 86.4% of monoclonal phages against p55 and Hand protein respectively bound specifically to the antigen in question, showing values more than 1.0 in an ELISA test at 405 nm.

6.3 Soluble scFv expression.

The *E. coli* strain HB 2151 was infected with monoclonal selected phages to express soluble scFvs. This experiment was done to select the scFv genes which can be expressed in soluble from (in media). The result showed that 0.04% of all monoclonal phage were able to be expressed in soluble form still giving positive results in ELISA. The 12 best scFvs which showed highest specificity for the antigens in question were selected for further experiments.

6.4 scFv characterisation

A first characterisation of the selected scFvs was performed by cross binding, westernblot and sequencing. The cross binding experiment was performed to detect the cross activity of the selected scFvs. Each scFv was incubated with 3 different fragments of the viral replicase (p55, Hand protein, 5 motifs (28H3) and the binding activity was detected by ELISA (fig.16). The result showed that the 12 selected scFvs can be categorised in 2 groups based on their binding region.

1. The scFvs which can bind with all given antigens, so the binding region should be the motif present in all 3 replicase fragments (motif B, C, D, E, F). These scFvs are scFv 1, 2, 3, 4, 5, 6, 8, 9, 11 and 12.

2. The scFvs which cannot bind with all given antigens, so the binding region may be somewhere out of the overlapping area. These scFvs are scFv 7, 10.

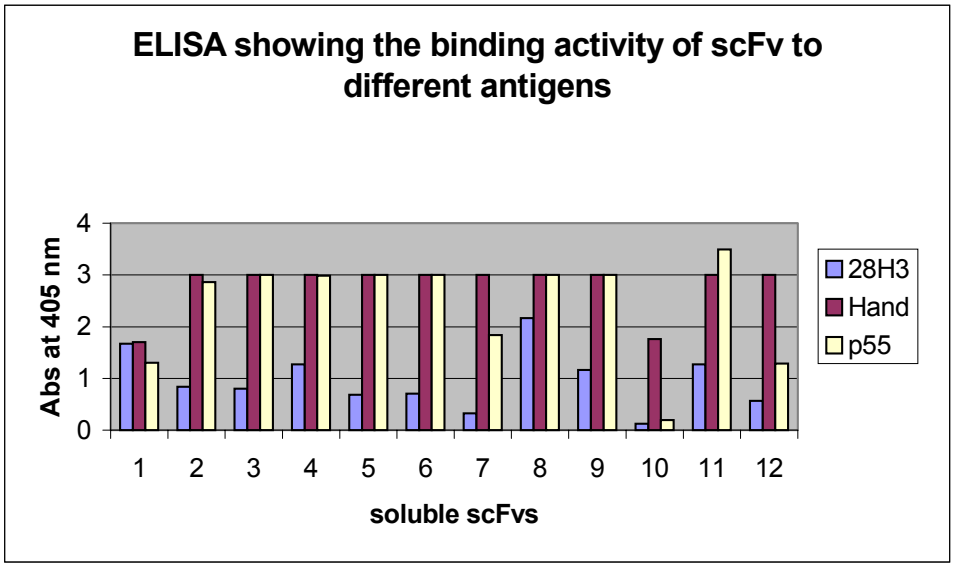


Fig.16 The ELISA result show the binding activity of scFvs to different antigens. Each expressed scFv was incubated with three different antigens. The binding activity was detected by ELISA. 1. = scFvp55/1/F8, 2 = scFvp55/2/A11, 3. =scFv p55/2/G11, 4. = scFvp55/2/G8, 5. = scFvp55/1/H9, 6 = scFvH1C5 , 7. = scFvH1G12, 8. = scFvH2C6, 9. = scFvH1E11, 10. =scFv H1F3, 11. = scFvH2F11, 12. = scFv28H3/A2.

The western blot result (fig.17) with these scFvs against the three different antigens yielded the same result as the ELISA (see fig.16). It can be concluded that all selected scFvs bind with 3 antigens in difference degrees (under 1.0 at 405 nm) and most of them bind poorly with antigen 28H3 (5 motifs).

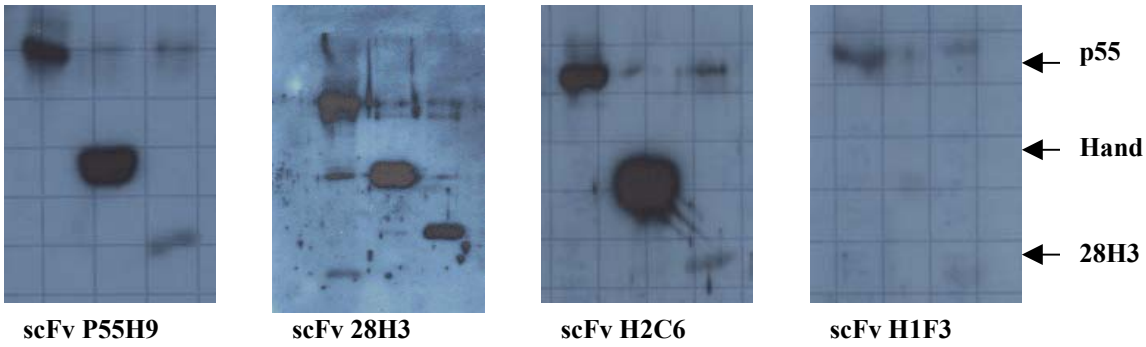


Fig.17 Western blots showing the cross binding of soluble scFv to 3 different antigens. One microgram of each antigens (p55, Hand and 28H3, 5 motifs) was electrophoresed in a 12 % SDS-PAGE. The gel was blotted onto a protein membrane and was incubated with 10 ml of supernatant from induced *E. coli* expressing different scFv genes. Western blot was carried out as described in Material and Methods.

Sequencing of the selected scFv genes was done to confirm that the different binding activity depended on the difference in the complementarity determining region (CDR) of each of the selected scFvs. The sequencing result of these scFvs show that all of selected scFvs have different CDRs (data not shown).

To investigate if the different binding affinity to the different antigen (fig.16 and 17) depends on the concentration of expressed scFvs from *E. coli*., the same amount of supernatant of induced *E. coli* expressing scFvs was precipitated with Stategene® resin and 10 µl of the resuspend resin was loaded in a 12% SDS-PAGE. A western blot was performed to detect the amount of expressed scFvs.

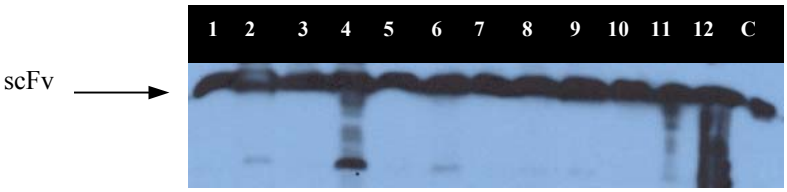


Fig.18 Western blot showing the level of the scFvs expression. TG1 cells infected by phagmids carrying scFvs genes were cultured and induced by 1 mM IPTG. The overnight induced culture cells were harvested. The supernatant which was expected to contain expressed scFvs was precipitated with Stategene® resin. 5 µl of the protein-resin complex were loaded into a 12% SDS-PAGE. The gel was blotted and the western blot was performed to detected the target proteins which were fused with a myc-tag at the c-terminus by using anti-myc antibody (1:100) and anti-mouse-POD conjugated antibody (1:2000). 1-12 are selected scFvs as in fig.17, C = a marker. The scFv band is indicated by an arrow.

Fig.18 shows that all selected scFvs were expressed at the same amount. Together with the ELISA (fig.16), western blot (fig.17) results and the sequencing data (data not shown) demonstrated that the different binding affinity to different antigens does not depend on the amount of the scFvs but depended on the different affinities to the targeted antigens.

6.5 ScFv purification

The selected scFvs were expressed and purified to be used for scFv-mediated inhibition RdRp in the next experiments. The scFv genes are fused with a myc-tag resulting in the expression of fusion proteins. Using the myc-tag for a purification strategy might result in copurification with unspecific bacterial proteins and reduction in the yield. Another strategy which uses Protein-L (Actigen) is more reliable and was therefore used to purify the scFv from the bacterial culture. Protein-L from *Peptostreptococus magnus* binds immunoglobulins (Ig) primarily through the light chain without interfering with the antigen binding site. It has been shown to bind strongly to human light chain subclasses I, III and IV and to some light chain from other species such as rat and mouse. Since the scFvs were selected from nonimmunised human scFv banks, Protein-L is the most attractive tool to purify scFvs from bacterial culture.

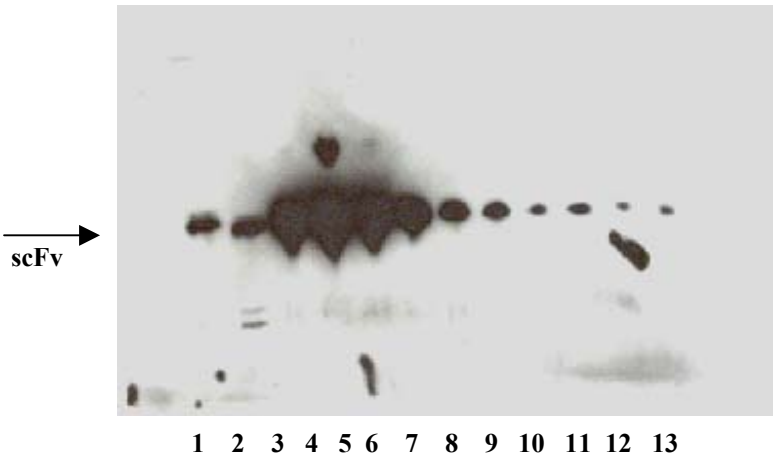


Fig.19 ScFv purification. The selected scFvs (in this case scFv P55H9) were expressed and purified by using Protein-L™ resin column (Actigen). Each fraction was collected and electrophoresed in 12% SDS-PAGE. The gel was transferred onto a PVDE membrane. The target proteins were detected by Western blot to quantify the amount and the purity of eluted scFv. The membrane was incubated with anti-myc antibody as a primary antibody and anti-mouse-POD as a second antibody. 1-13 are the number of eluted fractions. Arrow indicates the scFv band.

Fig.19 shows that the scFvs can be purified by using Protein-L resin and resulting in very high amount. The positive fractions were collected, dialysed against PBS and concentrated with PEG20000 at 4 °C. The purified scFvs were measured concentration by Bradford method and used for *in vitro* assays of scFv-mediated RdRp inhibition.

7. *In vitro* assays

7.1 ScFv mediated RdRp inhibition assay

The activity of selected scFvs to inhibit the function of partially purified viral RdRp was investigated *in vitro*. Since the scFvs were selected by using denatured *E. coli* expressed antigens, it is necessary to demonstrate that the selected scFvs can bind the native RdRp and show inhibition activity *in vitro*. In 2000, Nagy and Pogany had successfully purified and characterised Cucumber necrosis virus (CNV) and Tomato bushy stunt virus (TBSV) RNA-dependent RNA polymerase from infected plants. CNV and TBSV belong to the same group of viruses (Tombusviridea), and their RdRp are quite homologue (Koonin 1991). Comparison of the template usage of the TBSV and CNV RdRps reveals no significant differences between the two RdRps (Nagy and Pogany 2000). To study a broad rage inhibition of scFv, therefore, in this experiment the partially purified CNV RdRp was used to study the scFv-mediated RdRp inhibition. A control experiment was performed to proof that the purified scFvs and its buffers do not contain any compounds which could degrade the RNA template. 25 µl of each of the scFvs were incubated with *in vitro* transcribed RNA template (DI-72 minus strand) for 30 min on ice, then the RNA was phenol : chloroform extracted and precipitated with ammonium acetate. The pellet was resuspended with water and loaded in a PAGE gel. Fig.20 shows that the purified scFvs and its buffer do not degrade the RNA template.

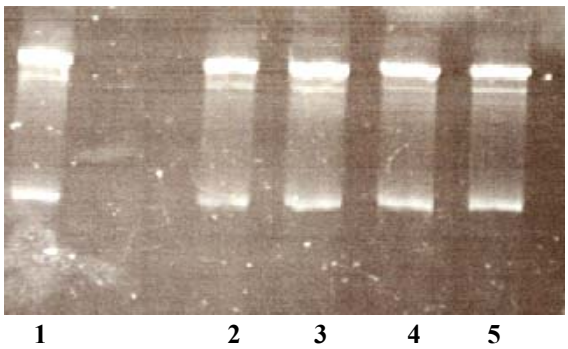


Fig.20 Influence of scFvs preparation on the RNA template. The RNA template (DI-72 minus strand) was incubated with scFvs for 30 min on ice. The RNA was extracted with phenol : chloroform, following by isopropanal precipitation. The RNA was visualized by running the RNA in 0.8% TBE gel following by Ethidiumbromide staining. The potential RNA degradation was compared with untreated RNA. Lane 1 = untreated RNA (without incubation with scFv), Lane 2,3,4,5 = the RNA template incubation with scFv 28H3, E11, H2C6 and P55 respectively. Arrow indicates the RNA template.

To investigate the potential of selected scFvs to inhibit the viral RdRp, detection of *de novo* products transcribed from the viral RdRp is the preferable method. ScFvs were incubated with CNV RdRp which was partially purified from infected plants for 30 min on ice, then the rest of the reaction mix was added. The radio labelled nucleotides which were incorporated into amplified products (*de novo*) during RNA synthesis were detected by a phosphor imager and the intensity of *de novo* bands were compared with a negative control. The results show that 4 of selected scFvs inhibit the CNV RdRp to different degrees (fig. 21).

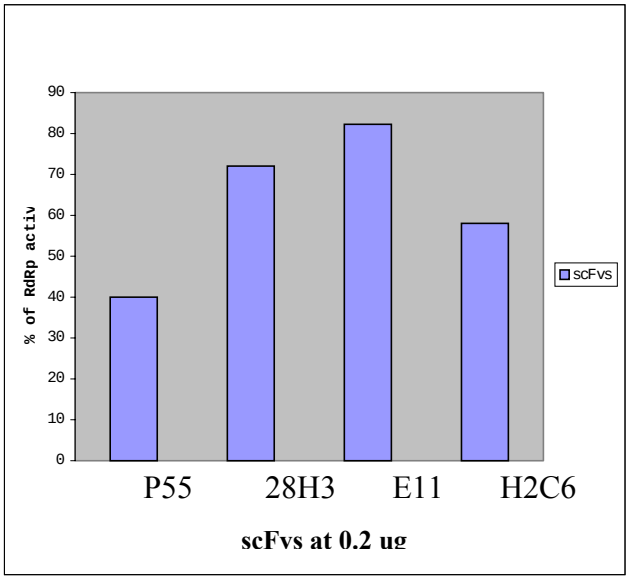


Fig.21 The bar graph shows % RdRp activity when partially purified CNV RdRp was incubated with scFvs prior to adding the reaction mix. The mixture was incubated at 4°C for 1 h, then the RNA was extracted, purified and electrophoresed in a PAGE. The incorporation of radio labelled UTP^{p32} into the RNA during RNA synthesis was visualised by a phosphor-imager. The intensity of the *de novo* band was estimated by a densitometer and compare with the *de novo* band resulting from a reaction without preincubation with scFv (negative control).

Another *in vitro* assay was performed to investigated the effect of the concentration of scFv on RdRp inhibition. Various amounts of scFvs were preincubated with defined amounts of CNV RdRp and the *de novo* products were detected as described. The results from this *in vitro* study clearly showed that the selected scFvs can inhibit the RdRp activity and the inhibition is concentration dependent (fig.22). ScFvP55 yielded the most efficient inhibition of CNV RdRp activity and therefore has been chosen for further investigations.

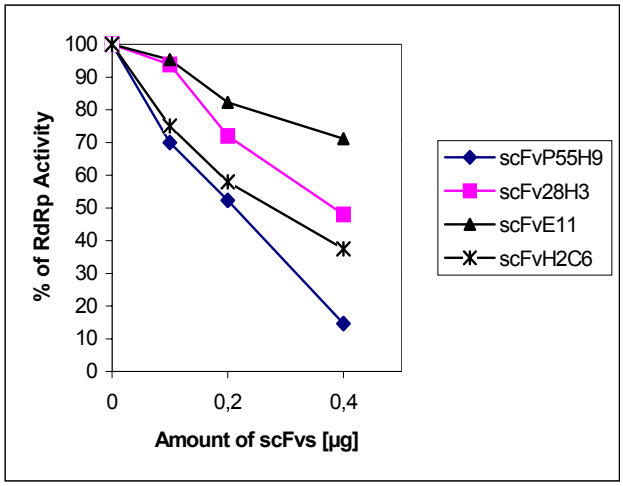


Fig.22 ScFv mediated inhibition of CNV RdRp *in vitro*. Different amounts of scFvs were incubated with defined amount of CNV RdRp for 30 min prior to the RdRp reaction. The remaining RdRp activity is counted in % of the RdRp activity without the addition of scFvs.

7.2 Effect of time and incubation temperature on scFv-mediated RdRp inhibition

To study the effect of different templates, temperature and incubation time, two experiments were designed. First to investigate the effect of minus and plus strand templates on RdRp inhibition was investigated by using partially purified CNV RdRp. Fig.23 shows that different templates do not effect the extend of inhibition of the RdRp and the minus strand is the best template for this study.

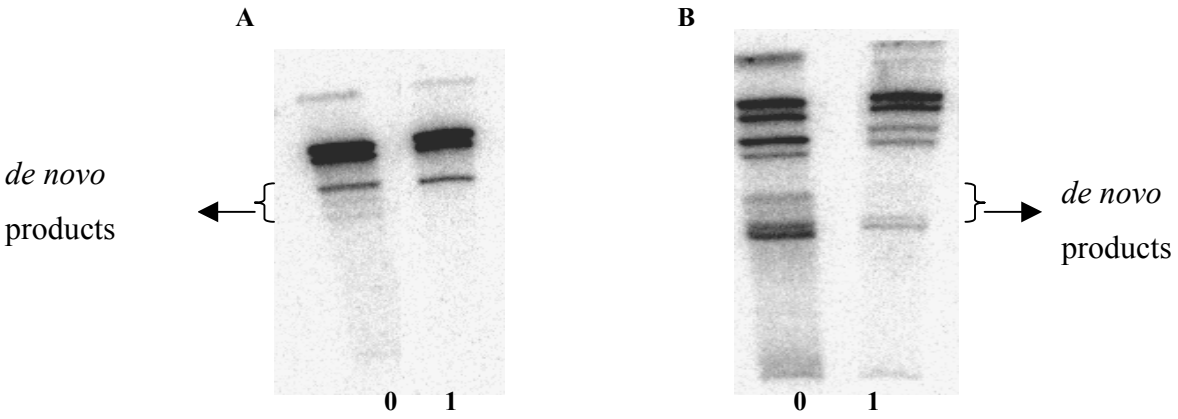


Fig.23 Effect of template on RdRp inhibition. DI-73 (+) (A) and DI-72 (-) (B) were used as the templates. The assay was carried out as described in Material and Method. 0 = no preincubated with scFv, lane 1 = the CNV RdRp was preincubated with scFvP55H9 prior to adding the template. The *de novo* products are indicated by arrow.

Since for the *in vitro* assay the incubation of scFvs and RdRp was done on ice which is not the condition met in plant cells, it is necessary to study the inhibition also at other temperatures. ScFv P55H9 was used for the experiments since it has a highest activity of all selected scFvs. ScFv P55H9 was incubated with CNV RdRp at 4°C and 25 °C (plant cell condition) for 30 and 60 min before adding the reaction mix.

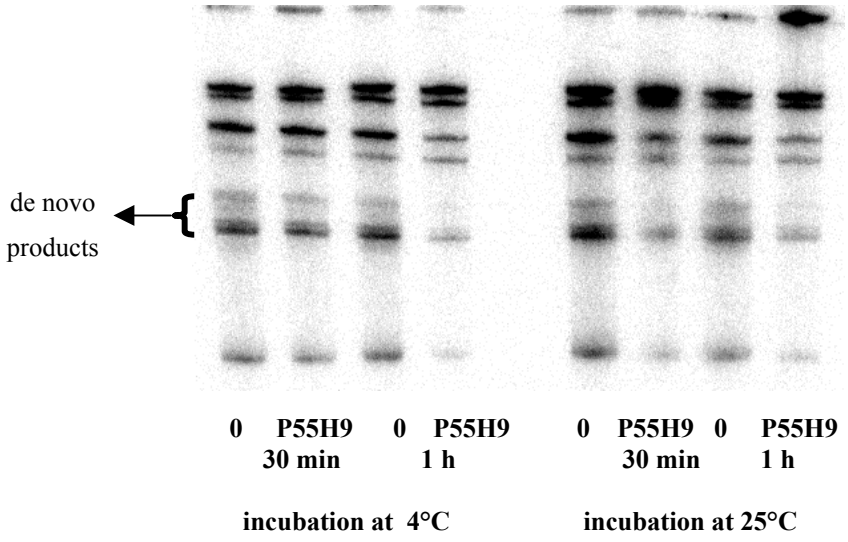


Fig.24 The effect of temperature and incubation time on scFv-mediated RdRp inhibition assay. ScFvP55H9 was preincubated with RdRp at different temperature and incubation time. The *de novo* products were extracted and electrophoresed in PAGE. The incorporation of $rCTP^{p35}$ during transcription by RdRp was detected by phosphor-imager. The *de novo* products are indicated by an arrow.

Fig. 24 shows that the inhibition effect increases with the incubation time and temperature. From this results it can be postulated that the inhibition activity of the scFv can also function in plant cells.

7.3 Mechanism of scFv-mediated RdRp inhibition.

To study the mechanism of scFv-mediated RdRp inhibition, two sets of experiments were performed. For the first experiment the RNA template was added at different time points after the incubation of the CNV RdRp with selected scFv. Fig.25 clearly shows that the RdRp activity was already completely inhibited after 30 min incubation with scFvP55H9.

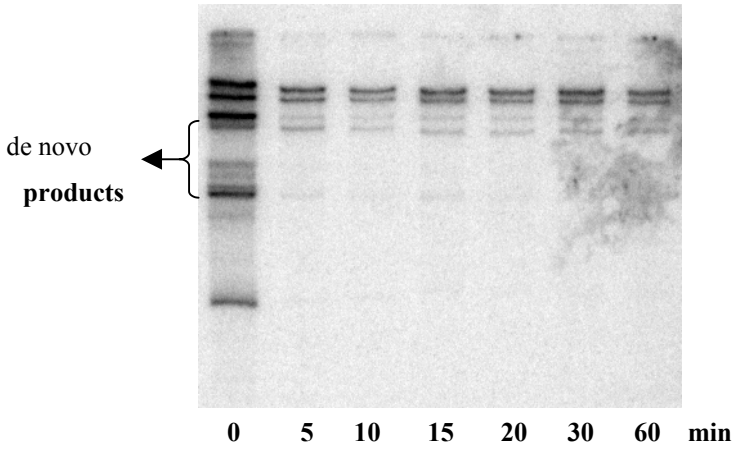


Fig.25 The effect of time point of addition of the RNA template. ScFvP55H9 was incubated with the CNV RdRp for 30 min prior to the addition of RNA template. After this incubation the RNA template was added at different time points (5, 10, 15, 20, 30, 60 min). 0 = No preincubation of the RdRp with scFvp55H9. The *de novo* products are indicated by an arrow.

A second experiment was performed to study the inhibition activity of scFvs when added after the start of transcription. In this experiment the scFv was not preincubated with the RdRp but added separately at different time points of the reaction. Fig.26 shows that the scFv cannot inhibit the RdRp activity when the transcription is already started.

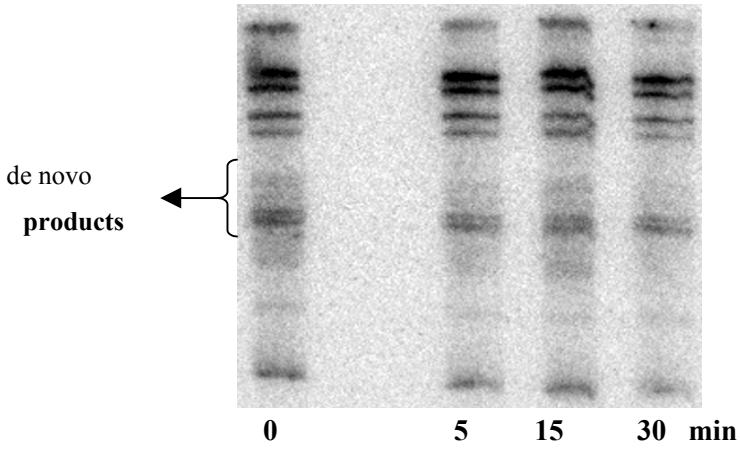


Fig.26 Inhibition of scFv of RdRp complex containing bound RNA template. The RdRp reaction was performed without preincubation of the RdRp with scFv. The scFvP55H9 was added after the transcription started 5, 15 and 30 min. 0 = no preincubation of RdRp with scFvP55H9. The *de novo* product was indicated by an arrow.

8. Epitope mapping

The *in vitro* assay clearly showed that the selected scFvs are able to bind to a partially purified CNV RdRp from infected plants resulting in reduction of its activity to amplify the RNA template. To exactly define the binding domain of the different scFvs, an epitope mapping was performed. Different fragments of p92 were cloned into an expression vector and expressed in *E. coli* BL21(DE3). The proteins were purified by using a NTA-column as described in Material and Methods. The binding activity of each of the scFvs to the different antigens was detected by ELISA.

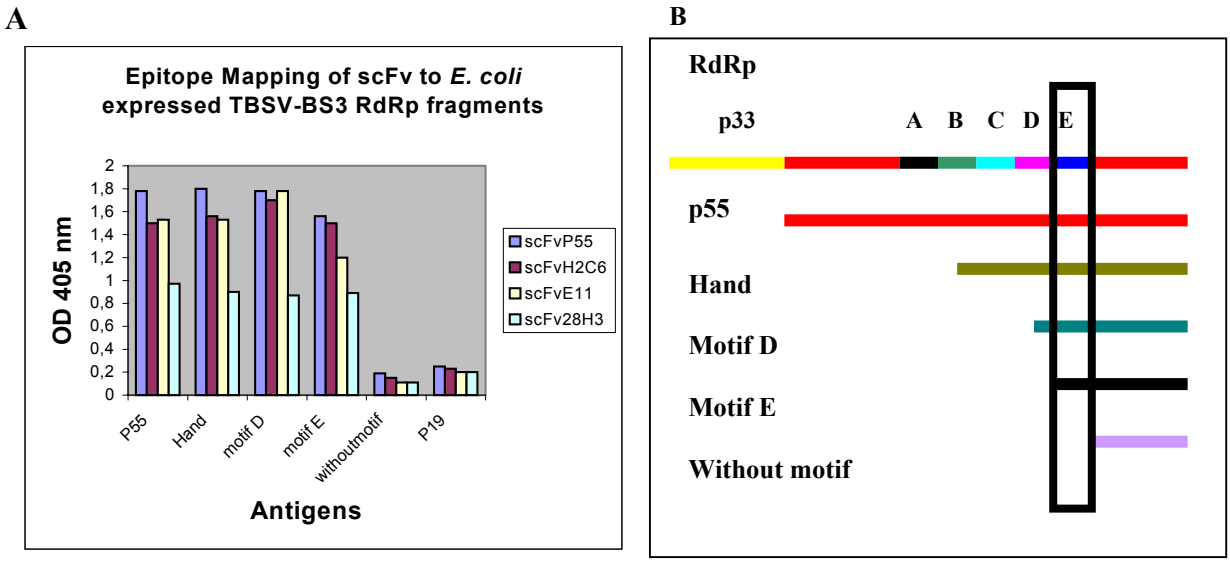


Fig.27 (A) Specificity of soluble single chain Fvs (scFvs) binding was determined by ELISA with a variety of antigens. p55 = RdRp without p33, Hand = p55 which motif A was deleted, Motif D = p55 which motif A , B and C were deleted. Motif E = p55 which Motif A, B, C and D were deleted, Without motif = p55 which all motifs were deleted and p19 = suppresser of gene silencing as a negative control. (B) shows the overlapping of the different RdRp fragment proteins.

The epitope mapping shows that all selected scFvs bind to motif E (fig.27). Moreover the binding is specific to the target antigen, since no significant signal can be detected when the P19 protein which is not a part of the RdRp was used as a negative control. Koonin (1991) demonstrated that TBSV RdRp belongs to the supergroup 2 of positive strand viral RdRps which comprises 4 different groups of viral polymerase. They are classified to (i) Tombus-, Carmo-, and Dianthovirus, (ii) Pestiviruses, (iii) Flaviviruses and (iv) ssRNA bacteriophage. Fig.28 shows the alignment of motif E among RdRps from different virus families. The motif E is quite homoluog in amino acid level among RdRps in supergroup 2. The Cystein residues

in this motif are contained in all RdRps of this supergroup. The E, V, E/P, F, C, Q/S residues in motif E are contained in all groups except group iv.

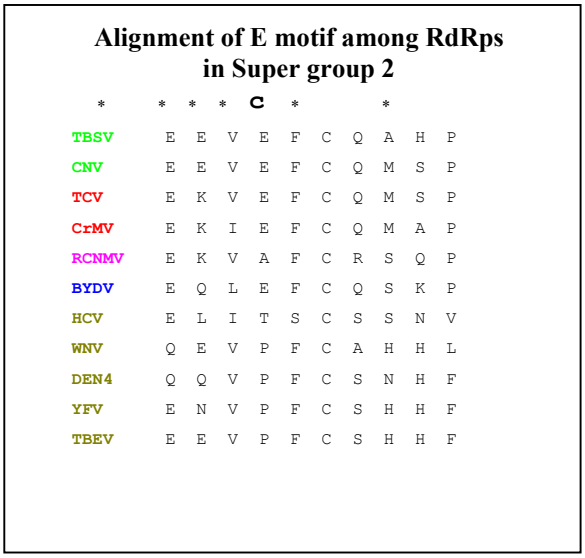


Fig.28 Alignment of the E motif of differenent groups of viruses. Tombusviruses, Carmoviruses, Ddianthoviruse, Pestiviruses and Flaviviruses

- These data were adapted from Koonin (1991).

From this data, it raises a question whether the selected scFv against the E motif of TBSV RdRp can inhibit other viral RdRps in this supergroup. Therefore the scFvP55H9 caused the most efficient inhibition of CNV RdRp activity *in vitro* has been chosen for further investigations using heterologous viral RdRps.

9. Inhibition activity of scFvs to *E.coli* expressed TCV RdRp.

To investigate further whether the selected scFvs can inhibit the RdRp from different groups of viruses, *E. coli* expressed functional TCV RdRp (Rajendran et al., 2001.) was used to study a scFv-mediated inhibition RdRp assay. TCV (turnip crinkle virus) belongs to the Carmovirus family. TCV has a small genome (4,054 bases) containing five genes of which two are required for replication, p28 and p88 (White et al., 1995). The p88 overlaps p28 and contains the signature RdRp motifs in the unique C-terminal portions. Rajendran et al. (2001) successfully expressed p88 in *E. coli* and demonstrated that the *E. coli* expressed RdRp has remarkable similarities with purified RdRp from infected plant in RNA template recognition and usage. To investigate further whether the selected scFvs can also inhibit RdRp of TCV (kindly provided by Rajendran), an *in vitro* assay with scFvP55H9 was performed. The

results clearly show that scFvP55H9 also strongly inhibits the activity of TCV RdRp *in vitro* (fig.29).

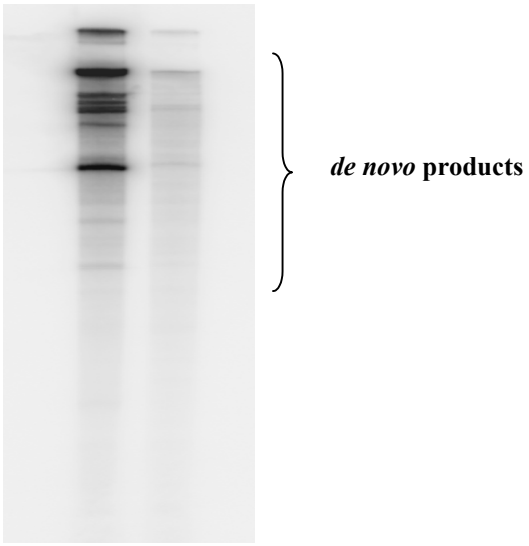


Fig.29 ScFv mediated inhibition of *E. coli* expressed TCV RdRp, *in vitro* assay. ScFvP55H9 was pre-incubated with *E. coli* expressed TCV RdRp 30 min prior to the RdRp reaction. Representative denaturing gel analyses of radio-labelled RNA products synthesised by *in vitro* transcription with *E. coli* expressed TCV RdRp (p88) in absence (lane 1) and presence (lane 2) of scFvP55H9. The reduced amount of replication product (*de novo* products) in lane 2 clearly shows the inhibition of RNA synthesis by scFvP55H9.

10. The binding activity of scFvs to HCV RdRp

The hepatitis C virus (HCV) is the major causative agent of parenterally transmitted and sporadic non-A, non-B hepatitis cases world-wide (Choo et al.,1989). It has been classified as a distinct member of the Flaviviridae family that also includes the flaviviruses and pestiviruses (Murphy et al.,1995). HCV contains a single strand RNA genome of positive polarity and approximately 9600 nucleotides length that encodes a polyprotein precursor of about 3000 amino acid (Bartenschlager and Lohmann 2000). HCV RNA replication proceeds via synthesis of a complementary minus-strand RNA using the genome as a template and the subsequent synthesis of genomic RNA from this minus-strand template. The key enzyme responsible for both of these steps is the virally encoded RNA-dependent RNA polymerase (RdRp), represented by nonstructural protein 5B (NS5B). HCV NS5B contains motifs shared by all RdRps and processes the classical fingers, palm, and thumb sub-domain. The alignment

data in fig.28 showed that the motif E of HCV RdRp shares some amino acid with TBSV RdRp. Mutation of amino acids in the region shows that hydrophobic residues in this motif are important for the interaction with the palm core structure (Hansen et al., 1997 and Jacoba-Molina et al., 1993). To investigate whether the selected scFvs can also bind to this RdRp, the binding activity of scFvs to HCV RdRp (kindly provided by Bartenschlager) was detected by ELISA.

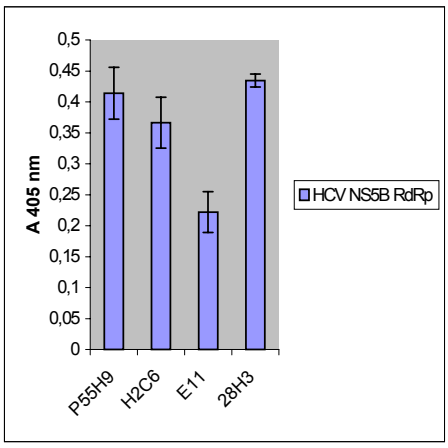


Fig.30 Binding of scFvs to NSB5 HCV *in vitro*. Immobilised NS5B was incubated with scFvs. Bound scFv proteins were detected after incubation with anti-myc followed by alkaline phosphatase-conjugated anti-mouse IgG and conversion of the phosphatase substrate p-nitrophenylphosphate by measuring the absorbance at 405 nm. Values are the mean of triplicate determination with standard deviations represented by error bars reduced by the mean value of the negative control (BSA).

The result in fig.30 shows that all selected scFvs can bind HCV RdRp (NS5B) to different degrees. ScFv28H3 showed the highest signal following by scFvP55H9, H2C6 and E11 respectively.

11. *In vivo* assay

The results of the *in vitro* assays clearly showed that the selected scFvs have the ability to inhibit the viral RdRps. To study scFv-mediated RdRp inhibition in plant cells two methods were developed to investigate the inhibition activity.

11.1 Agro-infiltration to transiently express scFvs in plant cells.

The scFv mediated inhibition of viral RdRp was investigated *in vivo* to confirm that the activity of the RdRp can also be inhibited in plant cells by selected scFvs. Two methods were developed to investigate the inhibition activity. First, intact leaves of wt *N. benthamiana* plants were infiltrated with an *Agrobacterium* suspension resulting in cytoplasmatic expression of the different scFvs. A control scFv not binding to the RdRp (scFv anti-Fus3, Fus3 being a seed specific transcription factor) was included in the assay to exclude the effect of the expression of heterogeneous proteins on viral infection. Four days after agroinfiltration TBSV virus particles were rub-inoculated onto infiltrated leaves. To confirm that the transient expression was functional, a leaf of each batch of infiltrated (non virus inoculated) plants was harvested 4 days after infiltration and protein expression (scFv) was determined by western blot. The results indicate that scFvs can be readily expressed in infiltrated leaves (fig 31C). Seven days after virus inoculation the plants expressing control scFv, scFv28H3 and scFvE11 developed systemic infection. The plants expressing scFvH2C6 and scFvP55H9 did not show, however, any symptoms of systemic infection at the same time point (fig.31A, B, data not shown for plants expressing scFv28H3 and scFvE11). At day 15, however, virus started to accumulate also in the scFvH2C6 and scFvP55H9 expressing plants. The delay of systemic infection in these plants is caused by the declining expression of scFvs in plant cells after agroinfiltration, since western blot analysis shows that the scFvs could not be detected anymore 15 days post infiltration (fig.31D).

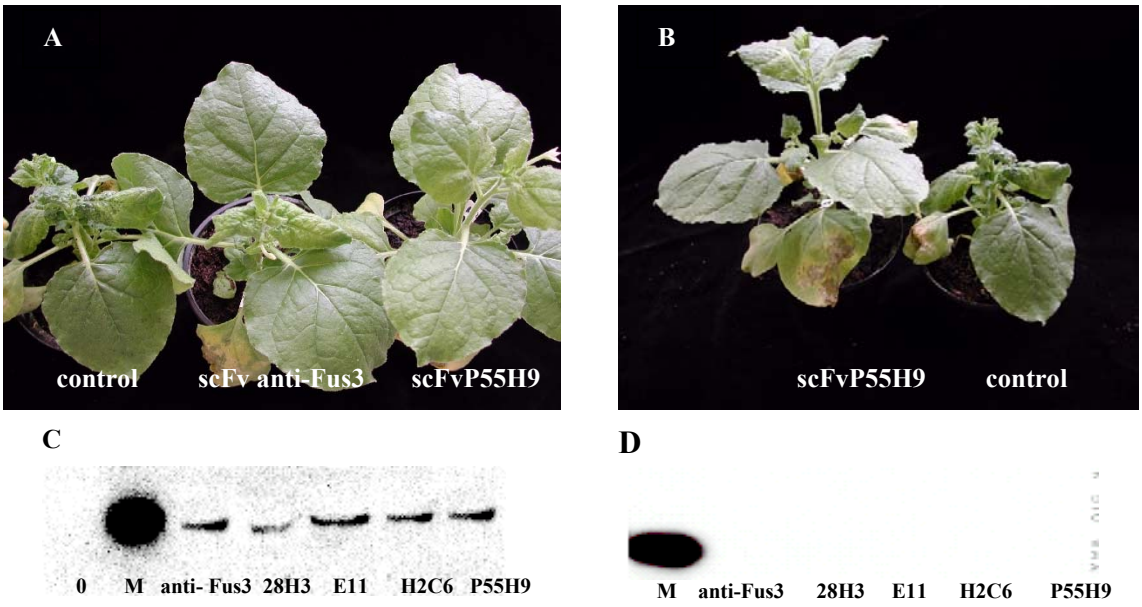


Fig.31 ***In vivo*** **assay.** The suspension of transformed *Agrobacterium* of scFvs was infiltrated into *N. benthamiana* leaves, 4 days after post infiltration the 1 µg of TBSV-virus particle were rub inoculated. Seven days post inoculate systemic infection was detected. Pic. (A) Comparison between systemic infection between Control, scFvanti-Fus3 and scFvP55H9, (B) shows the comparison of scFvP55H9 and control plant. The western blot show the expression of scFvs by Agroinfiltration. A infiltrated leaf of each constructs was harvested after 4 dpinf in (C) and 7 dpinf in (D). dpinf = day after post infiltration. M is a Marker.

11.2 Transient expression of scFvs in plant cells via a virus based vector.

In the second experiment, the scFv genes were cloned into an infectious clone of TBSV (provided by Galetzka, CGG) by replacing most of the ORF 3 coding for the coat protein of TBSV by the cDNAs in question (fig.32). This location in the TBSV genome was chosen since the coat protein is not necessary for systemic TBSV infection in host plants (Scholthof et al., 1995) and is therefore dispensable for the experiments. During the replication of the infectious clone the heterogeneous gene is expressed from the subgenomic RNA1 (sg RNA1) like the coat protein gene in the wild type virus (Scholthof 1999). Therefore, *in vitro* transcribed RNA carrying the different scFvs genes was used to rub-inoculated wild type *N. benthamiana* plants to test the inhibition activity of the different scFvs on the TBSV RdRp.

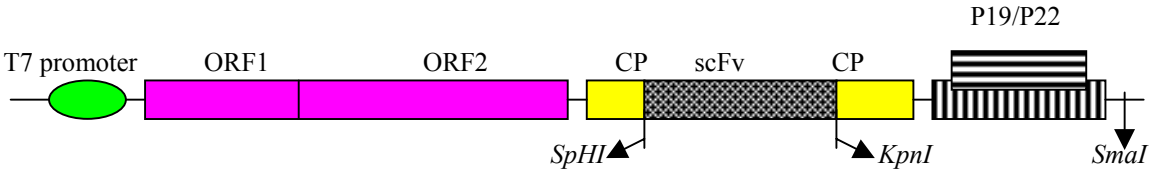


Fig. 32 Schematic drawing of FC8 infectious clone. The main part of coat protein was removed and substituted by scFv gene between *SpHI* and *KpnI* cloning site. The recombinant plasmid was linearized by digested with *SmaI* and used for RNA *in vitro* transcription by using T7 polymerase. The transcribed RNA was mechanically inoculated on *N. benthamiana*.

To obtain the viral RNA, the recombinant plasmid was linealized with *SmaI* and was used as the template for *in vitro* transcription as described in Materials and Methods. (see fig.33).

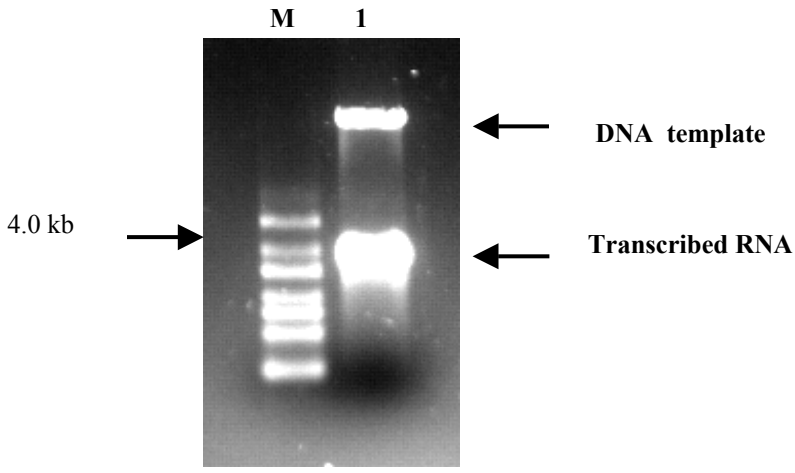


Fig.33 The result shows the *in vitro* transcription of the linearlized infectious clone. The viral genome was cloned behind the T7 promoter. Using T7 polymerase, the linearlized plasmid can be transcribed *in vitro*. The transcribed RNA products were rub inoculation on *N. benthamiana*. M = RNA ladder, lane 1 is the *in vitro* transcription. The DNA template is FC8 clone carrying a scFv gene.

5 µl of *in vitro* transcribed RNA was rub-inoculated on the *N. benthamiana*. The local and systemic lesions were observed and compared with the control scFv (anti-FUS3). Seven days after inoculation systemic virus infection was observed in inoculated plants expressing the control scFv and scFv28H3 but not in plants expressing the other scFv. Only a few local symptoms could be seen in leaves inoculated with infectious clones carrying scFvH2C6, scFvE11 and scFvP55H9 (fig.34A and B). Seven days after inoculation, a leaf of each inoculated plant was harvested to detect scFv expression. The western blot result showed that all selected scFvs were expressed in plant cells (fig.35A). This result supports that the plants

inoculated with infectious clones carrying the different scFv showed resistance to virus infection because of the expression scFvs. Moreover 21 dpi inoculated leaves of each batch of scFv expressing plants were harvested and the accumulation of scFvs was investigated by western blot. The results showed that only scFv anti-Fus3 from control plants could be detected 21 days after inoculation and seemed to accumulate compared to day 7 after inoculation (fig.35B).

To monitor the virus titre in inoculated plants, *N. benthamiana* wt plants were sub-inoculated with plant sap from apical leaves from each of the plants as this is the most sensitive method to detect the presence of infectious virus particles. Three days after inoculation only plants subinoculated with inoculum from plants expressing the control scFv and the scFv28H3 showed local symptoms in inoculated leaves and only plants expressing the control scFv showed systemic infection 4 dpi (data not shown).

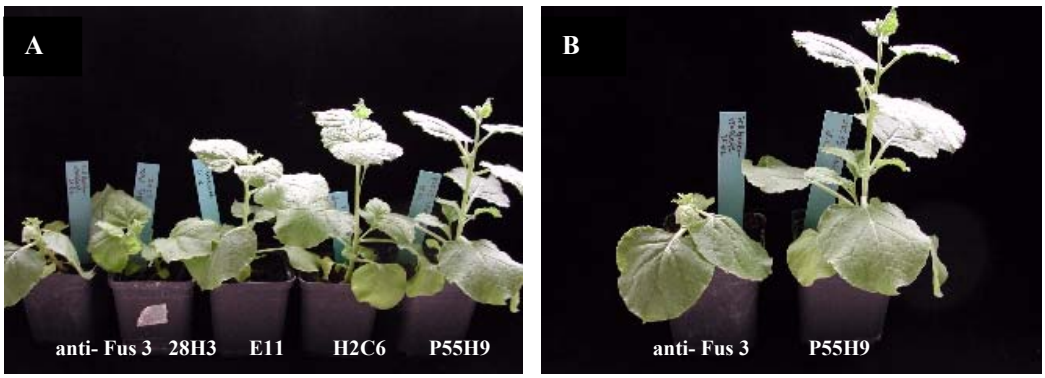


Fig.34 ScFv mediated inhibition of the replication of TBSV-BS3 wild-type virus, *in vivo* assays. (A) Symptom expression (7 days after inoculation) in plants inoculated with infectious TBSV-BS3 clones carrying different scFv genes. Plants infected with the clones expressing scFv 28H3 and scFv anti-Fus3 show systemic infection including stunted growth and malformation of apical leaves whereas plants infected with the clones expressing scFv E11, H2C6 and P55H9 remain symptom-free. (B) Symptom expression in plants expressing scFvP55H9 after agroinfiltration as well as in wt control plants 7 after virus inoculation. Apical leaves of wt and scFv anti-Fus3 expressing plants show typical malformation and mosaic symptoms whereas scFvP55H9 expressing plants remain symptom free after virus inoculation.

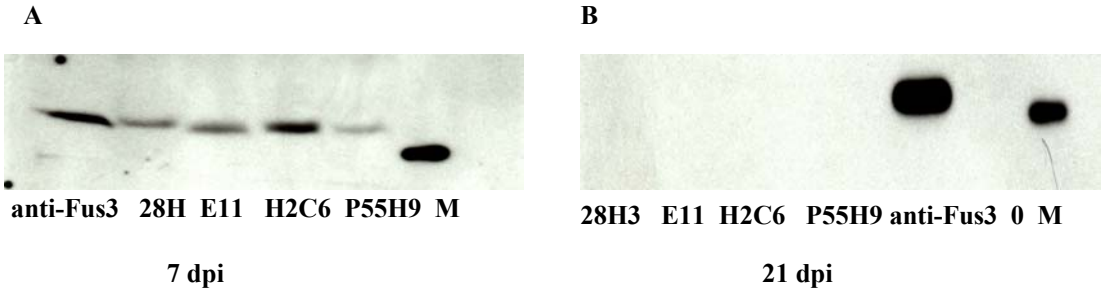


Fig.35 The western blot results show the expression of the scFvs from an infectious TBSV-BS3 clone carrying different scFv genes. (A) Western blot showing the expression of the different scFvs after agro-infiltration. infiltrated leaves were harvested 4 dpi (time point of infection. (B) Western Blot showing the scFv expression 21 days after inoculation with the infectious clones. Due to the inhibition of the replication of the infectious clone by the different scFvs only the expression of the control scFv could be detected 21 dpi.). dpi = day post infiltration. M = Marker (scFv anti-Fus3 expressed from *E. coli*).

12. Establishment transgenic plants expressing scFvP55H9

As the results of *in vitro* and *in vivo* experiments showed that scFvP55H9 is the most efficient scFv, it was transformed into *N. benthamiana* plants. The scFvP55H9 gene was cloned into pRTRA plasmid between *NcoI* and *NotI* restriction sites to express the scFv in the cytosol and to express the scFv in the ER, the gene was cloned behind the ER leader sequence at a *BamHI* restriction site (fig.36A). The whole cassette of two constructs was cloned into the binary vector pPZP 200 (Hajukiewicz et al., 1994) resulting in a recombinant plasmid named CytoP55 and ERP55 (fig.36B). Both of constructs contain a myc-tag and a KDEL tetrapeptide at 3´end of scFv for protein detection and stabilisation of scFv in the cytosol.

The recombinant plasmids were electrotransformed into competent *A. tumefaciens* ATHV. A single colony of each transformed bacteria was cultured overnight at 28°C and used for plant transformation as described in Materials and Methods. After regeneration, 9 lines and 8 lines from different transformation events of *in vitro* plants expressing scFvP55H9 in the cytosol and expressing scFv in ER respectively were obtained. Self fertilised seeds of each lines were germinated and used for testing viral resistance.

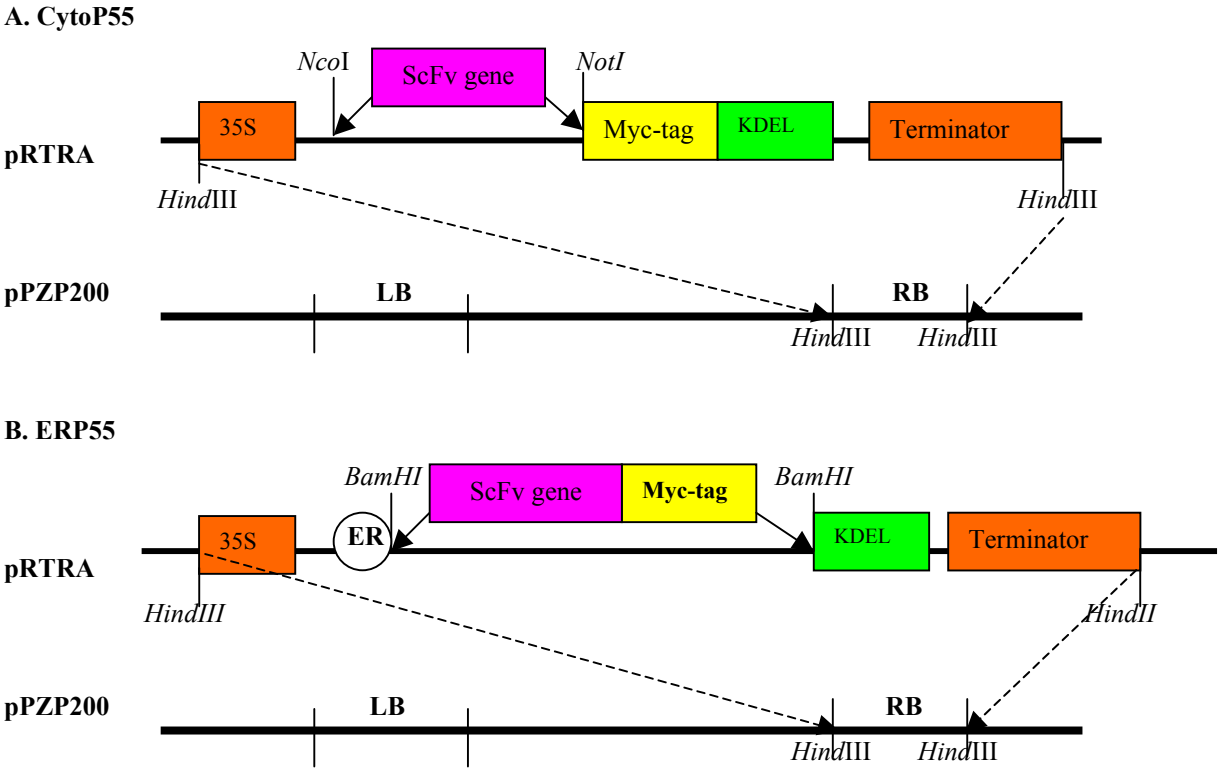


Fig.36 Schematic drawing shows the cloning profile of scFvs into the binary vector. The scFvP55H9 was cloned into pRTRA vector. The whole cassette was subcloned in the right border of pPZP200 at *Hind*III. The recombinant plasmid was electro transformed into competent *A. turmufacien* for plant transformation. (A) shows the cloning strategy of scFvP55H9 for expressing in cytocol. (B) shows the cloning strategy of scFvP55H9 for expressing in ER.

13. Challenging transgenic plants with two different virus families

Transgenic plants producing scFvs either in the cytoplasm or ER were regenerated to analyse the effect of antibody expression on virus infection. Seeds from self-fertilised transgenic lines carrying scFvP55H9 were germinated, selected against kanamycin and analysed for scFv expression by western blot analysis. 10 T_1 plants of 9 lines expressing the scFv in the cytoplasm (line 1-9CP) and 8 lines expressing the scFv in the ER (line1-8ER) were inoculated at the 4-6 leaf stage with 10 ng of purified virus particles (TBSV-BS3-Statice) per leaf. All wild type (wt) control plants of *N. benthamiana* infected with this inoculum concentration developed systemic symptoms of infection and died 2 weeks after inoculation. All transgenic T_1 plants from line 1, 2 and 4CP and line 1 and 6ER did not show, however, any symptoms of virus infection (fig.37A and C). To further monitor the virus titer in inoculated plants, *N. benthamiana* wt plants were sub-inoculated with plant sap from apical leaves of the transgenic

TBSV-inoculated plants as it was done in the transient assay. None of the subinoculated plants showed any local or systemic symptoms, indicating the complete inhibition of TBSV-BS3-Statice replication in the transgenic scFv expressing plants. Western blot results demonstrated that all of these transgenic plants expressed the scFv to a high level (fig.37D). All low or non-expressing scFv transgenic lines failed to show protection and became infected, including the inoculated wt plants.

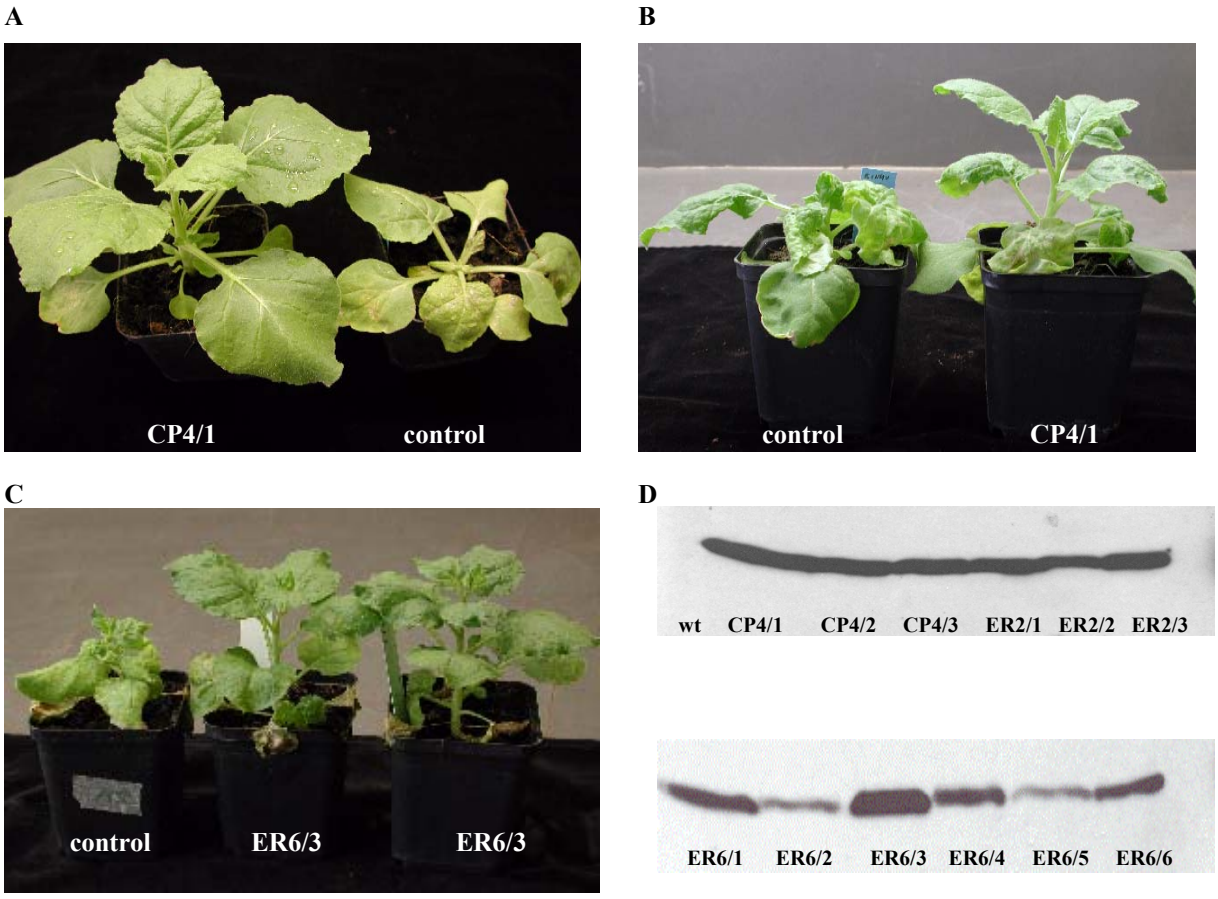


Fig.37 Transgenic *N. benthamiana* plants show resistance to RCNMV and TBSV. T_1 transgenic plants expressing scFvP55H9 (both in cytoplasm and ER) and untransformed wt control plants were mechanically inoculated with sap from RCNMV infected leaf tissue diluted 1:1000 or 10 ng of TBSV viral particles. (A) Plant from transgenic line CP4, showing no symptoms at all 10 dpi after inoculation with TBSV particles. (B) Plant from transgenic line CP4 inoculated with plant sap containing RCNMV particles, showing no systemic symptoms but some mild local symptoms 10 dpi. (C) Transgenic plants expressing scFv in the ER showed the same results after inoculation with TBSV (second plant from the left) and RCNMV (plant to the right). Control plant was inoculated with TBSV. The transgenic plants expressing scFv only to a low level (ER6/2, 5) did not resist to viral infection. Control plants of both experiments showed stunted growth, strong local symptoms and finally died 2 weeks after inoculation. (D) Western Blot of protein extracts from leaves of transgenic *N. benthamiana* lines expressing P55H9 either in the cytoplasm or ER. First number indicates the line, second number the plant. wt = untransformed *N. benthamiana* control plant.

Red clover necrotic mosaic virus is a dianthovirus and its RdRp is classified into the same group as the RdRps of Tombusviruses (Koonin, 1991). To investigate a possible broad range resistance of our transgenic lines, the same infection assays were done as for TBSV-BS3 with the exception that plant sap of RCNMV infected wt *N. benthamiana* plants diluted 1:1000 in inoculation buffer was used for infection. Control wt *N. benthamiana* plants showed systemic symptoms within 4-5 days and died 2 weeks after inoculation. ScFv expressing plants challenged with this inoculum showed local symptoms but no systemic symptoms 2 weeks after inoculation. (fig.37B and C). Some of these plants showed, however, mild systemic symptoms but did flower like control plants.

V. Discussion

Cloning of RdRp fragments

For the cloning, the different fragments of TBSV RdRp (p33, p55, p92 and only 5 motif) were amplified by PCR. *Nco* I and *Sal*I restriction sites were introduced at the 5´and 3´ end of each fragment respectively. Since *Nco*I restriction site are present not only at the 5´end of the p55 and p92 segments but also in frame, the PCR products of p55 and p92 had to be partially digested. The result showed that 1 µl of restriction enzyme (10U/µl *NcoI*), 1 µg DNA and around 10 minutes are the suitable conditions to obtain properly digested products in this case. All digested PCR products of each RdRp segment were cloned in various expression vectors as shown in table 4. The sequencing results confirmed that each plasmids carried the target genes in frame. Moreover a clone, 388 in 26b+ , which has a size of at about 35 kD and contains only a part of 5 motifs (starting from motif A-3´end of p92 gene) was also obtained. This part of p92 may have been non-specifically digested during the partial digestion step. To differentiate this segment from the others, it was named "Hand of the replicase protein (Hand)".

Production of recombinant proteins as affinity-tagged fusions in *E. coli* has become a standard laboratory procedure, which enables rapid purification. However, the choice of expression vectors and, hence, of the purification scheme is case-dependent. Among the factors affecting this choice are expression level and final yield, solubility of the fusion, specificity and affinity of the purification systems and the associated time and material costs. There are many fusion tags available to optimise the expression profile and the solubility of target proteins, such as the glutathione S-transferase fusion system, maltose-binding protein fusion and others. However these fusion tags are big and might change the intrinsic properties of the target proteins. Moreover using the target proteins fused with a large polypeptide as antigens for scFv selection might result in a large number of unspecific scFv which do not bind to the proteins in question but to the fused polypeptide. Therefore the best choice is to have a tag that is as short as possible to reduce the risk of altering the properties of the target proteins by the fused polypeptide and obtain scFvs which bind exactly to the proteins in question. Therefore expressing RdRp segments fused with a His-tag which is composed of only 6 amino acids was selected in this study. In primary studies two different kind of plasmids (pET 23a+ and 26b+) were used to express the RdRp segments in *E. coli*. The characteristic of these plasmids are shown in table 2.

Expression of RdRp fragments

All recombinant plasmids carrying RdRp segments were transformed into the different *E. coli* strains for protein expression. The many advantages of using *E. coli* expression system have ensured that it remains a valuable organism for the high level production of recombinant proteins (Georgiou and Valax 1996). However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism. Therefore it is worthwhile to combine different plasmids in different bacterial strains to optimise the expression profile. The characteristic of the plasmids used are shown in table 2.

In these experiments four different strains of bacteria were used for expression of the viral replicase proteins (see table 3). These hosts are lysogens of bacteriophage DE3, a lamda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lac*I gene, the *lac*UV5 promoter, and the gene for the T7 RNA polymerase (Studier and Moffatt 1986). The fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter directing transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). Addition of IPTG to a growing culture of the lysogen induces the T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid (see genotypes of each host stain in table. 3).

The expression results showed that the Hand of the replicase protein in pET 26b+ (38/8/26b+) and 5 motif in pET23a+ (111/23a+) can be expressed in BL21(DE3) and BL21(DE3)LysS bacterial strains and p55 in pET 23a+ (27/23a+) can only be expressed, however in BL21 LysS (see table 4 and fig.8 and 9). P33, clone 115 in pET 26b+ and p92 clone 38/6 in pET 26b+ could not be expressed in any bacterial strains, even through the sequences were correct and in frame. This may be due to the combination between the plasmid and the expression host and/or the target proteins themselves may interfere with gene expression or with the integrity of the cell. The protein of these segments may also be toxic to the expression hosts and may be destabilised in λDE3 lysogens by cAMP mediated derepression of the *lac*UV5 promoter, which may increase basal expression of the T7 RNA. Basal expression can take place by a leaking mechanism, meaning that the T7 RNA polymerase can be expressed without induction by IPTG (basal expression), this low amount of polymerase is then enough

to drive the transcription of target genes and finally translation. When a putatively toxic gene was cloned and expressed in this condition, the basal expression of this toxic protein may have resulted in the loss of plasmid-containing bacteria from the culture, and thus ultimately in a reduction of the amount of recombinant protein being produced (Suter-Crazzolara and Unsicker 1995). This hypothesis was supported by the successful expression of p55 in combination of 23a+ plasmid carrying a plain T7 promoter and BL21pLys bacterial strain. The pLys bacterial strain carries a pLys plasmid encoding the T7 lysozyme, which is a natural inhibitor of the T7 RNA polymerase and thus reduces its ability to transcribe the target genes in noninduced cells. However this strategy could not be applied to express p33 and p92 proteins, since these genes could not be expressed from this combination. Different concentration of IPTG (1:10, 1:100, 1:1000 of recommended concentration; 0.4 mM for pET 23a+ and 1.0 mM for pET 26a+ and different temperatures (26°C, 30°C and 37°C) were also applied to search for the optimal conditions for expression but variation of these factors did not give any expression of p33 and p92 (data not shown). An overnight expression of p92 was also tried. Since the p92 gene contains the amber stop codon, it may take longer time to express the whole segment. This attempt did not result in any expression of p92, since a target band could not be seen in the SDS-PAGE after induction of this clone overnight (data not shown).

In fact not every gene can be efficiently expressed in *E. coli*. This may be due to the unique and subtle structural features of the gene sequence in question, the stability and transitional efficiency of mRNA, the ease of protein folding, degradation of the protein by the host cell protease, the potential toxicity of the protein to the host and the major differences in codon usage between the foreign gene and the native *E. coli* (Makrides 1996). The major arginine $tRNA^{Arg\ (AGG/AGA)}$ has been shown to be a limiting factor in the bacterial expression of several mammalian genes (Brinkmann et al, 1989). This result was also confirmed when p33 and p92 which contain a arginine rich region were successfully expressed in combination of the pMal-cx vector (Biolab) and BL21-Codonplus *E. coli* strain (Stratagene) (Rajendran, personal communication). BL21-CodonPlus-is a *E. coli* strain which carry extra copies of the *argU, ileY*, and *leuW* tRNA genes. The tRNAs encoded by these genes recognise the AGA/AGG, AUA, and CUA codons, respectively. The presence of these additional tRNA genes resolves the issue of codon bias for organisms whose genome is AT-rich. However the purified p92 did not show activity to amplify a given template in an *in vitro* assay (Rajendran, personal communication). The reason might have been that the p92 was expressed as a MBP (maltose-

binding protein, size about 48 kD) fusion protein which might have changed the intrinsic property of p92.

Soluble and Insoluble protein investigation

To get properly folded proteins for selection of the antibodies, binding to the active centre of the protein in question, it is necessary to produce the proteins in the native soluble from.

Therefore two methods were used (shearing with syringe and using BugBuster® reagent from NOVAGEN) to investigate soluble and insoluble protein expression. By shearing the cells with the syringe method, most of the total protein remained in the insoluble part (pellet), they accumulated, however, more in the soluble part when the bacteria were extracted with the BugBuster® reagent (data not shown). It may be that the shearing force shearing with the syringe is not enough to break the bacterial cell wall and/or the soluble lysis buffer is not optimal. Therefore extraction of the induced cells was done in further experiments with the BugBuster® reagent. The results showed that most of p55, Hand protein (fig.10 and 11) and 5 motifs proteins (data not shown) remained in the insoluble part (inclusion body). Upon overexpression of recombinant proteins inclusion bodies can be observed in several host systems. Even endogenous proteins, if overexpressed can accumulate in inclusion bodies (Gribskov and Burgess 1983), suggesting that in most cases inclusion body formation can be the consequence of high expression rates, regardless of the system or protein used. There is no direct correlation between the propensity of inclusion body formation of a certain protein and its intrinsic properties, such as molecular weight, hydrophobicity, folding pathways, *etc*. Only in the case of disulfide bonded proteins inclusion body formation can be anticipated if the protein is produced in the bacterial cytosol, as formation of disulfide bonds does usually not occur in this reducing cellular compartment. The consequence is improper folding resulting in aggregation (Hauke et al., 1998).

Based on the information described above, various conditions were tested to increase the soluble protein fraction by slowing down the expression rate, such as decrease of temperature for induction from 37°C to 20°C and variation of the concentration of IPTG from 1:2, 1:10 of the recommended concentration (0.4 mM for the clone in 23a+plasmid, and 1.0 mM for the clone in $26b^+$ plasmid). However, the amount of the soluble protein could not be increased. In the case of the Hand protein (38/8) which was cloned in pET 26b+, the investigation of

soluble protein in the periplasmic space by using the Osmotic shock method was also done. In pET 26b+, there is the pelB leader gene upstream of the inserted viral replicase gene. This pelB gene codes for the protein which targets the target protein into the periplasmic space. In contrast to the cytoplasm, the periplasm of *E. coli* is an oxidising environment which contains enzymes that catalyse the formation and isomerization of disulfide bonds (Rietch et al., 1996; Raina and Missiakas 1997; Sone et al., 1997). Directing heterogeneous proteins to the periplasm is a common strategy when attempting to isolate active, folded proteins containing disulfide bonds. This is an option which may reduce the formation of inclusion bodies.

After expression of clone 38/8/26b+(Hand protein) in BL21(DE3) and extraction of the bacterial cells with Osmotic shock, the target protein could, however, not be detected in the soluble fraction. An explanation may be that this Hand protein is very toxic to the bacterial cells. So when this protein was over expressed in the bacterial cells, they might have tried to eliminate this foreign protein by missfolding, which caused the inclusion body formation before the protein could be transported to the periplasmic space (Snyder and Silhavy 1995).

The target proteins were apparently degraded after induction overnight (lane 8 and 10 in fig. 9) and data from western blot (the bands under 30 kD in lane 6 and 7 in fig.12) showed that the longer time for induction, the more target protein was digested by the bacterial protease, even the expression was done in BL21 strain which is deficient in the *lon* protease and lacks the *ompT* outer membrane protease that can degrade proteins during expression (Grodberg and Dunn 1988). Moreover there was a basal expression in clone 27/23a+(p55 protein) in BL21(DE3)pLysS, which can be seen from the western blot of non induced clones (lane 1 and 4 in fig.12). This might have occurred because the 23a+ plasmid contains the plain T7 promoter and the target protein was expressed in BL21(DE3) pLysS which corresponds to a mild stringent condition only. Even in the absence of IPTG, there is some expression of T7 RNA polymerase from the *lac*UV5 promoter in λDE3 lysogens which inducts the T7 promoter in the plasmid, and this causes basal expression.

Expression of these proteins under another soluble protein leader sequence such as NusA in the pET43^{+} vector and also in a new bacterial strain, Origami(DE3), was also tried. The NusA protein has been identified as having the highest potential for solubility (Davis et al., 1998). Expression of all target proteins fused with the NusA protein in the expression host listed in table 3 could not result, however, in any soluble protein expression. Origami™ is an expression host which carries mutations in both the thioredoxin reductase (*trxB*) and

glutathione reductase (*gor*) genes. These two proteins greatly enhance disulfide bond formation in the cytoplasm. Studies have shown that expression in this strain can yield 10 fold more active protein than in another host even though overall expression levels of the two proteins were similar (Prinz et al., 1997). All plasmids carrying the target proteins were also tried to be expressed in this bacterial strain. The result showed, however, that no soluble target proteins were obtained even by using this combination.

Purification of *E. coli* expressed RdRp fragments

To get the correctly folded proteins, it is very important to purify the proteins under native soluble conditions. Although most of all target proteins were present in inclusion bodies, there might have been some soluble material that can be purified in the native form when using large scale expression. The putative soluble proteins were therefore purified by Batch purification. The result shows, however, that this purification failed, since the bands of these proteins could be detected in binding and washing lanes but not in the elution lanes in SDS-PAGE and immunodetection (fig.13). Purification under non-denaturing condition can result in reduced yields if the His-tag is only partly exposed due to protein folding. Purification of 6X His-tagged proteins under native condition may allow co-purification of associated proteins such as enzymes subunits and binding proteins (lane E of fig.13). However, under native conditions, there is a higher potential for unrelated, non-His-tagged proteins to interact with the resin than under denaturing condition. Therefore the conditions for purification were also optimised, by increasing the concentration of NaCl from 300 mM to 2 M to prevent ionic interaction in the binding step and the pH of extracted proteins was adjusted to pH 8.0 before incubation with Ni-NTA Agarose. Other additives, like glycerol to prevent hydrophobic interaction between proteins, 10 mM ß-mercaptoethanol to prevent disulfide cross-linkages, were also added in the protein extraction solution and washing buffer, but all of these attempts did not affect the binding pattern. It may therefore be concluded that the His-tag of the p52 and Hand protein may be hidden in the protein molecules.

Taken altogether, the attempts to express the target proteins in soluble from failed. Therefore the inclusion body proteins had to be used for further experiments. The purification of target proteins under denaturing condition was successful and resulted in high yields of purified target proteins. Two method were applied to redenature the target proteins. Firstly the purified denatured proteins were dialysed against PBS buffer at 4°C. Secondly the proteins were

stepwise dialysed against serially diluted Urea buffer. However these attempts were failed to recover the native proteins (data not shown). Although there might have been some redenatured proteins below detection limit, they might fold in the wrong position. Moreover lacking the activity tests of these proteins, it is very difficult to guarantee that the redenatured proteins are still active after redenaturation. Therefore the denatured purified proteins were used to select scFvs in Phage display experiments.

Phage display for scFvs selection

The recombinant Phage Antibody system is designed to clone antibody genes and to express and detect functional antibodies. It is based on a phage-display system in which fragments of antibodies are expressed as fusion with phage proteins and displayed on the phage surface. In general, antibodies have been fused to the N-terminus of the pIII gene and displayed as Fab fragments or as single chain Fv (scFv) fragments in which the VH and VL domains are linked by a flexible polypeptide. Repertories of antibody fragments were first generated by polymerase chain reaction (PCR) from rearranged V-genes of populations of lymphocytes, sources including the spleens of immunised mice, the peripheral blood lymphocytes of immunised humans, and the bone marrow of infected humans. The use of immunisation greatly enriches for V-genes encoding antibodies complementary to the immunogen, leading to the isolation of antibodies with excellent binding affinities. However, such “immune” repertories, directed towards a single or limited number of antigens, are less suitable for making antibodies against a range of antigens, especially self-antigens. So the repertories which are built from the rearranged V-gene of peripheral blood lymphocytes of unimmunised human so call “ naive repertories” or by assembly from human V-gene segments rearranged *in vitro* “synthetic” repertories offer, however, a possibility to solve the limitation of immune repertories as mention above (Marks et al., 1991).

In this study two naive human repertories provided by MRC, Cambridge University were used. The process of recovering phages displaying a recombinant antibody by “ biological panning” entails the indirect immobilisation of phage to a solid surface or of the recoverable particle via an antibody-antigen interaction. In this study, the first approach was used. The purified denatured RdRp fragments were immobilised to a solid surface (polystyrene micro titer plate, Maxisorb™) and the phages became indirectly immobilised to the solid surface via the given antigens. After immobilisation, washing, and recovery, phages were eluted from the solid support, pooled and amplified in preparation for further rounds of affinity enrichment.

The results of panning showed that the purified denatured RdRp fragments could successfully be used as antigens to select scFvs from the repertories. In principle, each round of panning and amplification will result in enrichment of the phage population in favour of those that bind the immobilised antigens. Fig.15 shows the enrichment of the phage population for segment 28H3 and p52. However, in the case of the Hand protein the enrichment of phages dropped in the 2nd round of panning and increased again in the third round. Apparently the enrichment of a phage population is differed according to the antigens used. Not surprisingly, the conduct of a given experiments with respect to the kinetics of panning can vary widely between different antigens and different selection rounds (William and Schorr 1990). Such variability can be attributed to several factors inherent in the panning procedure including (a) different binding capacity and loading of individual antigens to the solid support surface, (b) the number of antigen-binding phages initially present in the library population (c) the affinity of phage binders for the selected antigen and (d) the stringency (volume and number) of washing conditions used prior to phage elution and amplification (William and Schorr 1990). After biological panning, the selected phage population was further selected for monoclonal phages and soluble scFv expression. The results showed that only 0.04% of monoclonal phages were able to express and excrete scFvs fused to pelB leader to the culture media, while still retaining the binding activity to the given antigens. It can be assumed that most of the expressed scFvs cannot be secreted to the media because of their intrinsic properties.

The monoclonal phages which are able to secret scFvs to the media and showing the best binding activity in ELISA were selected. 12 monoclonal phages were obtained and scFvs were further characterised. The characterisation data of these scFvs showed that all 12 selected scFv have differences in their binding domain (CDR) reflecting their binding activity (fig 16, 17 and 18).

As denatured RdRp protein segments were used for scFv selection, it was not clear if these selected scFvs would provide the same activity as scFv selected against the native viral RdRp. Therefore it was important to investigated if the selected scFvs show affinity to the viral native RdRp prior to transformation of these genes into plants.

***In vitro* assay of scFv-mediated RdRp inhibition**

In 2000, Nagy and Pogany had successfully purified and characterised CNV and TBSV RdRps from infected plants. These RdRps showed the activity to transcribe a given template

(DI-72) *in vitro*. Therefore, in the *in vitro* assays to study scFv-mediated RdRp inhibition, partially purified CNV RdRp was used. The results in fig.21 and 22 showed that all selected scFvs were able to inhibit the RdRp at different degrees. The reduction of *de novo* products was not due to a possible degradation of RNA by any compound in the scFvs preparation. This was supported by a control experiment as shown in fig.20. Moreover from fig.22 it was clear that the inhibition is concentration dependent. It can also be concluded that the degree of RdRp inhibition depends on the different complementary determining regions (CDRs) of each of the scFvs. The CDRs come together in the final tertiary structure to form an antigen binding pocket (Roitt et al., 2001). Therefore differences of these region of scFv result in different antigen binding activity *in vitro*. It is clear that scFv P55H9 is the best scFv giving the highest RdRp inhibition activity. Hence it was used for further experiments. Studying the effect of different templates and temperature condition on RdRp inhibition showed that the different templates do not effect the extend of inhibition of the RdRp. It seems, however, that the minus strand is the best template for RdRp recognition and amplification in *in vitro* studies (see fig.23). This is consistent with the fact that also in nature the minus strand of DI RNA is abundant in virus infected plants (Nagy, personal communication).

When scFvs and the RdRp were incubated at 25°C, the inhibition of the RNA synthesis was, however, more efficiently blocked than at 4°C (see fig.24). Considering the kinetic of enzymes, it might be postulated that the increasing temperature causes an increase of the reaction rate. However, when the temperature is too high, it may damage the enzyme molecules, resulting in a dramatically reduction of the reaction rate (Copeland, 2000). From this result it might be concluded that the selected scFvs may also efficiently inhibit viral replication in plant cells.

Mechanism of scFv-mediated RdRp inhibition

Interestingly, RNA synthesis could not be inhibited when scFvs were added only after the start of transcription of the template by the RdRp (fig.26). The observation that the scFv can inhibit the RdRp activity efficiently when applied prior or simultaneously with the template RNA (fig.25), and the fact that it cannot inhibit the RdRp activity if added subsequently suggest that the scFv may interfere with the binding of the template at the catalytically active site of the RdRp rather than inhibiting the RNA synthesis. This hypothesis was also supported by studies of Hayes et al. (1994) and Bates et al. (1995). It is also possible that the scFv binding to the RdRp may change the structure of the RdRp, making it unsuitable for initiation

of RNA synthesis. Based on the above results, it is therefore unlikely that the scFvs can inhibit the RNA synthesis during strand elongation.

Epitope mapping and inhibition of scFvs of different RdRps

To explain the mechanism of inhibition in detail it is important to know exactly the binding site of an scFv. Fig.27 shows that all 4 scFvs bind to the motif E of the RdRp. The motif E of TBSV RdRp is predicted to exhibit an α-helix conformation. However, it is unknown whether it can also act as a thumb clamp. The results show that binding of our scFvs to this region abolishes the activity of RdRp. However, after the transcription having started the scFvs cannot bind and inhibit the RdRp activity anymore (see *in vitro* results, fig.26). These data support the hypothesis that the motif E of the tested viral RdRp acts as a thumb clamp while binding to the template and is therefore highly important for the RdRp activity.

TCV is Carmovirus which also belongs to the family of Tombusviridae and its RdRp is in the same lineage as those from TBSV and CNV. Although the alignment result in fig.28 shows that the E motif of TBSV and TCV is almost identical, the percentage identity between these RdRps is only 36%. It is therefore worthwhile to investigate the inhibition activity of selected scFvs of the TCV RdRp. Recently it was demonstrated that TCV RdRp expressed in *E. coli*. shows RdRp activity *in vitro* (Rajendran et al., 2001). The results *in vitro* assay of scFv-mediated TCV RdRp inhibition clearly show that scFvP55H9 also strongly inhibits the activity of TCV RdRp *in vitro* (fig.29). It may be assumed from this result that the amino acid residues surrounding the E motif may not effect the conformation of this region in such a way that it would not be accessible to the scFv anymore.

Also the RdRps of a number of animal and human plus strand RNA viruses belong to the same supergroup as the tombusvirus RdRps. The RdRp of Hepatitis C (HCV RdRp) represented by the nonstructural protein 5B (NS5B) was successfully expressed *in vitro* and has been shown to be essential for viral replication in *in vitro* (Moradpour et al., 2001). HCV NS5B contains motifs shared by all RdRps. Epitope mapping showed that the binding site of all selected scFvs is in the motif E region (see fig.27), E motifs of TBSV and HCV RdRp sharing some amino acids homologies. The result in fig.30 shows that specific binding was clearly measured. These results open the way for further experiments to achieve broad range virus resistance by targeting highly conserved motifs in viral RdRps.

In vivo assays of scFv-mediated RdRp inhibition

The *in vitro* results clearly show that the selected scFvs can inhibit RdRps *in vitro*. However, this may not guarantee that the inhibition will be the same *in vivo*. Therefore it is necessary to investigate whether the selected scFvs function in plant cells.

Transiently expression of heterogeneous proteins via agroinfiltration or via virus based vectors offer attractive tools to study the pattern of protein expression and it's activity prior to transforming the target genes into plant cells. Based on these methods two sets of experiments were performed to study the inhibition activity of scFvs *in vivo*.

In the first experiment the scFvs were transiently expressed by infiltration of *N. benthamiana* leaves with agrobacterium suspension carrying the different scFv genes. *Agrobacterium*, infiltrated into plant leaves as a cell suspension, mediates the transfer from the T-DNA region of the bacterial Ti plasmid into plant cells (Kapila et al., 1997). Under control of the 35S promoter, the scFvs gene can be transcribed and finally translated in plant cells. Most of the plant cells in the infiltrated region showed to express the transgene. The results in fig.31C show that all selected scFvs were expressed by this method. Four days after infiltration, 1 µg of purified TBSV-BS3 virus particles was mechanically inoculated on the infiltrated leaves. This mimics the situation in a transgenic plant were leaves constitutively express the scFv in question. Seven days post inoculation systemic infection was observed only in plants which were infiltrated with agrobacterium carrying scFv28H3, E11 and anti-Fus3. ScFv anti-Fus3 is a scFv against a seed specific transcription factor (Fus3) and used as negative control. The reduced susceptibility to virus infection is not due to the stress induced upon the plants by agroinfiltration as showed by the plants infiltrated with the control construct. These plants showed infection like wild type *N. benthamiana* plants. The level of transgene expression usually peaks at about 3 days post-infiltration and declines rapidly thereafter (Voinnet et al., 2003). Therefore not surprisingly, virus started to accumulate also in the scFVH2C6 and scFvP55H9 infiltrated plants 15 days after infiltration. The delay of systemic infection in these plants may be caused by the declining expression of scFvs in plant cells. This interpretation was supported by a western blot analysis which showed that the scFvs could not be detected anymore at this time period. It was originally thought that the decline of gene expression was due to a inappropriate bacterial strain/host plant combination and that T-DNA

transfer might be sub-optimal. More recently, post-transcriptional gene silencing (PTGS) was proposed as another limiting factor (Johansen and Carrington 2001).

Plant virus based vectors have a number of advantages as gene expression tools including the ability of direct, rapid and high level expression of foreign genes. Therefore the inhibition activity of scFvs was furthermore studied by expressing these scFvs via a virus based vector in the second experiment. Scholthof et al. (1995) had demonstrated that the CP gene of TBSV is not necessary for systemic infection. Cloning of a heterogeneous gene in the CP region of TBSV resulted in expression of the gene while the infectious clone is replicating (Scholthof 1999). Therefore, *in vitro* transcribed RNA of the infectious clone carrying the different scFvs genes was used to rub-inoculate wild type *N. benthamiana* plants to test the inhibition activity of the different scFvs of the viral RdRp.

FC8 is a infectious clone of TBSV-BS3-statice from which the CP gene was partially removed and two restriction sites (*KpnI* and *SpHI*) were introduced (Galetzka, unpublished data). The selected scFvs were cloned into this vector resulting in recombinant plasmids (fig.32). After linearisation, the recombinant plasmids were transcribed *in vitro* under the control of the T7 promoter. *In vitro* transcribed RNA carrying the different scFvs genes was mechanically inoculated on wild type *N. benthamiana* plants. The western blot result in fig. 35A showed that the scFv genes were successfully expressed via this viral base vector. The results showed that 7 days after inoculation systemic virus infection was observed in inoculated plants expressing the control scFv (anti-Fus3) and scFv28H3 but not in plants expressing the other scFv. Only a few local symptoms could be seen in leaves inoculated with infectious clones carrying scFvH2C6, scFvE11 and scFvP55H9, respectively (fig.34A and B) This result supports that the resistance is due to scFvs expression. Qiu and Scholthof (2001) monitored the accumulation of genomic RNA (gRNA, expressing ORF for the viral RdRp), subgenomic RNA1 (sgRNA1, expressing ORF for coat protein) and subgenomic RNA2 (sgRNA2, expressing ORF for movement protein and gene silencing suppresser) of TBSV in infected protoplasts. The authors observed that sgRNA2 normally appears concurrently with gRNA and that sgRNA1 appeared a few hours later. It is known that the RdRp which is translated from gRNA regulates the transcription of sgRNA1 and 2. The scFvs were cloned and expressed via sgRNA1 which appears in plant cells after gRNA. Therefore the inhibition of the RdRp by scFv might not occur in the very primary phase of replication/infection and

thus not lead to complete RdRp inhibition and expression of local symptoms in this experiment.

To monitor the virus titre in inoculated plants, *N. benthaniana* wt plants were sub-inoculated with plant sap from apical leaves from each of the virus infected plants as this is the most sensitive method to detect the presence of infectious virus particles. The results showed that systemic symptoms were only observed in plants sub-inoculated with plant sap from scFv anti-Fus3 expressing plants. The plants which were sub-inoculated with sap from scFvP55H9, H2C6 and E11 never showed any local or systemic symptoms. However, the plants which were inoculated with sap from scFv28H3 expressing plants showed only some local symptoms. This demonstrates once more the absence of systemic virus infection in plants infected with the infectious clone expressing scFvP55H9, scFvH2C6 and scFvE11 and the inhibitory effect of these scFvs. ScFv28H3 may be less active in inhibiting the RdRp activity which leads in consequence to some accumulation and translation of gRNA and sgRNA1 in the infected plants. The low amount of viral RNA transferred in the sub-inoculation experiments and further down regulated by the scFv expressed from the viral genome prevented, however, the establishment of systemic infection in the wt *N. benthamiana* plants even in the case of scFv28H3. Moreover 21 dpi inoculated leaves of each batch of scFv expressing plants were harvested and the accumulation of scFvs was investigated by western blot. The results showed that only the control scFv could be detected 21 after inoculation and seemed to accumulate compared to 7 days after inoculation. The absence of scFvs expression from plants inoculated with infectious clones carrying the different scFv genes and unsuccessfully establishment of systemic infection clearly shows the inhibitory effect of these scFvs in plant cells.

This is the first report that antibodies can be directly expressed from the viral genome whose replication they are supposed to down regulate. This assay is especially useful because the viral RNA and the inhibiting agent can be delivered in a one step procedure simultaneously to the plant and the targeting of the antibodies is optimised. The results of the RdRp inhibition assays *in vitro* and *in vivo* clearly demonstrate that scFvP55H9 and scFvH2C6 inhibit the viral RdRps to a high extend, whereas scFvE11 and scFvs28H3 only partially inhibit the RdRp. So altogether scFvP55H9 and scFvH2C6 proved to be the best candidates for RdRp inhibition and the production of virus-resistant transgenic plants.

Establishment of transgenic plants expressing scFvP55H9

The discovery of *Agrobacterium*-mediated transformation of plants allows to establishment transgenic plants. *Agrobacterium* is a common lab tool for many plant molecular biologist, because it has evolved the unique capacity to transfer a piece of its own DNA, T-DNA (transferred DNA), into the nuclear genome of plant cells. This property has been extremely useful for the introduction of new genes into plants (Tinland 1996). In bacteria, T-DNA is localised on a large plasmid pTi (Tumour inducing plasmid). The T-DNA can be transferred into plant cells via the gene products of Vir genes which located elsewhere on pTi, the only cis-acting elements which are required during T-DNA transferring. This elements are two 25 bp direct repeat sequences, the border sequences, which delimit the region of DNA to be excised from pTi. Therefore it is possible to insert any DNA sequence to be introduced into plant cells between these two borders (Tinland 1996). pPZP200 is a binary plasmid (pTi) in which the Kanamycin resistance gene was cloned into the left border of T-DNA (Hajukiewicz 1999) for selection propose. Cloning of scFv genes into the right border of this vector resulted in recombinant plasmids (fig.36).

It is not clear yet in which cell compartment viral RdRps are translated. Hence to inhibit the RdRp at it's translated origin, the scFvs were designed to be expressed in the cytosol and in the endoplasmic reticulum (ER). For expressing scFvs in the plant cytosol, a tetrapeptide (KDEL) which is the signal for retaining proteins in the ER when translocated into the secretory pathway (Owen et al, 1992; Pelham 1989; Wandelt et al., 1992) was added at 3´end of scFv genes (fig.36A and B). It was demonstrated that fusing this peptide at the C-terminus of scFv genes resulted in significantly improved expression levels and protein stability in the cytosol (Jiang et al., 1995; Schouten et al., 1996). For expressing scFvs into ER, a ER leader sequence was added at 5´end of scFv genes (fig.36).

N. benthamiana is a plant which many groups of plant viruses including Tombusviridae can infected by mechanical inoculation and the transformation of this plant is well established. Therefore it was used to establish transgenic plants expressing scFvs. After transformation and selection, the calli were induced to regenerate explants. The shooting of explants from the callus which were carrying scFv anti-Fus3 gene occurred 2 month later calli carrying the other scFvs. Moreover these plantlets showed some changes in phenotype such as early flowering

and slowly growth. The reasons for this phenomenon is not clear yet. Because of the altered phenotype these were excluded from further experiments.

Challenge inoculation of transgenic plants with viruses from two different families

To investigate the effect of antibody expression on virus infection, the T_1 transgenic plants expressing scFvP55H9 in the cytosol and ER were mechanically inoculated with TBSV virus particles and RCNMV infected plant sap. The results showed clearly that transgenic plants expressing scFvP55 in both compartments (in the cytosol, line 1, 2 and 4CP and in the ER, line 1 and 6) resist to TBSV and RCNMV infections. The inoculated leaves showed however local symptoms when the transgenic plants were rub-inoculated with RCNMV infected plant sap. The possible explanation might be (a) the sap used as inoculum contained not only free RdRp but also template-bound RdRp. The template-bound RdRp which cannot be inhibited by the scFv could further transcribe viral RNA and thus cause local infection. The observation that the antibodies did not inhibit the activity of polymerase complexes containing bound RNA template is consistent with this hypothesis (Hayes et al., 1994; Bates et al., 1995 and *in vitro* assay of this study, fig.26). (b) The inhibition of RCNMV replication may be lower than that of TBSV replication, since the effectiveness of the scFv binding to the heterologous RCNMV RdRp may be lower than binding to the TBSV-BS3 RdRp, which was used in the scFvs selection process.

Interestingly the transgenic plants expressing scFv into ER resist to TBSV and RCNMV infection. This result raises the question how these transgenic plants resist to viral infection. It is known that the TBCV RdRp is translocated to peroxisomes which are used as replication site (Martelli et al., 1988). However the peroxisome targeting signal of the plant viral RdRp is not identical with two common ones (PTS type I and II) which were found in mammalian and yeast cells. Recent studies on the sorting and targeting of peroxisomal membrane proteins (PMPs) have led to modifications of the growth and division model, once again implicating the ER in the formation of peroxisome. These studies suggest that a subset of PMPs are sorted indirectly to peroxisomes via a specialised region of the ER and ER-derived vesicles (Mullen, et al., 2001). In this model, peroxisomal membrane proteins are initially synthesised on free polysomes in the cytosol and post-translationally sorted to peroxisomes (Titorenko et al., 1997). The initial post-translational sorting site is the ER rather than the peroxisomal boundary membrane. Once the protein inserted into ER, it would travel within the plane of the

ER membrane to a specialised region on a sub-domain of the ER (e.g. peroxisomal ER, pER) where expanding smooth vesicles destined for peroxisomes, are formed. The plants PMP-containing preperoxisomal vesicles are proposed to be transported to and fuse with pre-existing functional, mature peroxisomes (Mullen et al., 1999).

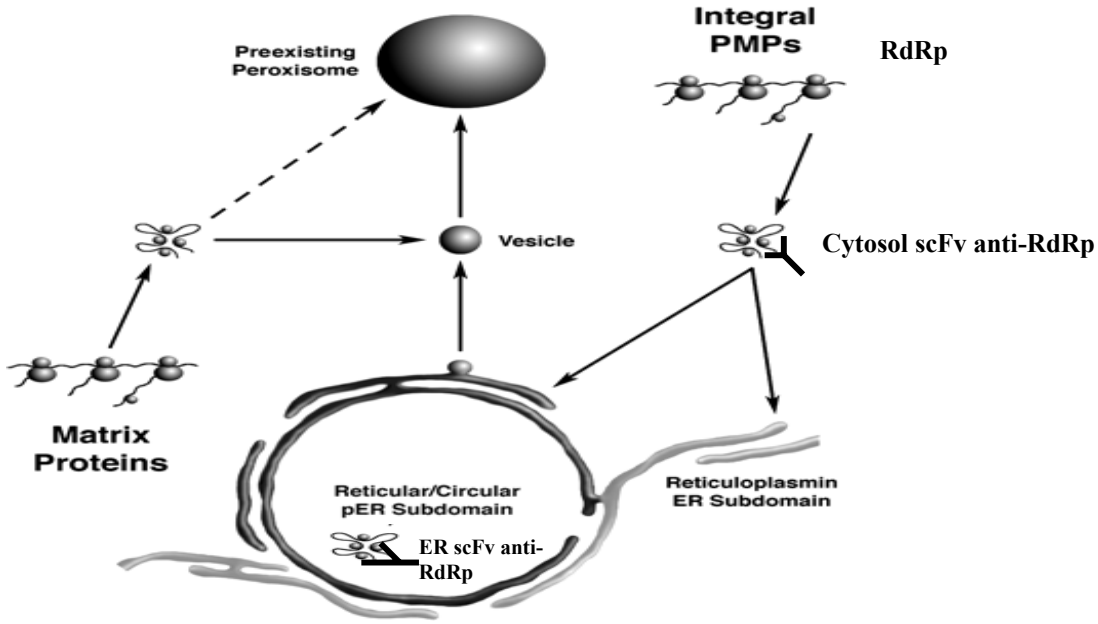


Fig.38 Model of the sorting of Membrane and Matrix Proteins to plant peroxisomes and the inhibition of viral RdRp by scFV. Membrane and Matrix proteins (TBSV-RdRp) synthesised on cytosolic polysomes appear to interact with molecular chaperones before post-translational organelle sorting. TBSV-RdRp (p92) may sort first to the reticuloplasmic-containing ER or to a peroxisomal ER (pER) subdomain (reticular/circular structure). Exit of the RdRp from pER seems to involve vescicles that are subsequently sorted to pre-existing peroxisomes. During the RdRp translocating to the pER, it may be inhibited by scFvs in the cytocol. The scFvs which are targeted to be expressed in the ER may also inhibit the RdRp after sorting to this organelle.

- This figure was adapted from Mullen et al., 1999.

By applying this new model of the biogenesis of peroxisomes to the explanation of why the transgenic plants expressing scFv in cytosol and into ER resist to TBSV infection, it might be postulated that the viral RdRp is initially synthesised in the cytosol on free polysomes, then transported to the ER and finally sorted to peroxisomes as described. Therefore after translation and during transportation to the ER it will be bound by the scFv in the cytosol. Moreover once the RdRp inserted into the ER, before forming ER-derived vesicles and sorted to peroxisomes, it will be bound with scFv which accumulated in the ER (fig.38).

This explanation is, however, only a hypothesis trying to illustrate the possible mechanism why scFvs expressed in the cytosol and in the ER can inhibit the viral infection. Certainly there are still a lot of open questions such as (a) where exactly the RdRp is synthesised and how it is transported to peroxisome. (b) If the scFvs fused to ER leader sequence are really translated and accumulate all in the ER or if they also accumulate in the cytosol. (c) Where does the RdRp bind to the RNA templates, after translation and transported as nucleo-complex to the transcription site or only at the transcription site, *etc*. More knowledge about RdRp translation and translocation will even improve the possibilities to target the scFvs to the critical compartments and lead to even more sophisticated resistance strategies.

Conclusion

Anti-RdRp antibody approaches will generate new strategies towards viral resistance. Despite the application of pesticides to reduce insect vector populations and conventional breeding approaches, virus diseases still present an important threat to the agricultural production of many crops. We have here demonstrated that scFvs directed against conserved domains of RdRps are highly efficient in inhibiting the replication of not only homologous but also heterologous plant viruses, confer virus resistance in transgenic plants and have even affinity for a human pathogenic virus. Wide-range virus resistance based on anti-RdRp adds a new tool to the repertoire for combating viruses that may have broad applications far beyond the area of plant pathology. Recombinant approaches, including transformation of viral sequences into the plants´ genome may be efficient in conferring virus resistance but are still controversially discussed by the public. Antibodies are known to be omnipresent in nature and to present not any threat to human health upon consumption. Plantibody-conferred virus resistance may therefore be the method of choice under technical and social aspects.

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Appendix I

A1. Solutions, buffers and medium for cloning

A1.1 10xTAE

48,4 g Tris-HCl, pH 8

11,5 ml Acetic acid, concentrated

25 ml 0,5 M EDTA

into 1 litre with ddH_2O

A1.2 LB medium

10 g Tryptone/peptone

5 g NaCl

5 g Yeast extract

(15 g micro agar for solid media)

into 1 litre with ddH_2O, adjust to pH 7.0, autoclave

For LB-Agar with Amp50, melt the LB medium with micro agar and, when it is cool to the touch, add 500 µl of filtered 50 mg/ml ampicillin. Pour the medium in Petri dishes.

A1.3 GTE solution

25 mM Tris

10 mM EDTA

5 mM Glucose

in ddH_2O, and autoclave.

A1.4 SOC media

20 g Trypton/peptone

5 g Yeast extract

0,2 g NaCl

0,2 g KCl

2 g $MgSO_4 7H_2O$

into 1 litre with ddH_2O, adjust to pH 7, autoclave.

Add 20 ml filtered 1 M glucose.

12,5 ml of 1 $MgSO_4$

10 ml of 2 M filter-sterilised glucose solution

Filter-sterilise.

A1.5 5x DNA Loading buffer

1 volume Bromophenol blue-xylene cyanole dye mixture

19 volume Formamide

A1.6 KZB for 100 ml

0.733 $CaCl_2$

1 ml 1M Tris HCl pH 7.8

5 mg Thymidin

Filter-sterilised

A2. Buffer for mechanical inoculation

A2.1 RNA inoculation buffer for RNA rub-inoculation

0.03 M $K_2HPO_43H_2O$

0,05 M Glycine

Adjust the pH to 9,2, then add 1% Benthonite and 1% Celite.

Autoclave.

A3. Solutions and buffers for protein analysis

A3.1 Soluble lysis buffer

50 mM Tris/HCl

2 mM EDTA

1/10 vol Triton X-100

A3.2 Total protein extraction buffer from plant

1 ml 10% Glycerol

1,4 ml 1,5 M Tris-HCl pH 8,8

0,5 ml ß-mercaptoethanol

5,1 ml ddH_2O

2 ml 10% SDS

A3.3 Soluble protein extraction buffer

1x PBS (see A3.2.10)

0,1% Triton X 100

A3.4 SDS polyacrylamide gel buffer

1,5 M Tris-HCl, pH 8,8

10% SDS

A3.5 12% Separation SDS-PAGE

For 40 ml :

16 ml Gel 30 (30% acrylamide: 0.8% bisacrylamind)

10 ml Separation buffer

13,6 ml ddH_2O

60 µl TEMED

300 µl 10% APS (Ammonium per phosphate)

A3.6 Collecting gel SDS-PAGE

For 10 ml :

1,55 ml Gel 30 (30% acrylamide: 0.8% bisacrylamind)

2,5 ml Collecting gel buffer

5,9 ml ddH_2O

22 µl TEMED

53 µl 10% APS

A3.7 Protein loading buffer

1 ml 10% Glycerol

1,4 ml 1,5 M Tris-HCl pH 8,8

0,5 ml ß-mercaptoethanol

5,1 ml ddH_2O

2 ml 10% SDS

0,001% Bromophenol blue

A3.8 1x Protein running buffer for SDS PAGE

For 1 Litre:

3,024g Tris

14,4g Glycine

0,1% SDS

A3.9 Transfer buffer (for transferring of proteins to membrane)

0,025 M Tris

0,192 M Glycine

0,1% SDS

20% Methanol

A3.10 Straining solution 500 ml

325 ml ddH_2O

125 ml Isopropanol

50 ml Acetic acid

0.2 g Coomassie blue

A3.11 Destraining solution

37.5 Methanol

25 ml Acetic acid

ddH_2O to 500 ml

A3.12 1x Marvel Buffer

20 mM Tris, pH 7,8

180 mM NaCl

A3.13 1x PBS for 1 L

8 g NaCl

0,2 g KCl

1,44 g Na_2HPO_4

0,24 g KH_2PO_4

Adjust to pH 7,4

A4. Solutions and buffers for RNA analysis

A4.1 5x TBE for 1 Litre

54 g Tris-HCl, pH 8,3

27,5 g Boric acid

20 ml 0,5 M EDTA

Autoclave.

A4.2 2x RNA Loading buffer

1 volume Bromophenol blue-xylene cyanole dye mixture (in ddH_2O)

19 volume Formamide

A4.3 PAGE

33 ml 5% Acrylamide (19:1 acrylamind:bisacrylamide)

300 µl 10% Ammonium persulfate

30 µl TEMED

A5 Media for establishment transgenic plants

A5.1 MS media for 500 ml

4,4 g Murashige and Skoog Medium (include vitamins)

20 g Saccharose

Adjust to pH 5,6- 5,8

5,5 g Microagar

A6 Culture media for Agrobacterium

A 6.1 YEB media

5 g beef extract

1 g yeast extract

5 g sucrose

Adjust to pH. 7.4

Autoclave

A 6.2 Induction media

YEB media supplement with 10 mM 2-(N-morpholino) ethansulfonic acid (MES). Adjust to pH 5.6

Autoclave and added with filter sterilised acetosyringone to final concentration of 20μM and $MgSO_4$ to final concentration of 2 mM

A 6.3 MMA media for 1 L.

MA salt

10 mM MES

20 g Sucrose

Adjust to pH 5.6 then Autoclave

Add filter-sterilised Acetosyringone to final concentration of 200 μM.

A7 Culture media for phage display

A 7.1 2XTY

16 g Tryptone

10 g Yeast extract

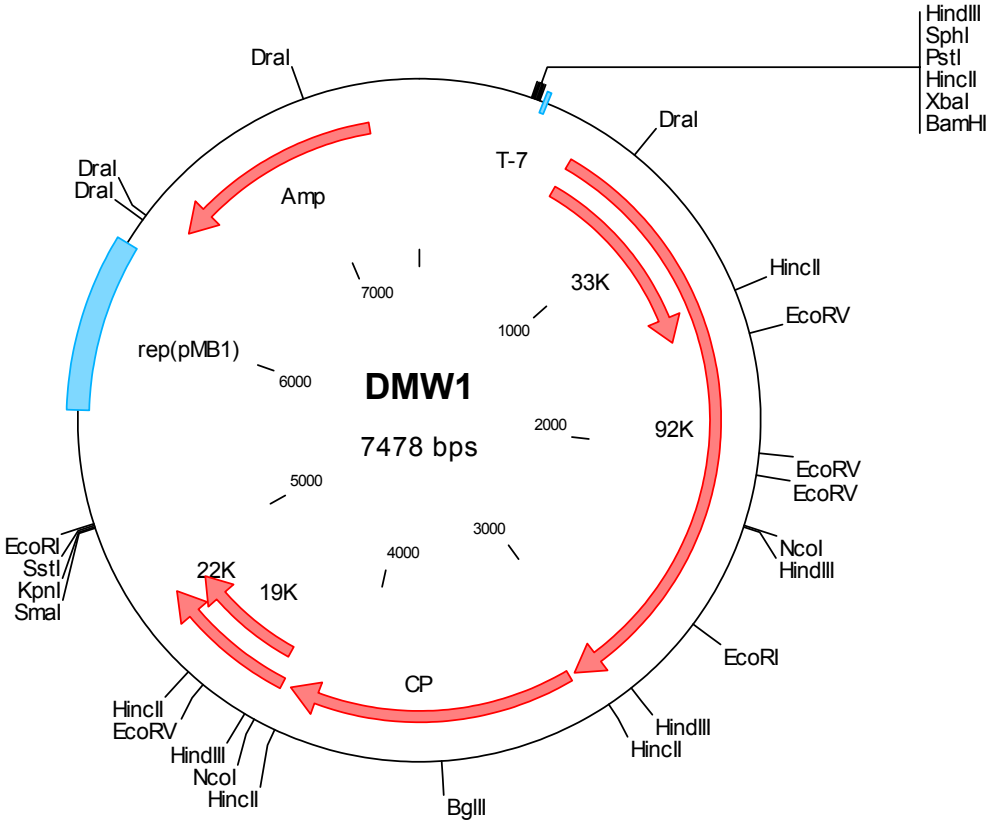
5 g NaCl

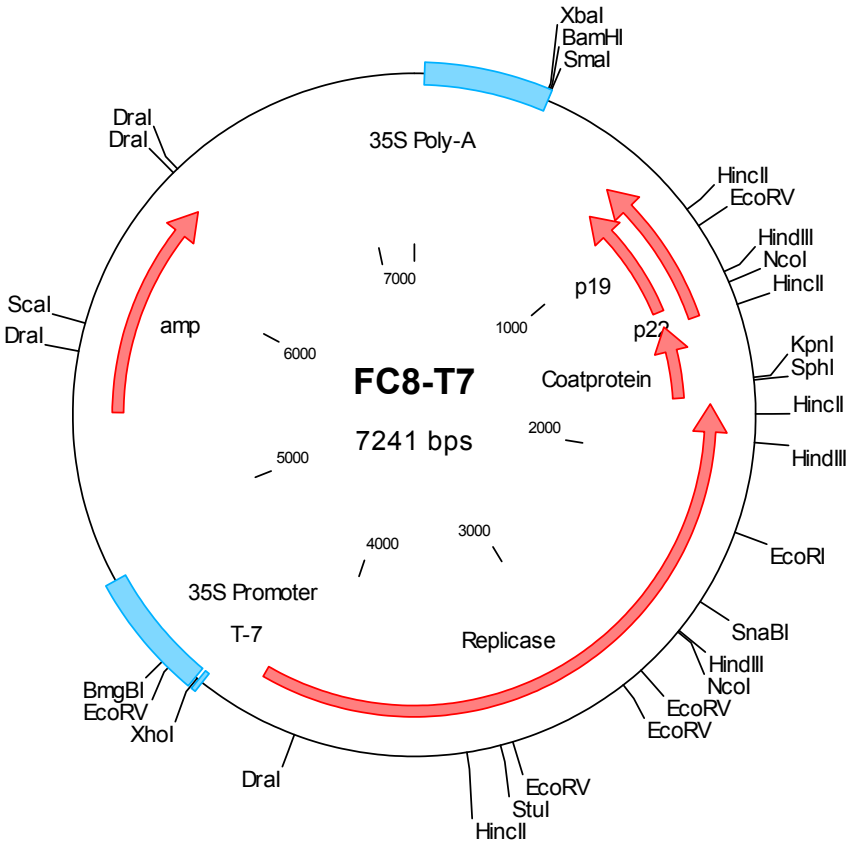
Autoclave

A 7.2 TYE for 1 L

15 g Bacto agar
8 g NaCl
10 g Tryptone
5 g Yeast extract
Autoclave

Appendix II plasmid maps

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EcoRV
HindIII
NcoI
HincII
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p19
1000
p22
ScaI
DraI
amp
6000
FC8-T7
Coatprotein
KpnI
SphI
HincII
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5000
HindIII
3000
4000
EcoRI
35S Promoter
T-7
Replicase
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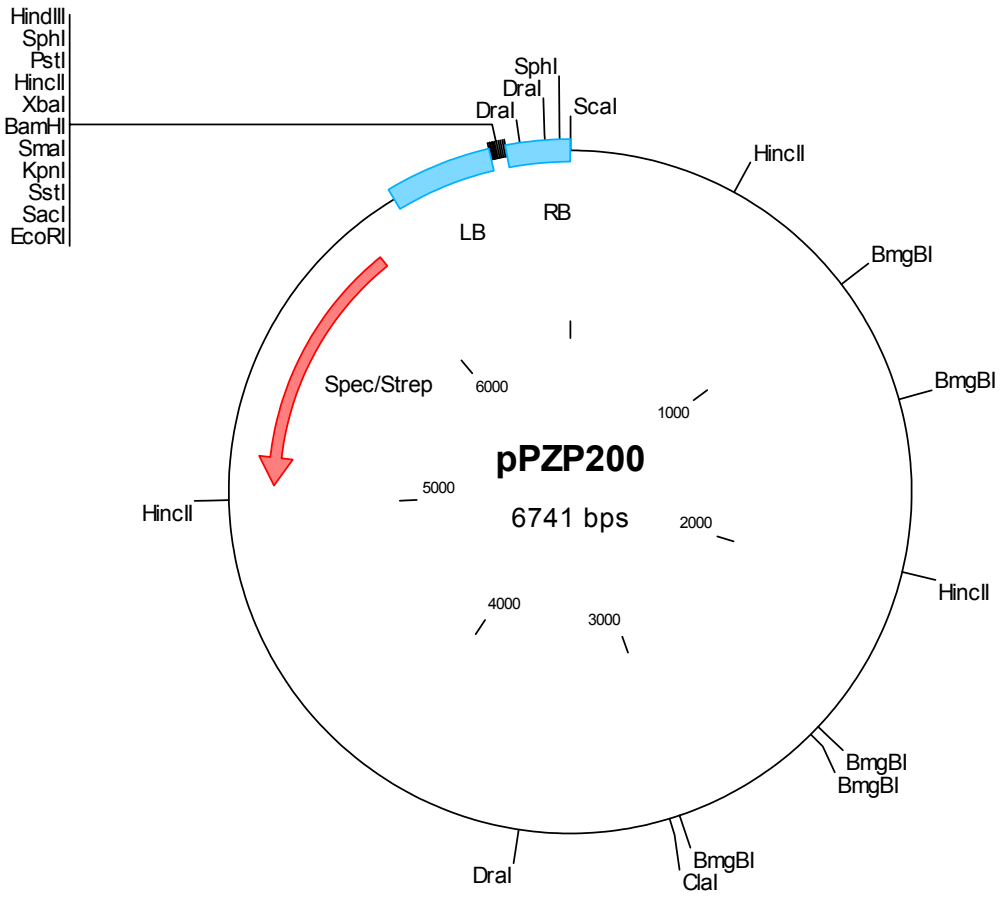
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SacI
EcoRI
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DraI
ScaI
HincII
RB
LB
BmgBI
BmgBI
Spec/Strep
6000
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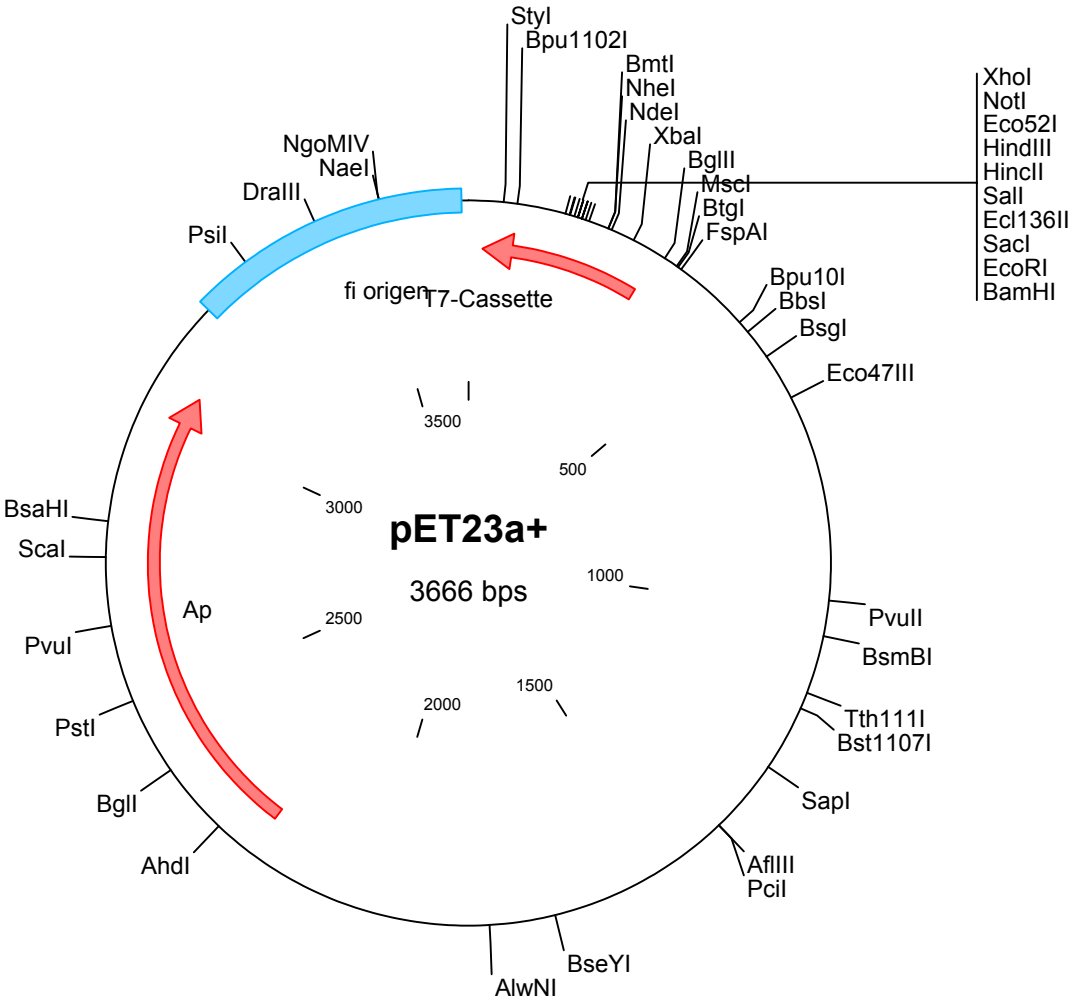
BmgBI
ClaI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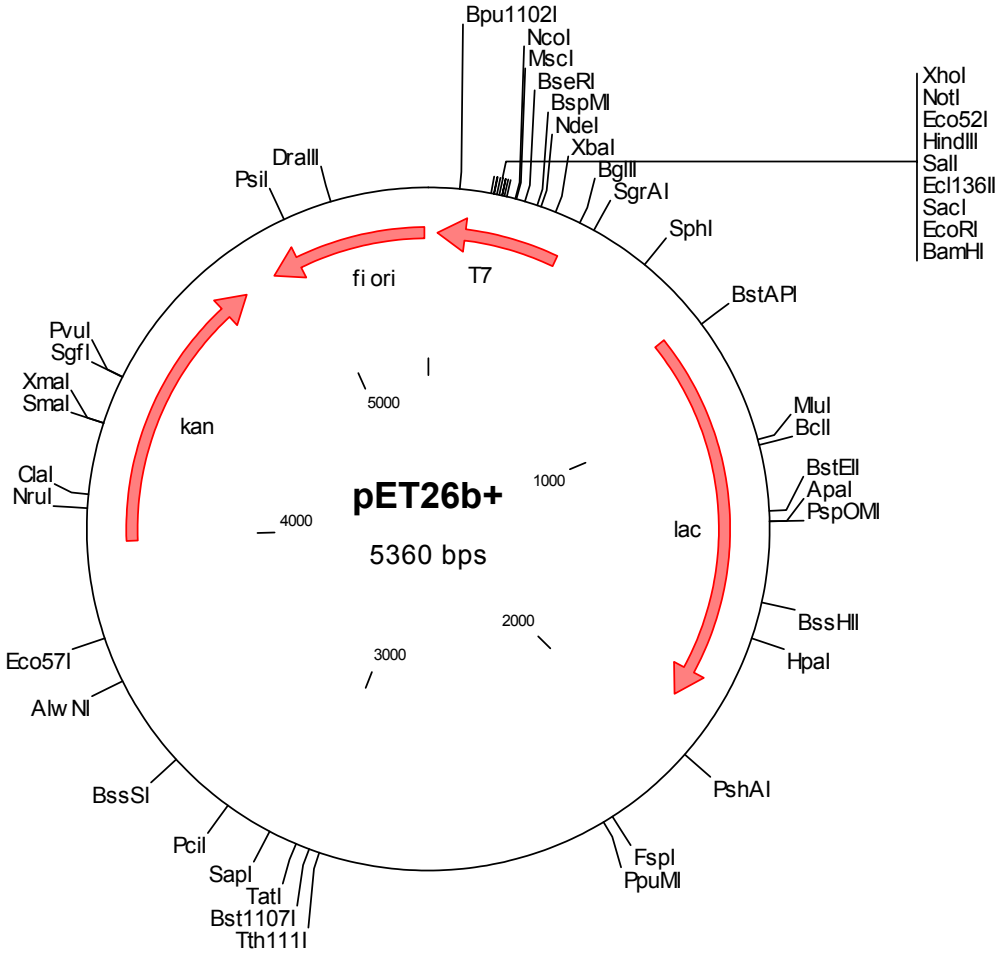
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BspMI
NdeI
XbaI
BglII
SgrAI
SphI
BstAPI
MluI
BclI
BstEII
ApaI
PspOMI
BssHII
HpaI
PshAI
FspI
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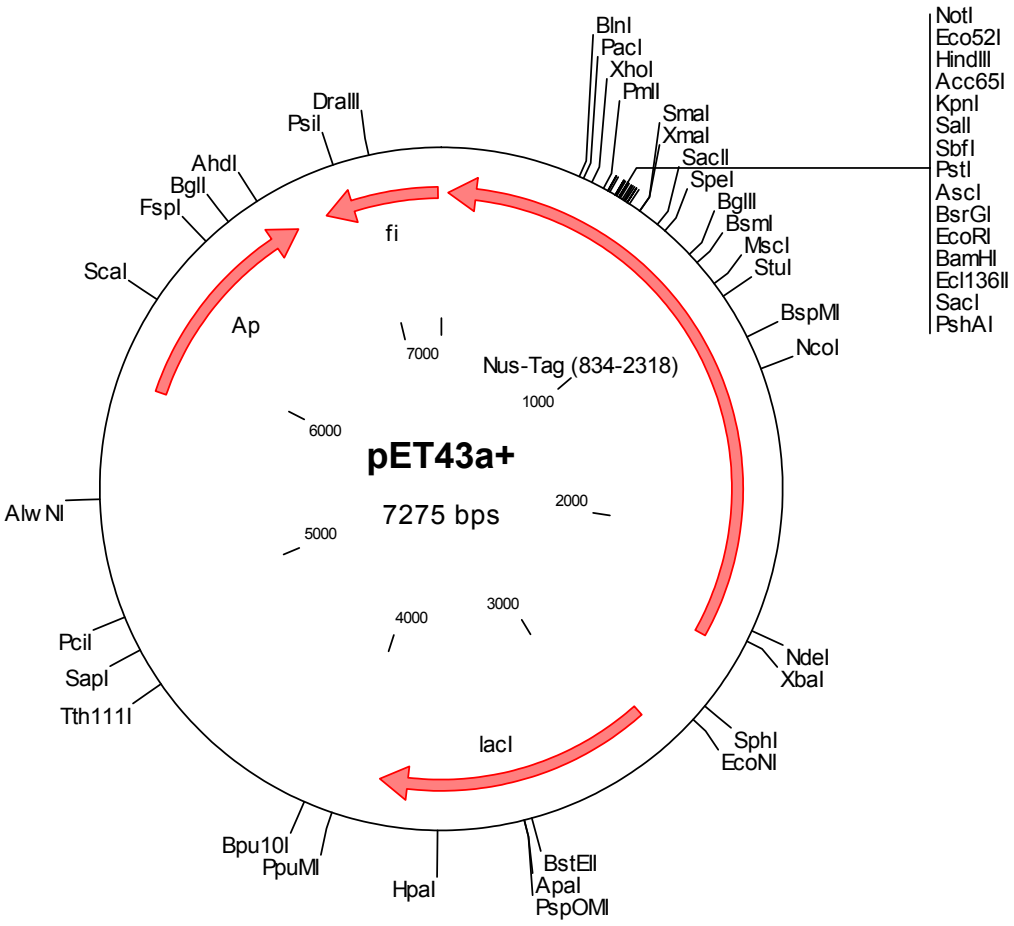
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Herewith I do confirm that the research carried out in this thesis was by myself with only the resources mentioned. This is the first doctoral thesis I do submit.

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Kajohn Boonrod

Curriculum Vitae

Personal information

Name: Boonrod

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Education

1977-1983: Primary school; Bhadungsitpitaya School, Bangkok, Thailand

1983-1988: Secondary school; Bhadungsitpitaya School, Bangkok, Thailand

Studies

1988-1993: Bachelor in Pharmacy, Silpakorn University, Thailand

1999-2001: Master of Science in Biotechnology, University of Applied Science, Mannheim

Jan 2001- March 2003: Ph. D. work at CGG Neustadt an der Weinstrasse.

Professional experience

1993-1995: Yasothon Hospital (Thailand)

1995-1997: Hoechst AG (Thailand)

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