Towards an Antibody-mediated Resistance against Geminiviruses

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

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PhD Thesis

Towards an Antibody-Mediated Resistance against Geminiviruses

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Zusamenfassung

In dieser Studie wurden "single chain fragment variable" (scFv) gegen Schlüsselproteine des *Tomato leaf curl virus-India* mit Hilfe des "Phage Display" und der Hybridoma Technologie hergestellt. Zur Immunisierung von Mäusen und zum Screenen der "Phage Display" Bibliotheken und der Hybridoma Klone wurden aufgereinigte virale Proteine (AV1: Hüllprotein; AC1: Replikase Enhancer) verwendet. Die Bindungseigenschaften der isolierten scFvs wurden durch Western Blot, ELISA und BIACORE charakterisiert.

Das fehlende Nukleotid, das in dem vom Hybridoma Klon abstammenden scFv-SAV gegen AV1 gefunden wurde, wurde wieder hergestellt und der resultierende scFv (scFv-RW-AV) zeigte bessere Bindungseigenschaften als der scFv-SAV.

Vier scFv gegen AV1 wurden aus der naïven Tomlinson Bibliothek erhalten. Zwei davon enthielten ein Stop-Codon, das *in vitro* so mutiert wurde, dass es entweder für Glutamin (Q) oder Glutaminsäure (E) kodierte. Der scFv mit der Q-Mutation zeigte höhere Expressionslevel aber geringere Bindungsaktivität als der mit der E-Mutation. Zusätzlich wurden zwei scFv gegen AC1 selektiert.

Alle scFv wurden als N-terminale GFP-Fusionen in Tabakblätter kloniert und cytosolisch exprimiert. Darüber hinaus wurden zwei scFv-GFP Fusionen durch ein N-terminales nukleares Lokalisationssignal (NLS) zum Kern dirigiert. Beide scFv-GFP Fusionsproteine wurden ausschließlich im Kern lokalisiert. Das AV1 Protein wurde daneben als N-terminale DsRed-Fusion in Tabakblättern exprimiert. Die AV1-dsRed Fusion war nur im Kern lokalisiert.

Die Bindungsaktivität der AV1-spezifischen scFvs wurde durch Koexpression der scFv-GFP-Fusion und AV1-dsRed getestet. Nur der vom Hybridoma Klon abstammende scFv zeigte Bindung und der Proteinkomplex wurde durch die AV1 NLS zum Kern geleitet.

Die Daten dieser Arbeit zeigen die erfolgreiche Expression von spezifischen und funktionalen scFvs gegen virale Proteine in der reduzierenden Umgebung des pflanzlichen Cytosols. Solch ein scFv ist ein potentieller Kandidat um den viralen Lebenszyklus zu beeinflussen und so eine virale Infektion zu verhindern oder zu verzögern.

Abstarct

In this study, single chains (scFvs) against key proteins of the *Tomato leaf curl virus-India* were generated using phage display and/or hybridoma technologies. Purified AV1 (coat protein) and AC1 (replicase enhancer) viral proteins were used for mice immunization and screening of phage display libraries and hybridoma clones. The binding properties of the isolated scFvs were determined by western blot, ELISA and BIACORE.

The missing nucleotide found in the hybridoma derived scFv-SAV against AV1 was restored and the resulting scFv (scFv-RW-AV) showed better binding properties than scFv-SAV.

Four scFvs against AV1 were derived from the naïve Tomlinson I library. Two of these scFvs carried a stop codon. The stop codon was mutated *in vitro* to either code for glutamine (Q) or glutamic acid (E). The Q-mutated scFvs showed higher expression level but less binding activity than the E-mutated scFvs. Additionally, two scFvs against AC1 were selected.

All scFvs were cloned and expressed in tobacco leaves as N-terminal fusion with the GFP for cytosolic expression in tobacco leaves. Additionally, two scFv-GFP fusions were targeted to the nucleus by a nuclear localization signal (NLS) introduced at the N-terminus of the scFvs. Both scFv-GFP fusion proteins were exclusively localized in the nucleus. The AV1 protein was also expressed in tobacco leaves as N-terminal fusion with DsRed. The AV1-DsRed fusion was completely localized in the nucleus.

The binding activity of the AV1-specific scFvs was tested by the co-expression of the scFv-GFP fusion and AV1-DsRed. Only the hybridoma derived scFv showed clear binding and the protein complex was targeted to the nucleus by the AV1 NLS.

The data presented in this study demonstrate the successful expression of specific and functional scFvs against the viral proteins in the reducing environment of the plant cytosol. Such a scFv is a potential candidate to interfere with the viral life cycle and thus abolishing or delaying the viral infection.

I Introduction

Higher plants can be infected by a large number of viruses, most of which have RNA genomes. There are, however, only two plant virus families, *Geminiviridae* and *Nanoviridae*, that have DNA genomes and replicate through DNA intermediates. *Geminiviruses* are, basically, characterized by two distinctive features: (i) the nature of their genetic material, consisting of one or two single-stranded DNA (ssDNA) molecules, 2.5–3.0 kb in length, and (ii) the morphology of the virion particle which is twinned (geminate), 30 nm long and 18-20 nm in diameter, and has the appearance of two quasi-icosahedral moieties with a total of 22 pentameric capsomers. Geminiviruses are responsible for devastating diseases in economically important crops of both monocotyledonous and dicotyledonous plants, in a variety of cereal, fibre, and vegetable crops worldwide, including maize, wheat, sugar bean, tomato, pepper, tobacco, bean, cotton, squash, beet and cassava.

I.1 Geminivirus classification

Geminiviruses are currently classified in the family *Geminiviridae*, which comprises four genera; *Mastrevirus* (subgroup I), *Curtoviruses* (subgroup II), *Begomovirus* (subgroup III) and *Topocuvirus* (subgroup IV) (Saunders et al., 2002; Fauquet and Fargette, 2005).
The differences in the genetic organization of geminiviruses as well as their host range and insect vectors serve as criteria for recognizing the four different genera (Table 1).

Table 1: Classification of the family *Geminiviridae*.

	Mastrevirus	***Curtovirus***	***Begomovirus***	***Topocuvirus***
Virus type species	*Maize streak virus* (MSV)	*Beet curly top virus* (BCTV)	*Bean golden mosaic virus* (BGMV)	*Tomato pseudo-curly top virus* (TPCTV)
Genomic organization	monopartite	monopartite	mono- or bipartite	monopartite
Host range	monocotyledonous	dicotyledonous	dicotyledonous	dicotyledonous
Insect vector	leafhoppers	leafhoppers, Treehoppers	whiteflies	treehoppers

I.2 Genome structure

The genomic structures of the four genera are shown in Fig. 1. Monopartite geminiviruses (i.e., *Mastreviruses*, *Curtoviruses*, *Topocuvirus*, and some old world *Begomoviruses*) contain four to six overlapping genes encoding all the viral proteins necessary for replication, transcription, virus movement, and encapsidation. These genes are arranged in two divergent clusters separated by a large intergenic region (LIR) that contains the promoters of both transcription units. Mastreviruses also contain a second smaller intergenic region (SIR) located at the end of the transcription units (Fig. 1). The genes on the right side (clockwise orientation) are called virion sense because they are transcribed from a DNA strand with the same polarity as the encapsidated viral DNA (also called plus strand). Similarly, genes on the left side (counter clockwise orientation) are called complementary sense (c-sense) because they are transcribed from a DNA strand that is complementary to the encapsidated DNA (minus strand) and present only in the dsDNA intermediate. In general, genes encoded in the complementary sense are involved in virus replication and transcription whereas genes encoded in the virion sense have a function in encapsidation and virus movement (Lazarowitz, 1992; Hanley-Bowdoin et al., 2000).

Bipartite begomoviruses have a genome composed of two ssDNAs (designated as components A and B) (Fig. 1), both of which are required for successful infection. They also display two divergent sets of genes separated by an intergenic region (IR) that includes segment of about 180 to 200 nucleotides (nt), called common region (CR), which is the only region highly conserved between both components. All of the cis elements required for viral replication reside within the CR, which varies from virus to virus, with the exception of a highly conserved 30 nucleotides-element with the potential to form stem-loop (hairpin) structure. Component A contains four or five genes. The coat protein gene (AV1) is the only gene found in the virion-sense orientation, whereas genes such as AC1 (replication initiation protein (Rep) gene), AC3 (replication enhancer (REn) protein gene), and (transcriptional activator protein (TrAP) gene) are found in the complementary sense. Another gene (AC4) has been described in some geminiviruses. However, its function is still not well understood. Component B, on the other hand, contains two genes, one of the complementary sense (BC1) and another in the virion sense (BV1). Both genes encode proteins involved in the movement of the viral DNA and necessary for systemic movement and symptom production. The BC1 gene product [movement protein (MP)] seems to be involved in cell-to-cell transport of the

virus, probably by changing the plasmodesmata (Pd) size exclusion limit. The BV1 gene product [nuclear shuttle protein (NSP)] accumulates in the nucleus and mediates nuclear import and export of DNA (Lazarowitz, 1992; Hanley-Bowdoin et al., 2000; Rojas et al., 2001).

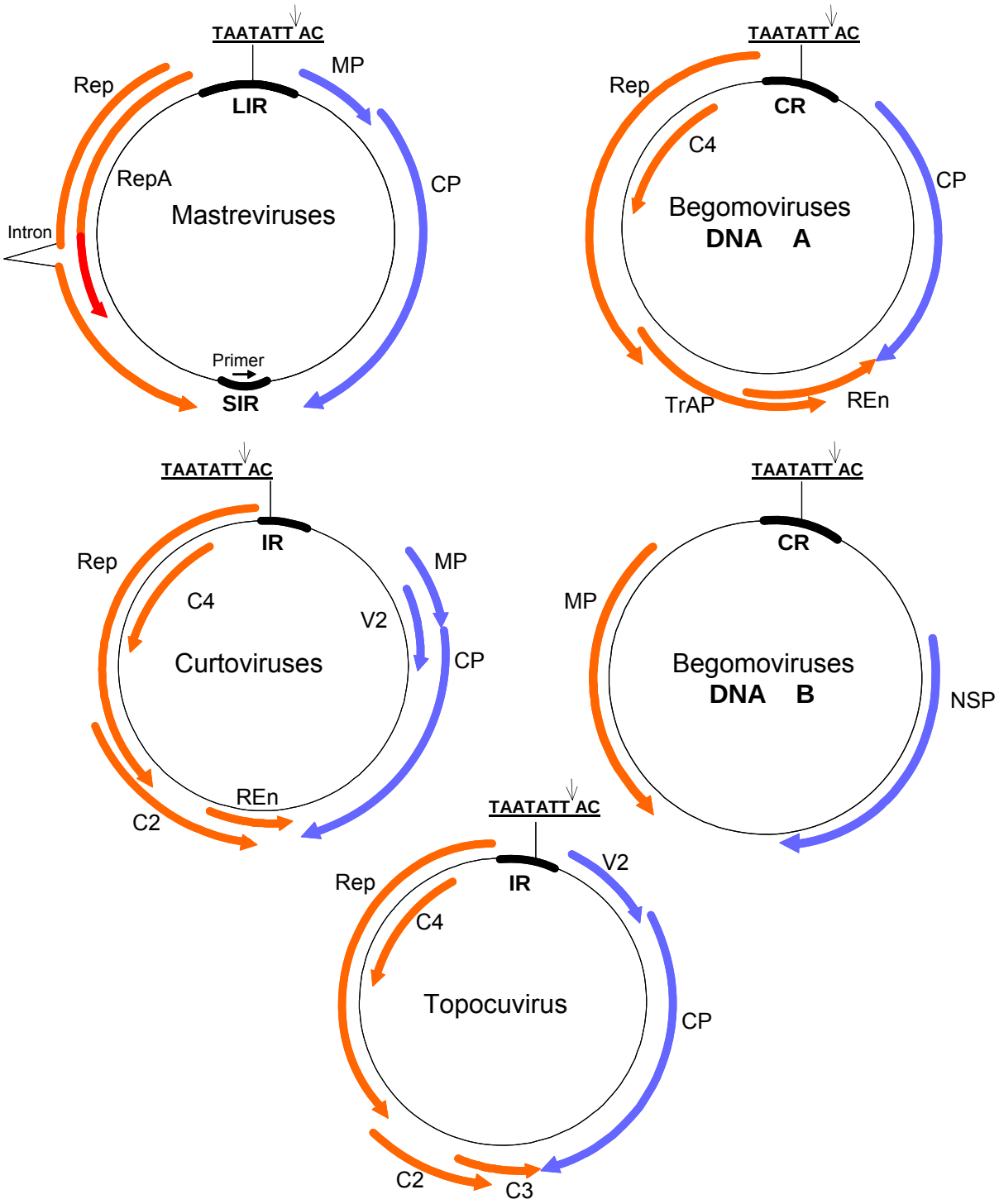


Fig. 1: Genome organization of the geminivirus genera. Each genus is represented by its type member (Table 1). The sequences regulating DNA replication and transcriptional activity are located in the intergenic regions. Mastreviruses contain a large (LIR) and a small (SIR) intergenic region, to which a small DNA molecule (primer) is associated. The invariant TAATATT↓AC sequence located in the LIR (mastreviruses), IR (curtoviruses) and CR (begomoviruses) containing the initiation site (↓) of rolling-circle DNA replication is shown. Arrows indicate the viral proteins. MP: movement protein; NSP: Nuclear shuttle protein; CP: capsid protein; Rep: replication initiator protein; TrAP: transcriptional activator; REn: replication enhancer; RepA: replication associated protein.

I.3 Geminivirus infection cycle

The first stage in the infection cycle involves the injection of viral ssDNA into a plant cell by an insect vector (Fig. 2). *Geminiviruses* replicate through a double-stranded (ds) DNA intermediate in the nucleus of the infected cells. Upon initial entry of a geminivirus into a host cell, there are no viral proteins present other than CP. Movement to the nucleus must thus be dependent entirely on the CP and exploitation of the host transport mechanism. It is not clear whether the virus inoculated into the host by the vector moves to the nucleus as an encapsidated virion or whether it decapsidates and moves as a nucleoprotein complex. Apparently, CP is involved during this transport stage, probably through interactions with the host transport network (Gafni and Epel, 2002). MSV CP gene expressed in tobacco protoplasts accumulated in the nuclei. Mutagenesis of a potential nuclear localization signal (NLS) in the CP resulted in cytoplasmic accumulation of the mutant protein. When co-injected with the CP, both ss and ds DNA moved into the nucleus suggesting that, in addition to entering the nucleus where it is required for encapsidation of the viral ss DNA, the MSV CP facilitates the rapid transport of viral (ss or ds) DNA into the nucleus (Liu et al., 1999).

Once in the nucleus, the viral ssDNA is converted into a transcriptionally active dsDNA intermediate that acts as a template for both transcription and replication. This complementary DNA synthesis is accomplished entirely by host proteins. This viral dsDNA is associated with histones and packaged into so-called minichromosomes (Pilartz and Jeske, 1992). Similar to other viral systems, the expression of geminiviral genes seems to follow a finely tuned temporal sequence. It is believed that the genes encoding proteins involved in replication and transcription (e.g., Rep, TrAP, and REn) are expressed earlier than the virion-sense genes (e.g., CP and NSP genes). After the expression of the early viral genes (left side or complementary sense), the multiplication of the virus genome by a rolling-circle (RC) mechanism generates new viral ssDNA molecules from the dsDNA intermediate. An ssDNA

molecule produced in this process has two fates depending upon the stage of infection. In the early stage, viral ssDNA can be converted, by host machinery, to dsDNA forms. These molecules will then be directed to the transcription and/or replication processes, amplifying the viral genome within the cells (a necessary condition to initiate a systemic infection) (Timmermans et al., 1994). The second alternative occurs in a later stage when the late gene products CP and NSP are present and can bind viral ssDNA either to encapsidate it or to transport it out of the nucleus. Once in the cytoplasm, the virus moves to neighbouring cells through the plasmodesmata, and finally to the phloem for long distance transport. In the bipartite geminiviruses, proteins associated with viral movement are encoded by the DNA B genome. In the case of the *Bean dwarf mosaic virus* (BDMV), movement of single-stranded and double-stranded viral DNA out of the nucleus is mediated by the NSP protein, while the intercellular transport of double-stranded viral DNA is mediated by the MP protein (Noueiry et al., 1994; Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996; Sanderfoot and Lazarowitz, 1996). The monopartite geminiviruses such as TYLCV do not code for BV1 and BC1 homologs and therefore other viral protein(s) (CP and Pre-CP) must fulfil these functions (Kunik et al., 1998). It has been shown that some viruses require the CP for systemic spread in some hosts, whereas in other hosts the CP is dispensable. For example, *Pepper huasteco virus* (PHV) mutants that cannot produce CP can systemically infect pepper and *Nicotiana benthamiana* plants. However, when the mutants are inoculated onto *N. tabacum* plants, the virus replicates only in the inoculated cells; it does not spread throughout the plant (Guevara-González R. G., 1999).

The last stage of the cycle corresponds to the uptake of the virions by the insect vector (Fig. 2). In this case, it has been shown that the CP and, probably, virus particles are indispensable for insect transmission.

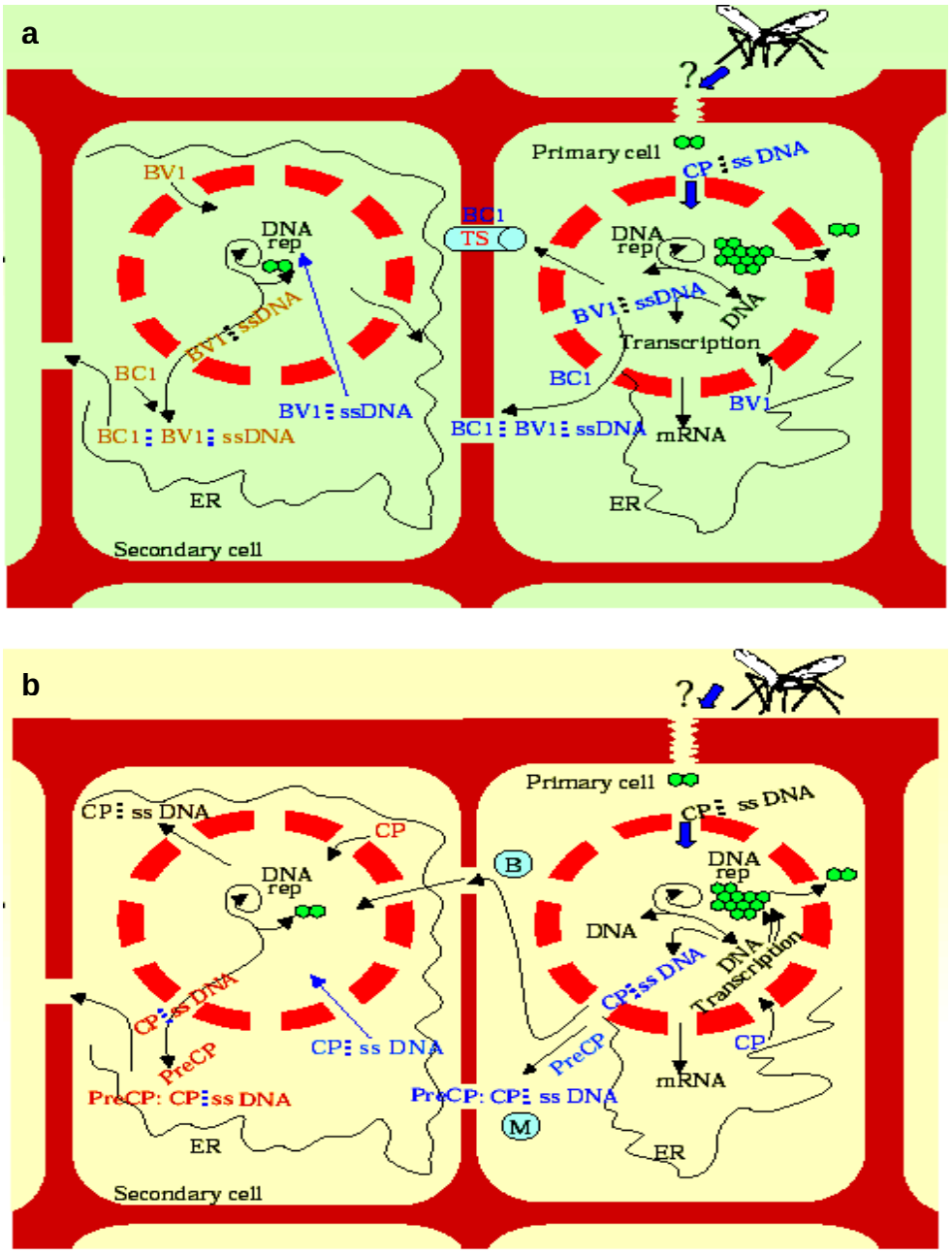


Fig. 2: A model for geminivirus intra- and inter-cellular movement. a: Virions of bipartite-genome geminivirus are inoculated by vector insects into the initial host cell. A complex of the ssDNA and the CP is formed which enters the nucleus for replication and transcription of viral genome. BV1, translated from viral mRNA, enters the nucleus to form a complex with ssDNA that then exits the nucleus and interacts with BC1. The BC1:BV1: ssDNA complex is directed to Pd to be transferred to an adjacent cell. For SLCV, it was suggested that BC1 associates with ER membranes forming unique tubular structure (TS) which function in the translocation from cell to cell of the BV1:ssDNA complex. In subsequently infected cells, BV1 functions in shuttling the ssDNA to the nucleus for another cycle of replication and transcription. b: Virions of monopartite-genome geminiviruses enter the primary host cell by insect inoculation. A complex of the ssDNA and CP is formed and enters the nucleus for replication and transcription of the viral genome. CP is also proposed to function in nuclear export of the CP:ssDNA complex. Two routes are proposed to distinguish between the cell to cell movement of mastreviruses (pathway M) which involves Pre-CP (MP) and CP, and that of begomoviruses (pathway B) for which only CP involvement has been conclusively demonstrated to date. Host protein involvement is not shown (Gafni and Epel, 2002).

I.4 Selected viral proteins

I.4.1 The AC1/AL1/Rep protein

Rep, the replication initiator protein, is encoded by open reading frame (ORF) C1 (also called AL1 or AC1 in geminiviruses with bipartite genomes). It localizes to nuclei of infected plant cells (Nagar et al., 1995; Hanley-Bowdoin et al., 2000) where it plays a key role in the viral life cycle. Rep is an ~ 40kDa multifunctional, highly conserved protein, involved in viral replication (Morris et al., 1992; Laufs et al., 1995; Gutierrez, 1999), autoregulation of its own gene transcription (Stanley, 1993), activation of virion sense transcription in some geminiviruses (Hofer et al., 1992; Hanley-Bowdoin et al., 2000), and activation and recruitment of host encoded proteins related to DNA synthesis (Hafner et al., 1997). It also induces the expression of the proliferating cell nuclear antigen in non-dividing plant cells (Nagar et al., 1995), possibly through interaction with the plant cell cycle machinery (Hanley-Bowdoin et al., 2000). It is the only viral protein that is absolutely required for viral replication (Timmermans et al., 1994; Sanderfoot and Lazarowitz, 1996).

During replication, Rep specifically recognizes the viral origin (Fontes et al., 1994) and acts as an endonuclease and ligase to initiate and terminate rolling-circle replication (Laufs et al., 1995; Orozco and Hanley-Bowdoin, 1998). Rep protein has an ATP-dependent topoisomerase activity (Pant et al., 2001) and has been proposed to function as a DNA helicase during replication (Gutierrez, 1999). In addition to its catalytic and DNA binding activities, Rep is involved in several protein interactions, including oligomerization, binding to the viral replication enhancer AC3, and interaction with the retinoblastoma host protein (pRb in animals and RBR1 in plants) (Xie et al., 1995; Settlage et al., 1996; Ach et al., 1997).

I.4.2 AV1/Coat protein

The second-largest gene residing on the genome of geminiviruses is the V1 coding for the coat protein (CP) that is located on the virion strand. The *Tomato leaf curl virus-India* (ToLCV-India) CP has a length of 260 amino acids (aa) (~ 30 kDa); it is a protein rich in arginine, valine, serine and lysine, has a positive charge at neutral pH, and represents the only known building block of the viral capsule. Sequences important for transmission have been mapped in the central part of the protein (Hofer et al., 1997; Noris et al., 1998; Hohnle et al., 2001) whereas the DNA binding domain was localised to the N-terminal half of the protein (Liu et al., 1997; Qin et al., 1998). N-terminal and C-terminal sequences as well as aa in the

insect-transmission domain seem to be also involved in multimerisation (Hallan and Gafni, 2001).

Apart from viral ssDNA, CP is the only known structural component of the virus particles (Lazarowitz et al., 1992). One hundred ten CP monomers are arranged in 22 pentameric capsomers producing the geminate particle morphology. Although coat proteins of different genera show only limited sequence homology (Padidam et al., 1995), it is thought that all members of the family *Geminiviridae* share more or less the same overall coat protein and geminate particle structure (Unseld et al., 2004).

The coat protein of geminiviruses is a multifunctional protein. It determines vector specificity (Hofer et al., 1997; Hohnle et al., 2001) and protects viral DNA during transmission by the insect vector (Azzam et al., 1994) or mechanical transmission (Frischmuth and Stanley, 1998). Thus far, no evidence exists to support suggestions that the CP possesses an enzymatic function, but it is able to interact with other macromolecules as well as to bind ssDNA and to self associate (Gafni, 2003).

Although the majority of plant viruses do not replicate in the nuclear compartment, geminiviruses do. After injection of a geminivirus into a plant cell by its insect vector, the viral genome must be imported into the nucleus where viral replication and transcription take place. Microinjection experiments with MSV and *Tomato yellow leaf curl virus* (TYLCV) suggest that nuclear import of the viral DNA upon initial infection is mediated by the coat protein (Liu et al., 1999; Rojas et al., 2001). After translation in the cytoplasm, newly synthesized CP has to enter the nucleus to encapsidate viral ssDNA.

For karyophilic proteins, nuclear import is generally mediated by NLS sequences. Most NLSs belong to one of two groups: (i) the monopartite SV40 large T antigen NLS (P***KKKRK***V) motif (Kalderon et al., 1984); or (ii) the bipartite motif, consisting of two basic regions separated by a variable number of spacer aa, exemplified by the nucleoplasmin NLS (***KR***PAATKKAGQA***KKK***) (Robbins et al., 1991). On the other hand, nuclear export of proteins is generally mediated by nuclear export signal (NES) sequences. Although these NESs are not so well defined as NLSs, a common motif rich in leucine residues has been proposed, exemplified by the HIV-1 Rev molecule (***L***PP***L***ER***L***T***L***) (Fischer et al., 1995).

Nuclear import has been demonstrated and putative nuclear localization signal (NLS) sequences have been identified in the coat proteins of both mono- and bipartite geminiviruses (Kunik et al., 1998; Qin et al., 1998; Liu et al., 1999; Kotlizky et al., 2000; Unseld et al.,

2001; Unseld et al., 2004). Three regions with the ability to direct GFP to the nucleus were identified within the coat protein of *African cassava mosaic virus* (ACMV), a bipartite begomovirus, that mediate the nuclear import of a GFP fusion protein: one region is located in the N-terminus; a second is located in the central part, and a third is in the C-terminus (Unseld et al., 2004). Scanning of the N-terminus of ACMV-CP revealed a potential bipartite NLS within the first 20 aa. These 20 aa failed, however, to import the GFP into the nucleus. Nuclear import was observed only when the fused CP part was extended to the first 54 aa, including a further basic region. This is in contrast to TYLCV CP in which the first 36 aa, excluding the region 48-54 which contains three basic aa, were sufficient to accomplish nuclear import (Kunik et al., 1998). Another NLS was found at the C-terminus, (the C-terminal 58 aa). This is in contrast to TYLCV CP, where no nuclear import has been assigned to this region (Kunik et al., 1998). The ACMV CP100-127 domain is also able to direct GFP to the nucleus. Nuclear targeting function has also been assigned to this region of *Squash leaf curl virus* (SqLCV); aa 100-200) (Qin et al., 1998) and TYLCV (aa 36-193) CPs (Kunik et al., 1998). The ACMV CP100-127 was also found to facilitate nuclear export. A third function of this CP domain (aa 100-119) was found, which is targeting the GFP fusion proteins to the cell periphery (Unseld et al., 2001; Unseld et al., 2004). The accumulation of three NLS-containing domains might be explained by different functions of the CP during the life cycle of geminiviruses (Unseld et al., 2004). The viral genome has to be transported into the nucleus after insect transmission to plants. If the virus particles enter phloem cells, the CP might be the mediator for the transport of the viral genome to the nucleus (Unseld et al., 2004). The geminivirus genome is replicated and transcribed in the nucleus of infected cells. The CP transcript is translated in the cytoplasm, thus CP must enter the nucleus to encapsidate viral DNA. One or more of the characterized NLSs might facilitate these different steps. NLSs often overlap or lie near the nucleic acid binding motifs (LaCasse and Lefebvre, 1995) and this seems to be true for the coat protein of geminiviruses. The DNA binding domain has been mapped in the N-terminal part of the proteins (Liu et al., 1997; Qin et al., 1998). Although it has been shown in some cases that NLS and DNA binding motifs can be mutated independently without disrupting the other function (Matheny et al., 1994), it has been postulated that the basic nature of some NLS sequences might enhance DNA or RNA binding (LaCasse and Lefebvre, 1995). DNA binding may mask the N-terminal NLS. Furthermore, protein conformation, protein-protein interaction, and posttranslational

modification of the CP could be involved in utilizing one or the other NLS for nuclear import (Unseld et al., 2001). Phosphorylation of the *Abutilon mosaic virus* (AbMV) CP has been demonstrated in the heterologous organism *Escherichia coli (E. coli*) (Wege and Jeske, 1998), indicating possible involvement in functional regulation of the CP (Unseld et al., 2001).

The coat proteins of several geminiviruses have been found to bind single- and double-stranded DNA *in vitro* in a sequence nonspecific manner (Ingham et al., 1995; Liu et al., 1997; Palanichelvam et al., 1998).

Little is known about the multimerisation of geminivirus coat proteins. CP forms the capsule that encapsidates the viral genomic ssDNA. Analysis of the homotypic interaction capacity of TYLCV-CP revealed that full-length CP interacts with itself (Hallan and Gafni, 2001). Using an *in vitro* binding assay, the multimerisation domain of SqCLV CP was mapped in the N-terminal 97 aa, overlapping the DNA binding domain (Qin et al., 1998; Hallan and Gafni, 2001). Truncation of the protein from the C-terminus led to a diminution of the self-interaction process. In addition, the N-terminal region of the CP seemed to be necessary for the interaction as two C- and N-terminal deletion mutants interacted successfully with the wild-type protein, although they failed to self-interact. It was suggested that the N-terminal aa interact with aa of the C-terminal region of the CP of another subunit (Hallan and Gafni, 2001). Aa in the central portion of TYLCV CP interfere with particle formation (Noris et al., 1998), and they influence the homotypic interaction (Unseld et al., 2004).

I.5 Resistance strategies

Geminiviruses are versatile, infecting multiple hosts from monocots such as maize to dicots such as cassava and tomato. The transportation networks that are spreading the virus from tropical to temperate regions have also brought different species (there are more than 66 of them) into close contact. This may have set the stage for the viruses to swap DNA. So far, the researchers have identified more than 1000 recombinant strains, in some cases involving species whose DNA sequences indicate that they are not closely related.

And while new virulent strains are generated, measures to control it are falling short. Plowing under plant debris after harvest limits the food supply for the white flies and the virus, but the

measure has limited effectiveness, as do insecticides. Whiteflies are developing resistance to commonly used chemicals (Moffat, 1999).

Resistant cultivars have been created either by classical crossbreeding or through genetic engineering. Crossbreeding has produced more resistant cassava, beans, and tomatoes, although the new resistant varieties have such drawbacks as small fruit or poor taste. And although modern gene transfer techniques are faster and more precise than classical plant breeding, the diverse, changeable viruses have already demonstrated that they can outwit the genetic engineers (Moffat, 1999). Transgenic expression of pathogen-derived sequences has been extensively used to obtain virus-resistant plants. These strategies have variously explored and exploited the general idea that transgenic expression of virus-derived sequences may interfere with the viral life cycle (Beachy, 1997). It was shown that transforming *Tomato leaf curl virus*-infected plants with the homologous replicase gene constructs that produce RNAs capable of duplex formation confers gene silencing and results in recovery of infected plants. It was proposed that the antisense suppression in the virus-infected plants provides a threshold level of dsRNA needed to induce gene silencing leading to the virus suppression (Praveen et al., 2004).

However, the observation that the predicted molecular interference mechanisms have not always coincided with those operating in resistant transgenic plants has revealed the complexity of the interaction between the transgene and the challenging virus. It has become clear that a given transgenic sequence can act via a protein mediated mechanism or by posttranscriptional gene silencing (PTGS), depending on its molecular fate, and that infection can induce transgene silencing (virus-induced gene silencing [VIGS]), resulting in a recovery phenotype (Beachy, 1997; Baulcombe, 1999). To counterattack the RNA silencing defence mechanism, most plant viruses have evolved , like RNA viruses, RNA silencing suppressors of PTGS (Voinnet et al., 1999). Moreover, geminiviruses can silence via VIGS trans- and endogenes when homologous sequences to these genes are expressed from their genomes (Kjemtrup et al., 1998; Peele et al., 2001; Lucioli et al., 2003; Noris et al., 2004). (Lucioli et al., 2003) showed that transgenic expression of a truncated form of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) Rep protein (Rep-210) confers resistance to viral infection (Brunetti et al., 1997; Brunetti et al., 2001) and that resistance is lost when TYLCSV shuts off transgene expression by PTGS. The ability of TYLCSV to spread in silenced transgenic plants may reflect a balance between silencing, silencing suppression (Voinnet et al., 1999;

Van Wezel et al., 2002), and virus replication (Lucioli et al., 2003). It was suggested that if the virus reaches a threshold level of expression/replication in the initially infected cells then virus spreading can no longer be prevented (Noris et al., 2004).

Researchers are also attempting to endow plants with broad-scale resistance to the rapidly expanding family of geminivirus pathogens. One strategy being explored is to equip plants with a protein from a bacterial virus that binds viral DNA and prevents movement of geminiviruses within plants, thereby blocking infection. Another strategy is to arm susceptible plants with a protein that attacks a structural element common to the replicase proteins of many geminiviruses (Moffat, 1999).

Ectopic expression of recombinant antibodies (rAbs) (immunomodulation) has great potential to prevent viral infection (Boonrod et al., 2004). The feasibility of the immunomodulation approach was shown for human viruses (Marasco, 1995), but application in plant virology have been limited. Different rAbs were successfully expressed in transgenic plants (Hiatt et al., 1989; Fecker et al., 1996; De Jaeger et al., 2000) and methods were developed for their accumulation in different plant organs and plant cell compartments (Conrad and Manteuffel, 2001). Expression of rAbs against the coat protein *Tobacco mosaic virus* (TMV) resulted in reduced susceptibility of tobacco plants to TMV (Voss et al., 1995). Also, the intracellular expression of scFv against either TMV or *Artichoke mottle crinkle virus* (AMCV) coat proteins yielded improved, but not complete, resistance against TMV and AMCV, respectively (Tavladoraki et al., 1993). Delay and suppression of virus symptoms were observed against *Beet necrotic yellow vein virus* (Fecker et al., 1996) and TMV (Schillberg et al., 2001), by using either full size or recombined fragments of antibodies from hybridoma cell lines. It was shown that scFvs directed against the conserved motif of viral RNA-dependent RNA polymerase (RdRp) are able to confer resistance in transgenic plant to homologous and heterologous viruses (Boonrod et al., 2004).

I.6 Engineering of recombinant antibodies by phage display technology

I.6.1 General structure of typical antibody molecule

Antibodies are the secreted form of the B-cell receptor. They belong to a family of glycoproteins known as immunoglobulins, which are found in the fraction of serum known as

the gamma globulin fraction. Antibody molecules are Y-shaped molecules consisting of three equal-sized portions, loosely connected by a flexible tether. A typical IgG antibody is a large molecule (~150 kDa), composed of polypeptide chains linked by disulphide bridges. There are two identical light (L) chains of about 220 amino acid residues (~25 kDa each) and two identical heavy (H) chains of about 450-600 amino acid residues (~50 kDa each). Each H and L chain consists of homologous segments of about 110 amino acid residues that form independently folded domains. The light chain is made up of two such domains, whereas the heavy chain contains four (Fig. 3). There are two types of L chain, kappa (κ) or lambda (λ) and a given immunoglobulin either has κ chains or λ chains.

The amino-terminal sequence of both H and L chains varies considerably in antibodies of different specificity. This variability is limited to approximately the first 110 aa, corresponding to the first domain; these make up the variable regions, V_H and V_L that are responsible for the antigen-binding diversity of antibodies. One V_H and one V_L fold together to form an antigen-binding site, so each IgG molecule has two identical antigen-binding sites. The amino acid sequence of the carboxyl half of the L chain and three-fourths of the H chain show relatively limited variability, and make up the constant regions (C_H and C_L). Each light chain has one C_L domain, and each heavy chain has three or four C_H domains. The hinge region is a more extended region (not folded into a domain) between H chain C_H1 and C_H2. It allows the two antigen-binding regions of each antibody molecule to move independently to bind antigen.

The antigen binding site (paratope) of most antibodies is formed by six loops called ‘complementarity determining regions’ (CDRs) (Kabat et al., 1977; Kabat, 1978), three from the V_L domain (L1, L2, L3) and three from the V_H domain (H1, H2, H3) (Al-Lazikani et al., 1997). The six CDRs exhibit hypervariability and form hypervariable loops at the end of the two variable domains. The CDR with the greatest variation in terms of length and sequence is the heavy-chain CDR3 (HCDR3) (Sanz, 1991). The regions of variable domains outside these loops are called framework. They are highly conserved in sequence and their conformation can be predicted using standard homology modelling techniques (Morea et al., 1998; Morea et al., 2000).

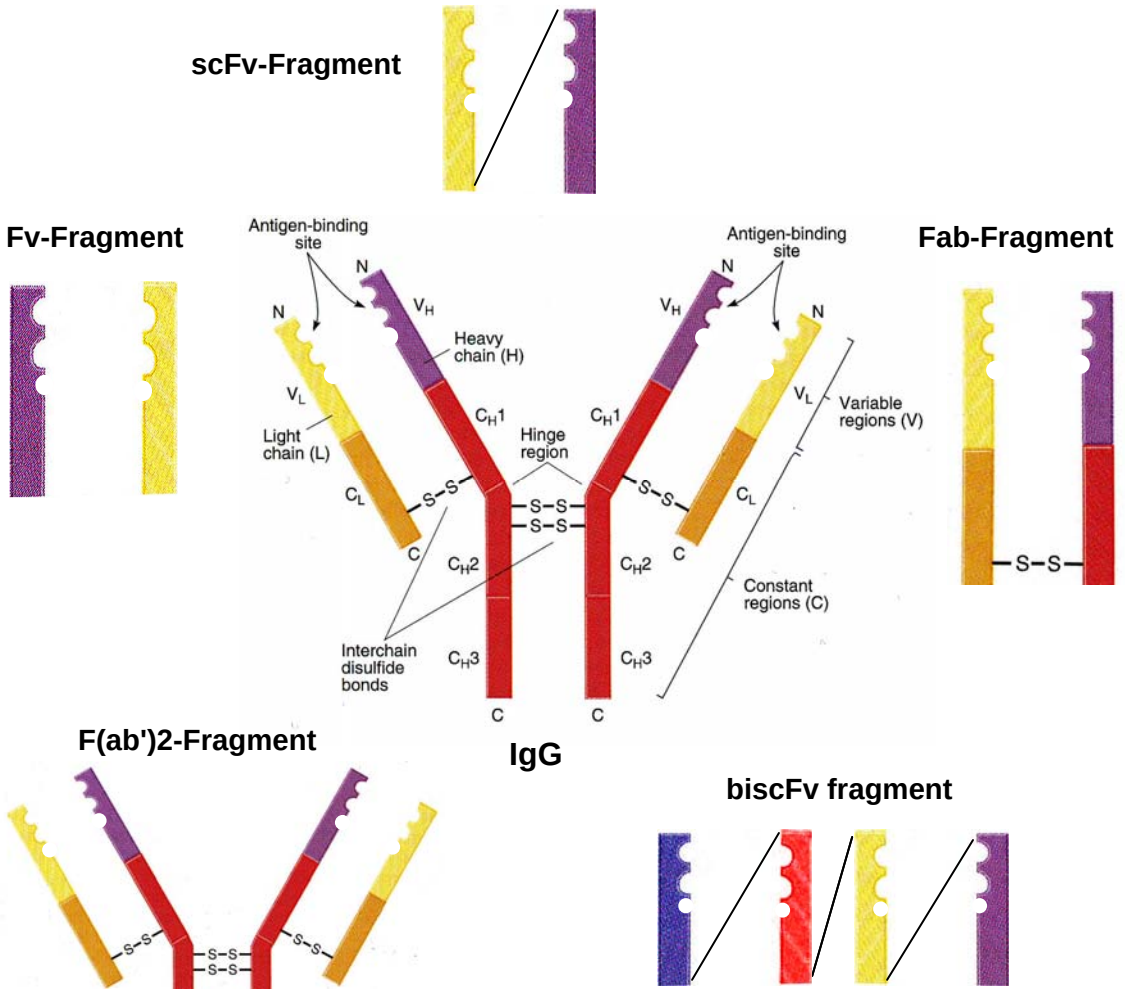


Fig. 3: Schematic presentation of IgG molecule and antibody fragments. For details see (I.6.1)

As antibodies are very large molecules, the use of small fragments can be advantageous for research and *in vivo* applications, due to their ability to penetrate tissues and faster clearance from tissues and serum (Cumber et al., 1992; Yokota et al., 1992). The recombinant antibody fragment most commonly used in research and therapy is the single-chain variable fragment (scFv) (Bird *et al.*, 1988; Huston *et al.*, 1988; Skerra and Plückthun, 1988). ScFv (~30 kDa) consist of the V_L and V_H domains of immunoglobulins linked by a flexible peptide (usually 15-20 aa long, e.g. of the sequence (Gly4Ser3) (Whitlow et al., 1993; Plückthun, 1994). If no reorientation of the two domains occurs they should have the same monomeric binding affinity as the parental monoclonal antibody as experimentally demonstrated in a number of cases (Glockshuber et al., 1990; Bird and Walker, 1991; Huston et al., 1991). The hydrophobic interaction between the VH and VL domains is not very strong. This can lead to

dimer formation where the VH of one molecule interacts with the VL of another and *vice versa*. Consequently, most scFv fragments are found in two forms: monomer and dimer (Kortt et al., 1994; Chames and Baty, 2000). This tendency to dimerize has been exploited to create bivalent or bispecific diabodies (Holliger and Winter, 1997; Plückthun and Pack, 1997) and triabodies (Iliades et al., 1997).

Single-chain fragments can be expressed in a variety of hosts (Verma et al., 1998), including bacteria (Plückthun et al., 1996), yeast (Fischer et al., 1999 a) and plants (Fischer et al., 1999 b; Fischer et al., 1999 c; Fischer et al., 1999 d; Smith and Glick, 2000). Single-chain fragments have found broad applications in medicine (Huston et al., 1993; Hudson and Kortt, 1999) and have also great potential in biotechnology. The unique and highly specific antigen-binding ability might, for example, be exploited to block specific enzymes, bacteria, viruses or to detect environmental pollutants present in very low concentrations (Wörn and Plückthun, 2001).

I.6.2 Antibody libraries

The advent of the polymerase chain reaction (PCR) and the increased knowledge of antibody gene sequences have made it possible to produce large repertoires of antibody fragments (Tomlinson et al., 1996). Using sets of oligonucleotide primers that bind the highly conserved terminal regions of the V_H and V_L genes of different animal species, the antibody genes from mice (Clackson et al., 1991; Hoogenboom et al., 1991), human donors (Vaughan et al., 1996; Sheets et al., 1998), rabbits (Ridder et al., 1995) and camels (Hamers-Casterman et al., 1993) have been amplified by PCR. The products were then assembled in plasmid vectors to produce repertoires of antibody fragments. Sources of immunoglobulin genes include the spleens and peripheral blood lymphocytes of immunized animals (Clackson et al., 1991) or the bone marrow, tonsils and peripheral blood lymphocytes from non-immunized human donors (Vaughan et al., 1996; Sheets et al., 1998). Phage display libraries can be derived from V-gene repertoires of ‘immunized’ or ‘naïve’ human or animal donors (Clackson et al., 1991; Marks et al., 1991). The phage display libraries from immunised donors contain representatives of antibody genes after their rearrangement and after somatic mutation of the germline sequences *in vivo*. These repertoires are biased towards antibodies specific to the immunized antigen. Moreover the antibodies having undergone affinity maturation also have high affinity for the antigen. This advantage is offset by the need for making a specific phage display library for each antigen. On the other hand, the naïve library, constructed from non-

immunized donors, contains population of antibody sequences that correspond to those of primary repertoire as well as memory B-cells (the antibody sequence after somatic mutation of the germline cells) of the individual from which the samples were taken. The antibodies sequences in this type of libraries are affected by the medical history of the donors, but they are not intentionally biased towards any particular antigen. The naïve libraries offer the possibility to select antibodies to a large panel of antigens, including self, non-immunogenic and relatively toxic antigens without the need for immunization (Marks et al., 1991; Griffiths et al., 1993; Vaughan et al., 1996). Alternatively, synthetic or semi-synthetic human antibody V-gene repertoires have been made by *in vitro* assembly of V gene segments and D/J fragments (Griffiths et al., 1994; De Kruif et al., 1995). In these, framework V_H and V_L sequences have been combined with synthetic CDR sequences that comprise mixtures of trinucleotide cassettes, which are biased against stop codons and which contain codons favoured by bacterial protein translation system (Krebs et al., 1998).

I.6.3 Selection of recombinant antibodies by phage display

Phage display is a powerful technique for identifying proteins or peptides that bind to particular molecules of interest. Antibody phage display has become a popular method for identifying and selecting novel molecules for product development. The development of phage display vectors (Smith, 1985) has allowed expression and presentation of antigen-binding antibody fragments on the surface of filamentous phages. In 1990, McCafferty and colleagues demonstrated in a model experiment that it was possible to select antibody fragments from a large population of proteins expressed by filamentous bacteriophages. A protein, in this case an antibody fragment, is located on the surface of a phage particle by cloning the antibody fragment into the phage genome, fused with a coat protein (pIII, pIV or pVIII). Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the binding structures being present on the phage surface, while its genetic material resides within the phage particle. This relation between genotype and phenotype allows enrichment of specific phages (Barbas, 1993; Burton and Barbas, 1994; Hoogenboom, 1997), which are selected on immobilised targets by a simple *in vitro* selection procedure called ‘biopanning’. Phages that display relevant binding structures will be retained, while non-adherent phage will be washed away. Bound phages are recovered from the surface and used for reinfection of bacteria. The success of ligand phage display hinges on the combination of this display and enrichment method with the synthesis of large

combinatorial repertoires on phage (Hoogenboom et al., 1998; Chames and Baty, 2000). Large libraries displaying peptide and proteins have been made using pIII as fusion partner (Smith and Scott, 1993; Winter et al., 1994), leading to the development of a number of techniques for selecting the molecule(s) desired from such libraries (Clackson and Wells, 1994; Hoogenboom, 1997). Peptides and proteins have also been fused to the amino-terminal part of the major capsid protein pVIII (Iannolo et al., 1995). Heavy and light-chain variable regions have been fused to the amino terminus of pVII and pIX and displayed on phages showing that these two minor coat proteins can also be used for display (Gao et al., 1999). Initially, phage vectors that carried all the genetic information required for the phage life cycle were used (McCafferty et al., 1990; Clackson et al., 1991). Display of antibodies on smaller filamentous phage-like particles using phagemids (Bass et al., 1990; Breitling et al., 1991) advanced antibody engineering. A phagemid is a type of plasmid which contains the filamentous phage intergenic region with its origin of replication. When the host bacterium is infected with "helper" phage, the single stranded phagemid DNA is replicated along with the phage DNA and packaged into filamentous phage-like particles. The phagemid also contains an origin for plasmid replication and a resistance marker. The phagemid can maintain itself as a plasmid and function as a bacterial expression vector if desired. A helper phage, such as M13KO7, has a defective packaging signal so that the majority of phages produced contain the phagemid single-stranded DNA (Russel et al., 1986). Bacteria infected with this phage can be selected via their antibiotic resistance. The phagemid DNA can then be propagated again by infection of the bacteria with helper phage. In phagemids, the scFv may be fused at the N-terminus of the mature gene III protein (McCafferty et al., 1990; Hoogenboom et al., 1991) or at the N-terminus of truncated pIII lacking the first two N-terminal domains (Barbas et al., 1991; Garrard et al., 1991). Phagemids have higher transformation efficiency and they are suited for generating very large repertoires. They may also be formatted for direct secretion of the unfused antibody fragment without subcloning (Hoogenboom et al., 1991).

I.7 Research objectives

In this study the generation of specific rAbs in form of scFvs directed against two key viral proteins, the Rep (AC1) and the CP (AV1), of ToLCV-India was anticipated.

The major objective of this resarch was to interfere and/or block essential functions of AC1 and AV1 viral proteins by using genetic engineering and antibody technology. The final aim of the study was the generation of AC1 and AV1 specific scFvs interfering *in vitro* and *in vivo* with the viral proteins' functions and to engineer virus resistant plants.

Our strategy was based on cytoplasmic and/or nuclear expression of scFvs binding to these essential viral target proteins within transgenic plants to ideally block viral functions involved in coating/uncoating, transmission, replication, and nuclear import and export.

We investigated whether scFvs directed against ToLCV-CP/Rep were capable of neutralising virus infectivity or inhibiting virus spread *in planta* or its transmission by the vector.

Cloning of the AC1 and AV1 genes as fusion proteins with DsRed was performed to monitor and localize these proteins in the transgenic plant cells. Theoretically, the AV1-DsRed fusion protein should accumulate and fluorescence in the nucleus since AV1 carries well-characterized NLS. Also AC1 should enter the nucleus where the geminiviruses replication and transcription take place. However, the presence of rAbs directed against the AV1 or AC1 may prevent the fusion protein from entering the nucleus and the fluorescence will be seen exclusively in the cytoplasm.

A schematic overview of this Ph.D thesis is presented in Fig. 4.

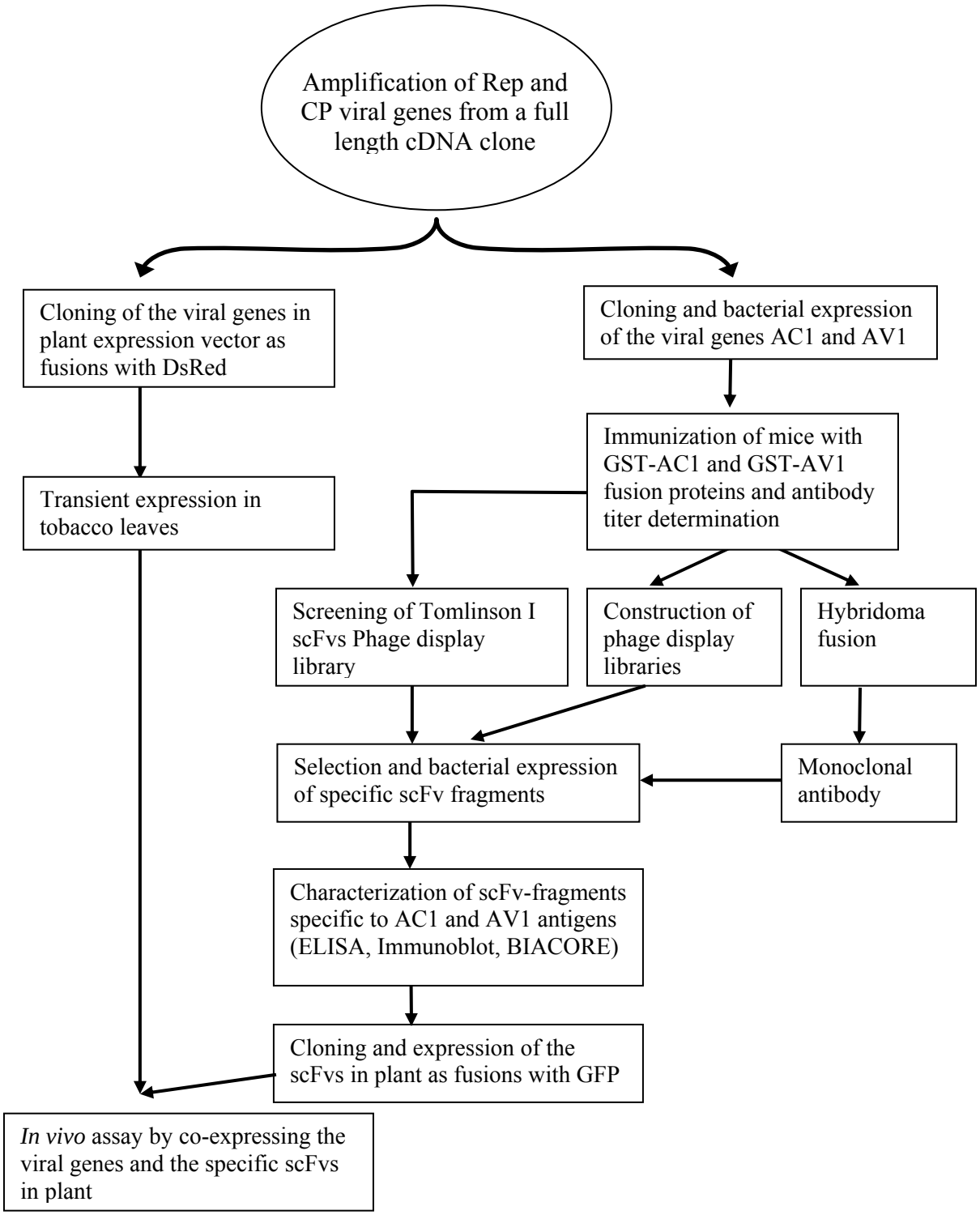


Fig. 4: A schematic overview of this study.

II Material and Methods

II.1 Material

II.1.1 Chemicals and consumables

The chemicals used throughout the work were purchased from the following companies: Bio-Rad (München), Roche (Mannheim), Fluka (Neu-Ulm), Gibco BRL (Eggenstein), Merck (Darmstadt), Amersham Pharmacia Biotech (Freiburg), Roth (Karlsruhe), Serva (Heidelberg), Sigma (Deisenhofen). The consumables were from: Amicon (Witten), Biozym (Hess. Oldendorf), Eppendorf (Hamburg), Greiner (Solingen), Kodak (Stuttgart), Millipore (Eschborn), Nunc (Bieberach), Schott Glaswerke (Mainz), Serva (Heidelberg), USB/Amersham (Braunschweig), Whatman (Bender & Hobein, Bruchsal) and Zeiss (Oberkochem).

II.1.2 Enzymes and reaction kits

Restriction enzymes either from New England Biolabs (Schwalbach) or GibcoBRL were used for DNA digestion. *Taq* DNA polymerases from Gibco BRL or Roche were used for amplification of mouse heavy and light chain fragments and for amplification of cloned DNA (‘‘check-PCR’’).

The following kits were used:

Plasmid isolation kits (Mini, Midi, Maxi) Qiagen (Hilden)

QIAquick gel extraction kit Qiagen

QIAquick PCR purification kit Qiagen

RNeasy mini kit Qiagen

SuperscriptTM preamplification system kit GibcoBRL

QuikChange Mutagenesis Kits – Stratagene, La Jolla, CA

II.1.3 Primary antibodies, secondary antibodies and substrates

Mouse anti-GST monoclonal antibody, provided by Dr. Michael Monecke (RWTH Aachen, Institut für Biologie VII, Germany), was used for analyses of GST fusion protein expression. Mouse anti-c-myc tag monoclonal antibody (9E10) (Kindly provided by Gottfried Himmler, Institute of Applied Microbiology, Vienna, Austria) was used for detection of scFv-fragments

by immunoblot (II.2.4.3) and ELISA (II.2.9.1). Mouse anti-His (Qiagen or Sigma) was also used for detection of scFv fragments in transient protein expression experiments.

Polyconal antibody against the geminivirus African Cassava Mosaic Virus (ACMV) was used for AV1 detection.

Alkaline phosphatase (AP) or horseradish peroxidase (HRP)-conjugated to goat anti-mouse (GAM) IgG (H+L, Fc) (Dianova) antibodies were used as secondary antibody in immunoblot analysis (II.2.4.3) and ELISA (II.2.9.1). NBT/BCIP (Bio-Rad) and pNPP (Bio-Rad) were used as substrate for detection of immobilized proteins in Immunoblot (II.2.4.3) and ELISA (II.2.9.1), respectively.

II.1.4 Bacterial strains

E. coli strains DH5α , XL10-Gold and XL1-blue were used as a host cells for all intermediate cloning constructs; BL21 was used for expression of GST/MBP fusion proteins (II.2.3.1 and II.2.3.2, respectively); TG1 was used for generation of phage-displayed antibody libraries used in solid-phase panning (II.2.7). HB2151 was used for expression of soluble scFv-fragments (II.2.9.1) (Table 2).

Agrobacterium tumefaciens (A. tumefaciens) GV 3101 (pMP90RK Gm^R, Km^R, Rif^R (Konz and Schell, 1986) was used for *Agrobacterium*-mediated gene transfer (II.2.1.6).

Table 2: Names, suppliers and genotypes of *Escherichia coli* strains used throughout the work.

Strain	Source	Genotype
DH5α	Ausubel et al.,1994	F- (*f80d Lac* 2Δ*M*15) Δ(*LacZYA-argF*) *U*169*end A*1 *rec*1 *hsdR*17(*rk- mk*+) *deoR thi*-1 *supE*44 *gyrA*96 *relA1 λ*-
HB2151	Ausubel et al.,1994	K12, *ara,* Δ(*lac-pro), thi*/ F´ *pro A+B+, lacIqz*Δ*M15* BL21(λDE3) Novagen F- *ompT hsdSB* (rB - mB-) *gal dcm* (DE3)
XL1-Blue	Stratagene	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F′ *proAB lacIIq ZΔM*15 Tn10 (Tetr)**]**
XL10-Gold	Stratagene	Tetr Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*qZΔ*M15* Tn*10* (Tetr) Tn5 (Kanr) Amy].
TG1	Stratagene	*supE thi*-1 Δ(*lac-proAB*) Δ(*mcrB-hsdSM*) 5(*rK- mK*-) [F´*traD*36 *proAB lacIq ZΔM*15]
BL21(λDE3)	Novagen	F- *ompT hsdSB* (rB- mB-) *gal dcm* (DE3)

II.1.5 Plants and animals

Nicotiana benthamiana, grown from seeds, was used for transient protein expression.

6-8 weeks old female BALB/c mice were used for immunization with GST-AC1 and GST-AV1 fusion proteins (II.2.3.1).

II.1.6 Helper Phages

M13KO7 helper phage (Amersham Pharmacia Biotech) is an M13mp1 derivative containing a modified gene 11 [G changed to T at position 6125, giving a methionine to isoleucine change at codon 40 of the gene 11 protein]. The p15A origin of replication and the kanamycin resistance gene from Tn903 are present in M13KO7. Self-made helper phage was also used with Tomlinson I library (II.2.7.1). The original stock is M13 VCS, Stratagene.

II.1.7 Vectors

Schematic presentations of the vector maps are provided in the Appendix.

pGEX-5X-3 from Amersham Pharmacia Biotech was used for subcloning of AC1 and AV1 viral genes and expression of the GST fusion protein.

pMAL-cx2 from New England Biolabs. The vector expresses the male gene fused to the lacZα gene. Restriction sites between malE and lacZα are available for inserting the coding sequence of interest. The cloned gene is inserted downstream from the malE gene of *E. coli*, which encodes maltose-binding protein (MBP), resulting in the expression of an MBP fusion protein.

pHENHI phagemid vector containing *Sfi*I-*Nco*I, *Not*I cloning sites was used to establish the mouse phage display libraries. The pHENHI vector contains an N-terminal *pel*B leader peptide that targets the expressed protein into the periplasmic space and a C-terminal His6 tag for purification via IMAC (Ni-NTA). The pHENHI is modified version of pHEN4II vector (Zhang et al., 2001), which is a derivative from the pHEN4C vector (Hoogenboom et al., 1991).

pTRA-kc (Thomas Rademacher, Institut für Biology VII, RWTH Aachen, Germany) is an optimized plant expression vector containing the 35SS promoter and the pA35S untranslated region (UTR) from CaMV. A matrix attachment region was introduced to improve transcription.

pIT2 (HIS myc tag) is the phagemid vector that Tomlinson I library is constructed in. It contains *Sfi*I/*Nco*I-*Xho*I, *SalI-Not*I cloning sites.

II.1.8 Oligonucleotides

Oligonucleotides used for sequence analysis and amplification of DNA are listed below. Oligonucleotides synthesized by MWG (Ebersberg, Germany) are:

1. Primers used for amplification of AC1 and AV1 genes from Full-length cDNA clones of ToLCV-India (MAHYCO) for subsequent cloning into pGEX-5X-3:

- AC1 primers:

5′- Primer (*Sal* I / *Eco*RI)

5′- AC CTA GTC GAC AGG AAT TCC GCG AAG CGA CCA GCA GAT ATC -3′

3′- Primer (*Not* I)

5′- TG GAT CCA GCG GCC GCC ATT TGT TAC CGA ATC ATA GAA AT -3′

- AV1 primers:

5′- Primer (*Sal* I/*Bam* HI):

5′- AC CTA *GTC GAC* G*GG ATC C*CC GCT CCG CCA CGT CGT TTC AGA A -3′

3′- Primer (Not I):

5′- TG GTT CCA *GCG GCC GC*C ACT CGC CTC CTG AGA ATG CTC TT-3′

2. Primers used for PCR amplification of AC1 gene for cloning into pMAL-c_2x:

5′- AC1 *Bam*HI:

5′- GGC GCG GAT CC*G TCG AC*G CCG CTC CGC CAC GTC GTT TCA GAA TAA ATG CAA AAA ACT ATT TC -3′

3′- AC1 *Hind*III:

5′- CCC *GAC AAG CTT* GCG GCC GCC ACT CGC CTC CTG AGA ATG CTC TTC TTC TTC TTC TGG GGG -3′

3. Primers used for amplification and cloning of AV1 gene as fusion with DsRed in pTRA vector:

5′- AV1 (*Nco*I):

5′-ACC TAA GCC ATG GCC GCG AAG CGA CCA GCA GAT ATC ATC ATT TC-3′

3′- AV1 (*Nco* I):

5′- T GGA TCC ACC ATG GCA TTT GTT ACC GAA TCA TAG AAA TAG -3′

4. Splice overlap extension (SOE) phage display primer sequences for first strand cDNA generation from total RNA in 5′-3′ orientation.

mVHI Back	CAG GTG CAG CTK SWG SAG TCW GG
mVHII Back	CAG GTG CAG CTG SWV SAG YCT GG
mVHIII Back	CAG GTG CAG CTB VAG SAG TCD GR
mVHIV Back	CAG GTG MAG CTG AWG GAR TCT GG
mVHV Back	CAG GTC CAG CTR CAR CAR TCT GG
mVHVI Back	CAG GTC CAR CTG CAG SAR YCT GG
mVHVII Back	CAG GTG AAG CTG GTG GAR TCT GG
mVHVIII Back	CAG GTG AAG STY MTC GAG TCT GG
mVHIX Back	CAG GTG AAG CTK GAK GAG WCT GR
mVHX Back	CAG GTG CAR CTK GTT GAG TCT GG
mVHXI Back	CAG GTT ACT CTR AAA GWG TST GG
mVHXII Back	CAG GTG AAC TTG GAA GTG TCT GG

mVH1Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTK SWG SAG TCW GG
mVH2Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG SWV SAG YCT GG
mVH3Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTB VAG SAG TCD GR
mVH4Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG MAG CTG AWG GAR TCT GG
mVH5Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTC CAG CTR CAR CAR TCT GG
mVH6Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTC CAR CTG CAG SAR YCT GG
mVH7Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG AAG CTG GTG GAR TCT GG

mVH8Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG AAG STY MTC GAG TCT GG
mVH9Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG AAG CTK GAK GAG WCT GR
mVH10Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAR CTK GTT GAG TCT GG
mVH11Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTT ACT CTR AAA GWG TST GG
mVH12Sfi Back	ATG GCT CAG GGT GCG GCC CAG CCG GCC ATG GCC CAG GTG AAC TTG GAA GTG TCT GG

mV_kI Back	GAC ATT GTG ATG ACC CAG TCK CCA DCW HYC
mV_kIa Back	GAC ATT GTG ATG ACC CAR TCK CCA WCC TYC
mV_kII Back	GAC ATT GTG ATG ACC CAR WCT CCM RAA TYC
mV_kIIa Back	GAC ATT GTG ATG ACC CAG ACT CCA YTC WCY
mV_kIII Back	GAC ATT GTG ATG ACC CAG ACT MCA YYM TCY
mV_kIIIa Back	GAC ATT GTG ATG ACC CAG TCT CCA RCM MYM
mV_kIV Back	GAC ATT GTG ATG ACC CAG TCT CCW GCM HYC
mV_kIVa Back	GAC ATT GTG ATG ACC CAG ACT ACA KSC WYA
mV_kV Back	GAC ATT GTG ATG ACC CAR TCT CCA KCH WMT
mV_kVa Back	GAC ATT GTG ATG ACC CAR TCT CCA GCA MTC
mV_kVI Back	GAC ATT GTG ATG ACC CAG GCT GMM YYC TCY
mV_kVIa Back	GAC ATT GTG ATG ACC CAG WCT SCA RCM ACY
mV_kVII Back	GAC ATT GTG ATG ACC CAG TCT CMM RAM YTC
mV_kVIII Back	GAC ATT GTG ATG ACC CAG TCT CCA RHM MYC

mVλI Back	CAG GCT GTT STK AST CAG KMA TCT
mVλII Back	CAG GCT GTT STK AST CAG CCA AGC
mVλIII Back	CAR SYT GTK STS ACT CAG KAA T
mVλIV Back	CAR SYT GTK STS ACT CAG KCA T

mV_k1link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG TCK CCA DCW HYC
mV_k1alink Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAR TCK CCA WCC TYC
mV_k2link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAR WCT CCM RAA TYC
mV_k2alink Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG ACT CCA YTC WCY
mV_k3link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG ACT MCA YYM TCY
mV_k3alink Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG TCT CCA RCM MYM
mV_k4link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG TCT CCW GCM HYC
mV_k4alink Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG ACT ACA KSC WYA
mV_k5link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAR TCT CCA KCH WMT
mV_k5alink Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAR TCT CCA GCA MTC
mV_k6link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG GCT GMM YYC TCY
mV_k6alink Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG WCT SCA RCM ACY
mV_k7link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG TCT CMM RAM YTC
mV_k8link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	GAC ATT GTG ATG ACC CAG TCT CCA RHM MYC
mVλ1link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA
	CAG GCT GTT STK AST CAG KMA TCT

mVλ2link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA CAG GCT GTT STK AST CAG CCA AGC
mVλ3link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA CAR SYT GTK STS ACT CAG KAA T
mVλ4link Back	CG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCA CAR SYT GTK STS ACT CAG KCA T
mVhI For	TGA GGA GAC GGT GAC CGD AGT BCC TKS RCC
mVhII For	TGA GGA GAC GGT GAC TGA RGT BCC TKS RCC
mVhIII For	TGA GGA GAC GGT GAC HRD GGT TCC TKS RCC
mVh1link For	CC CGA CCC ACC ACC GCC CGA GCC ACC GCC ACC TGA GGA GAC GGT GAC CGD AGT BCC TKS RCC
mVh2link For	CC CGA CCC ACC ACC GCC CGA GCC ACC GCC ACC TGA GGA GAC GGT GAC TGA RGT BCC TKS RCC
mVh3link For	CC CGA CCC ACC ACC GCC CGA GCC ACC GCC ACC TGA GGA GAC GGT GAC HRD GGT TCC TKS RCC
mV_kI For	TTT GAT CTC CAG CTT GGT VCC NSM DCC G
mV_kII For	TTT GAT CTC CAG CTT GGT GCC HSM DSC G
mVλI For	GGG CTG RCC TAG GAC AGT SAS YTT GGY TCC
mVλII For	RCC TAG GAC AGT SAS YTT GGT
mVλIII For	GGA TAC AGT TGG TGC AGC ATC AGC
mVλIV For	AAG CTC YTC AGA GGA AGG TGG AAA
mV_k1Not For	GAG TCA TTC TCG ACT TGC GGC CGC TTT GAT CTC CAG CTT GGT VCC NSM DCC G
mV_k2Not For	GAG TCA TTC TCG ACT TGC GGC CGC TTT GAT CTC CAG CTT GGT GCC HSM DSC G

mVλ1Not For	GAG TCA TTC TCG ACT TGC GGC CGC GGG CTG RCC TAG GAC AGT SAS YTT GGY TCC
mVλ2Not For	GAG TCA TTC TCG ACT TGC GGC CGC RCC TAG GAC AGT SAS YTT GGT
mVλ3Not For	GAG TCA TTC TCG ACT TGC GGC CGC GGA TAC AGT TGG TGC AGC ATC AGC
mVλ4Not For	GAG TCA TTC TCG ACT TGC GGC CGC AAG CTC YTC AGA GGA AGG TGG AAA

Note: degeneracy codes: K= G or T; M = A or C; S = C or G; R = A or G; W = A or T

5. Primers used for DNA sequencing [LI-COR IR2-DNA sequencer, labelled with IRD 700 or IRD 800 (USB/Amersham)]. Sequences are given in 5′-3′ direction.

Universe	GTT GTA AAA CGA CGG CCA GT
Reverse (P4)	ACA CAG GAA ACA GCT ATG AC
pHEN forward	GCC GCT GGA TTG TTA TTA CTC GC
pHEN backward	TTT CAA CAG TCT ATG CGG CCC C
pSS 5′	ATC CTT CGC AAG ACC CTT CCT CT
pSS 3′	AGA GAG AGA TAG ATT TGT AGA GA
pGEX forward	GGG CTG GCA AGC CAC GTT TGG TG
pGEX backward	TTT CAA CAG TCT ATG CGG CCC C

6. Primers used for DNA sequencing, sequences given in 5′-3′ direction.

pSS 5′	ATC CTT CGC AAG ACC CTT CCT CT
pSS 3′	AGA GAG AGA TAG ATT TGT AGA GA
LMB3	CAG GAA ACA GCT ATG AC
pHEN seq	CTA TGC GGC CCC ATT CA

Oligonucleotides synthesized by Sigma-Genosys are:

1. Primers used to restore the point deletion in scFv-SAV:

5` scFv1:

5′- CAG CTT CTG CAG TCA GGG ACT GCA CTG GCA AAA C -3′

3` scFv1:

5´- G TTT TGC CAG TGC AGT CCC TGA CTG CAG AAG CTG -3´

2. Primers used to exchange the internal stop codon from the scFvs SCR-AV3 and SCR-AV6 with amino acid Glutamine (Q):

5` aAV3:

5´- TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC -3´

3` aAV3:

5´- GGT GAC CAG GGT TCC CTG GCC CCA GTA GTC AAA -3´

3. Primers used to exchange the internal stop codon from the scFvs SCR-AV3 and SCR-AV6 with amino acid Glutamate (E):

5` aAV3-For:

5´- TTT GAC TAC TGG GGC GAG GGA ACC CTG GTC ACC -3´

3` aAV3-back:

5´- GGT GAC CAG GGT TCC CTC GCC CCA GTA GTC AAA -3´

4. Primers used to clone the scFvs into the vector pTRA as fusion with GFP:

5` Cyt-F:

5'- GCTGAGGTACCGAATTC GCC ATG GCC GAG GTG CAG CTG TTG – 3'

3` NLS-b:

5'- CGC TGC ATA CTG CGA CAT GTG ATG GTG ATG ATG ATG AGC GGC CGC CCG TTT G- 3'

NLS-For:

5'- G CTG AGG TAC CCC ATG GCG CCA AAG AAA AAG AGG AAA GTG GCC GAG GTG CAG CTG TTG GAG TC – 3'

II.1.9 Buffers, media and solutions

All standard solutions, buffers, and media were prepared according to (Ausubel et al., 1995; Coligan et al., 1995; Sambrook et al., 1996). Compositions of non-standard solutions or buffers are listed at the end of the respective method section. Media for cultivating bacteria were sterilized by autoclaving (121°C/1-2 bar), all other solutions were sterile filtered (0.2 µm). Thermo labile components such as antibiotics were sterile filtered and added to the media after autoclaving and cooling to 50°C.

II.1.10 Matrices and membranes

Glutathione sepharose 4B from Amersham Pharmacia Biotech was used for purification of GST fusion proteins (II.2.3.1).

Ni-NTA agarose matrix from Bio-RAD was used for purification of scFv fragments from large scale cultures by immobilized metal ion affinity chromatography (IMAC) (II.2.3.3). ImmobilonTM-P transfer membrane (PVDF) (0.45µm) from Millipore, HybondTM-C nitrocellulose membrane (0.45µm) from Amersham Life Science and Whatman no.1 paper from Whatman (Maidstone, England) were used in immunoblot analysis (II.2.4.3).

II.1.11 Equipment and applications

Cameras: MP4 (Polaroid, Cambridge, MA, USA). E.A.S.Y 429K camera (Herolab, Wiesloch).

Centrifuges: AvantiTM 30 and AvantiTMJ-25 (Beckman, California, USA), Biofuge A (Heraeus, Hanau), Sigma 3-10 and Sigma 4-10 (Sigma, St. Louis, Missouri, USA), RC5C and RC5B plus (Sorval instruments, Du Pont, Bad Homburg). Rotors: F0650, F2402H, JLA 10.500 and JA 25.50 (Beckman), #1140 and #11222 (Sigma), RLA-300, SS-34 and GS-3 (Du Pont).

DNA gel electrophoresis apparatus: wide mini and mini cells for DNA agarose electrophoresis and power supplies (Bio-Rad).

DNA-sequencing machines: LI-COR IR2-4200 Sequencer (LI-COR, MWG-Biotech) and Base ImageIRTM 4.0 software (LI-COR). ABI Sequencer (Applied Biosystems, Foster City, CA), Big Dye kit v. 3.1.

Electroporation apparatus: “Gene pulserTM”, “Pulse controller” unit, Extender unit (BioRad) and 0.2 cm cuvettes (Bio-Rad).

InnovaTM 4340 incubator shaker (New Brunswick Scientific, Nürtingen).

PCR Thermocyclers: Primus and Primus 96 plus (MWG-Biotech).

Photometers: Spectrophotometer Uvikon 930 (Kontron, Neufahrn) and multi-channel spectrophotometer Spectromax 340 (Molecular Devices, Sunnyvale, Kalifornien).

Probe sonicator: (Braun Biotech, Melsungen).

Protein gel electrophoresis equipment: Mini PROTEAN IITM from BioRad. Gel Air Dryer (Bio-Rad). Protein gel – Novex Mini-Cell, Invitrogen, and X Cell II Blot Module.

Surface plasmon resonance: BIAlite™ X, semiautomated SPR system, (BIACORE, Uppsala, Schweden) + PC, Windows NT 4.0 operating system (Microsoft) and BIAEvaluation 4.1 Software.

UV-Transilluminators: wavelength 302 nm and UVT-20M (Herolab). UV-chamber (Bio-Rad).

Microprojectile bombardment: as described by Gal-On (1997) 13-mm Plastic Swinney Filter holder, PALL Gelman Laboratory, Ann Arbor, MI. Gold: Biolistic R, 1.0 Micron Gold, Bio-Rad, Hercules, CA.

Software: Windows NT 4.0 operating system (Microsoft); Microsoft Office 2000 (Microsoft); Adobe Photoshop 6.0 (Adobe); Adobe Photoshop 7.0 software (Mountain View, CA); Chromas; Origin 6.0 (Data analysis and technical graphics, Microcal Software, Inc.); BIAEvaluation 4.1 Software (BIACORE); and GCG (Wisconsin Package TM of Genetic Computer Group).

Plant growth cabinets: Snijder climatic cabinet (Snijder, Tilburg, Netherlands) Photoperiod 16h, light intensity 400 uE m^{-2} s^{-1}.

Confocal microscopy: Leica TCS SP spectral confocal laser-scanning microscope. (Leica Microsystems, Heidelberg) water-dipping lenses.

II.2 Methods

All experiments related to the genetic engineering were performed according to the regulations of "S1-Richtlinien" and were officially approved by the "Regierungspräsidium des Landes NRW" (RP-Nr.: 23.203.2 AC 12, 21/95) and "BGA" [AZ 521-K-1-8/98:AI3-04/1/0866/88 (S1) and 55.8867/-4/93 (greenhouse)].

General recombinant DNA techniques, i.e. PCI (phenol/chloroform/isoamyl alcohol) and CI (chloroform/isoamyl/alcohol) extraction, DNA precipitation, restriction enzyme digestion, DNA ligation, DNA agarose gel electrophoresis, were according to the standard protocols described in (Ausubel et al., 1995; Sambrook et al., 1996).

II.2.1 Recombinant DNA technologies

II.2.1.1 Competent cells for RbCl-mediated transformation

E. coli strain DH5α competent cells were prepared for RbCl-mediated transformation by heat-shock as described by (Hanahan, 1985). A single bacterial colony was inoculated in 5 ml

of LB broth and cultured at 37°C overnight (o/n). 0.5 ml of the o/n culture was transferred into 50 ml of LB broth containing 20 mM $MgSO_4$ and 10 mM KCl. The cells were cultured at 37°C for 3-4 hours until the OD600nm reached 0.4-0.5 and then transferred to an ice-cold tube. After incubation on ice for 10 min, the cells were recovered by centrifugation (2000g/4°C/10 min). The pellets were resuspended in 15 ml ice-cold TfB-I solution by gentle vortexing and stored on ice for 10 min. The cells were recovered by centrifugation as described above and resuspended in 2 ml ice-cold TfB-II. 200 µl- aliquots of the suspension were dispensed into prechilled microcentrifugation tubes, frozen immediately in liquid nitrogen and stored at -80°C.

TfB-I pH 5.8:

Potassium acetate	30 mM
$MnCl_2$	50 mM
$CaCl_2$	10 mM
Glycerol	15% (v/v)

TfB-II pH 6.8:

MOPS	30 mM
$CaCl_2$	75 mM
RbCl	10 mM
Glycerol	15% (v/v)

II.2.1.2 Transformation of *E. coli* by heat-shock

As soon as the competent cells (II.2.1.1) were thawed, plasmid DNA (up to 100 ng) (II.2.1.9) or ligation products (Sambrook et al., 1996) were mixed gently with the competent cells and incubated on ice for 30 min. The cells were then exposed to 42°C for 90 seconds and placed on ice for 2 min. 800 µl of LB medium were added to the tubes and incubated at 37°C for 45 min. 200 µl of cells were plated onto a LB-agar plate supplemented with appropriate antibiotics and incubated at 37°C overnight.

When the commercial strain XL10-Gold was used, 45 µl of the cells were mixed with 2 µl of the β-ME mix provided with the cells kit. The cells were then incubated on ice for10 minutes with gentle swirling every 2 minutes. The DNA was then added and the mixture was incubated on ice for 30 min. The cells were then exposed to 42°C for 30 seconds and placed on ice for 2 min and continued as with other strains.

II.2.1.3 Preparation of electrocompetent *E. coli*

Electrocompetent *E. coli* were prepared from the following strains, DH5α, BL21(DE3), XL1-blue, and TG1 as described by (Dower et al., 1988). A single bacterial colony from an LB plate was inoculated in 5 ml LB-broth and cultured at 37°C o/n. Three ml of fresh o/n culture was transferred into 500 ml of LB broth. The cells were cultured at 37°C for 3-4 hours until the mid-log phase (OD_{600nm} = 0.5-0.8). Then the cells were placed on ice for 15-20 min and harvested by centrifugation (3000g/4°C/10 min). Cells were washed three times with sterile water and resuspended in ice-cold 10% (v/v) glycerol to a 300-fold concentration from the original culture volume (at $>10^{10}$ cells/ml). 40 µl aliquots were stored at -80°C.

II.2.1.4 Transformation of *E. coli* by electroporation

Electrocompetent cells (II.2.1.3) were thawed on ice and mixed with 1 pg to 300 ng of DNA in sterile dH_2O. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of SOC medium and incubated at 37°C with shaking for 1 h. Finally, 100 µl of the cells were plated onto LB agar containing appropriate antibiotics and incubated at 37°C o/n.

II.2.1.5 Preparation of electrocompetent *Agrobacterium* cells

A single colony of *A. tumefaciens* strain GV3101 grown on YEB-agar plate containing 100µg/ml rifampicin (Rif) and 25µg/ml kanamycin (Km) (YEB-Rif-Km) was inoculated in 5 ml of YEB-Rif-Km medium in a 100 ml Erlenmeyer flask and incubated at 28°C for two days with shaking (250 rpm). 1 ml of the culture was transferred into 100 ml of YEB-Rif-Km medium and cultivated at 28°C for 15-20 h with shaking (250 rpm) until the OD600nm reached 1-1.5. The cells were chilled on ice for 15 min and spun down by centrifugation (4,000g/4°C/5 min). The culture medium was decanted and the cells were washed three times with 10 ml of dH_2O by centrifugation and resuspended in 500 µl of sterile 10% (v/v) glycerol. 45 µl-aliquots of the suspension were dispensed into prechilled microcentrifugation tubes, frozen immediately in liquid nitrogen and stored at -80°C.

YEB-Rif-Km medium:

Nutrient Broth	0.5% (w/v)
Yeast Extract	0.1% (w/v)
Peptone	0.5% (w/v)
Sucrose	0.5% (w/v)

2 mM MgSO4, 100 µg/ml rifampicin, 25 µg/ml kanamycin were added after autoclaving and cooling.

II.2.1.6 Transformation of *Agrobacterium* by electroporation

0.2-1.0 µg of plasmid DNA (II.2.1.9) in sterile dH_2O was added to a thawed aliquot of electrocompetent *Agrobacterium* cells (II.2.1.5) and incubated on ice for 3 min. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of SOC medium in a 4.0-ml tube and incubated at 28°C with shaking (250 rpm) for 1 h. Finally, 1-10 µl of the cells were plated on YEB-agar containing 100 µg/ml rifampicin (Rif), 25 µg/ml kanamycin (Km) and 100 µg/ml carbenicillin (Carb) (YEB-Rif-Km-Carb) and incubated at 28°C for 2-3 days. As a control transformation of *Agrobacterium* cells with H_2O was performed.

II.2.1.7 Culturing of *E. coli* and glycerol stock preparation

Individual colonies of all strains were obtained by plating the pertaining strain on LB agar plates. Strains carrying an F′ factor were spread on M9 plates. Incubation was performed at 37°C. The plates were stored at 4°C for short periods (less than 2 weeks). LB medium containing the suitable antibiotics and 2% glucose was inoculated with a single recombinant colony of *E. coli* and grown o/n at 37°C with vigorous shaking (225 rpm). Glycerol stocks were prepared by mixing 600 µl of a fresh overnight culture with 600 µl of 40% (v/v) sterile glycerol. Bacteria glycerol stocks were stored at -80°C.

II.2.1.8 Growth of recombinant *A. tumefaciens* and preparation of glycerol stocks

Single colonies of *A. tumefaciens* were examined for the presence of plasmids by control PCR (II.2.1.12). Positive colonies were inoculated in 10 ml of YEB-Rif-Km-Carb medium and cultivated at 28°C for 2-3 days with vigorous shaking at 250 rpm. The culture was transferred to Falcon tubes and *Agrobacteria* cells were pellet by centrifugation at 4000g for 10 min at 15°C. The cells were resuspended in a 1:1 volume of YEB Rif-Km-Carb medium and Glycerol 50% (v/v). The suspension was aliquoted (100µl) and stored at –80°C for further experiments.

II.2.1.9 Isolation of plasmid-DNA from *E.coli*

Recombinant plasmid DNA was purified with the Qiagen plasmid DNA Mini-and Midiprep kits according to the manufacturers' instructions based on the alkaline lyses method

(Sambrook et al., 1996). Quality and yield of plasmid DNA was examined by reading the absorbance at 260 nm and 280 nm in a spectrophotometer according to (Müller et al., 1993); (Sambrook et al., 1996). The integrity of DNA was verified by a control restriction digest followed by agarose gel electrophoresis (II.2.1.10). Isolated plasmid DNA was stored at -20°C.

II.2.1.10 Agarose gel electrophoresis of DNA

Plasmid DNA and PCR-fragments were separated in 0.8-1.2% (w/v) agarose gels. Preparation of agarose gels and electrophoresis of the samples were carried out as described by (Sambrook et al., 1996). Ethidium bromide was added to the gel solution and TBE electrophoreses running buffer prior to the experiment. Known amounts of DNA molecular markers such as 1 Kb ladder, 100 bp ladder and λ-digested with *Pst*I were used for evaluation and determination of DNA concentration and size. The DNA bands were visualised directly upon illumination with a UV transilluminator at 302 nm. Documentation of the DNA gels was performed by using a black and white E.A.S.Y 429K camera (Herolab) and a photo printer (Mitsubishi).

II.2.1.11 Preparative agarose gel electrophoresis

Preparative gel electrophoresis was used for large scale purification of a particular DNA fragment from a mixture of DNA fragments after restriction enzyme digestion. The agarose containing the DNA fragment of interest was excised from the gel on an UV transilluminator with a sterile scalpel. The DNA extraction was performed with QIAquick Gel extraction kit according to the manufacturer's guidelines. The concentration of recovered DNA was measured by spectrophotometer and /or determined by agarose gel electrophoresis (II.2.1.10) and was used in further experiments.

II.2.1.12 PCR amplification

Polymerase chain reaction (PCR) was used for amplification of different genes using synthetic oligonucleotides. DNA was amplified, based on the protocol of (Sambrook et al., 1996), with *Taq* DNA polymerase and DNA polymerase buffer from Roche. The reactions were performed in 0.2 ml PCR reaction tubes (Biozym Diagnostik GmbH, Hessisch Oldendorf), using a DNA thermal Cycler (MWG). The cycler contained a heated lid to avoid the use of mineral oil.

For rapid identification of recombinant *E. coli* and *Agrobacteria* control-PCR was carried out to detect plasmids as described by (Jesnowski et al., 1995).

PCR reactions were carried out in a total volume of 50 µl as described below:

Components	Volume	Final concentration
10X PCR buffer	5 µl	1X
50 mM $MgCl_2$	1.5 µl	1.5 mM
10 mM dNTPs	1 µl	0.2 mM each
10 pmol forward Primer	0.5-1 µl	10 pmol
10 pmol backward primer	0.5-1 µl	10 pmol
Template DNA	0.5-5 µl	10-100 ng
Taq DNA polymerase (5U/µl)	0.25 µl	1.25 units
dd H_2O	To 50 µl	

Amplification was carried out under the following conditions:

10 min 95°C
1 min 94°C
1 min 55°C } x 20-35
1 min 72°C
10 min 72°C

The annealing temperature and the time for denaturation were experimentally optimized.

The performance of each PCR reaction was checked by running 5µl of each reaction on agarose gels (II.2.1.11), with appropriate DNA markers.

II.2.1.13 DNA sequencing

Fluorescently labeled primers were used for sequence analysis by chain terminating inhibitors (Sanger et al., 1977) using the "Thermosequenase sequencing kit'' and the LICOR 4200 IR2 automated DNA sequencer. For evaluation of sequencing data the Base ImageIR 4.0 software package was used.

pGEX forward and pGEX backward primers were used for sequencing the viral genes genes cloned in the pGEX-5X-3 vector. Forward and backward pHEN primers were used for sequencing of scFv-fragments in the pHEN4II phagemid vector. pSS 5` and pSS 3` were used for sequence analysis of genes in pTRA vector.

II.2.1.14 Sequence analysis

Chromas and Bioedit software packages were used for displaying the chromatogram files from LI-COR automated DNA sequencer and ABI Sequencer, respectively.

II.2.1.15 *In vitro* site-directed mutagenesis

QuikChange® II and QuikChange® II XL Site-Directed Mutagenesis Kits (Stratagene) were used for DNA mutation according to the manufacturer's protocols.

The QuikChange® II kit was used with small plasmids (pHEN and pIT2). The sample reaction was prepared as follow:

Components	Volume	Final concentration
10X PCR buffer	5 µl	1X
dsDNA template	*X* µl	5–50 ng
10 mM dNTPs	*1* µl	0.2 mM each
oligonucleotide primer #1	*X* µl	125 ng
oligonucleotide primer #2	*X* µl	125 ng
PfuUltra HF DNA polymerase 2.5 U/µl	1 µl	2.5 units
dd H_2O	To 50 µl	

Amplification was carried out under the conditions described in table 3.

Table 3: Cycling Parameters for the QuikChange II Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12	95°C	30 seconds
		55°C	1 minute
		68°C	4 minutes

QuikChange® II XL was used with pTRA vector. The sample reaction was prepared as follow:

Components	Volume	Final concentration
10X PCR buffer	5 µl	1X
dsDNA template	*X* µl	10 ng
10 mM dNTPs	*1* µl	0.2 mM each
oligonucleotide primer #1	*X* µl	125 ng
oligonucleotide primer #2	*X* µl	125 ng
QuikSolution	3 µl	
PfuUltra HF DNA polymerase 2.5 U/µl	1 µl	2.5 units
dd H_2O	To 50 µl	

The reaction was cycled using the cycling parameters outlined in table 4.

Table 4: Cycling Parameters for the QuikChange II XL Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	10 minutes
3	1	68°C	7 minutes

After the DNA amplification (for both kits), 1 µl of the *Dpn* I restriction enzyme (10 U/µl) was added directly to the amplification reaction and gently and thoroughly mixed. The reaction was then incubated at 37°C for 1 hour to digest the parental (i.e., the non-mutated) supercoiled dsDNA. 2µl were then used for transformation into XL10-Gold competent cells (II.2.1.2).

II.2.2 Transient expression assay in tobacco leaves

II.2.2.1 Preparation of recombinant *Agrobacteria*

10 ml of YEB-Km-Rif-Carb medium was inoculated with 50 µl of glycerol stock of the selected recombinant *Agrobacteria* carrying a plant expression vector. The culture was grown at 28°C o/n with shaking at 250 rpm. Next day the cells were diluted 1:10 in YEB-Km-Rif-Carb medium containing 10mM MES and 20µM Acetosyringone and cultivated at 28°C o/n with shaking at 250 rpm. *Agrobacteria* cells were centrifuged (4000g/15-25°C/15 min) and

resuspended in 10 ml of MMA solution and kept at RT for 2 h. The OD600nm was measured after 1:10 dilution and the cell suspension was adjusted to an OD600nm of 0.8-1. The diluted cell suspension was used for tobacco leaves infiltration of plant leaves (II.2.2.2).

MMA solution:

$MgCl_2$	2 mM
MES	10 mM
Acetosyringone	150 µM

II.2.2.2 Infiltration of tobacco leaves

Transient expression in *N. benthamiana* leaves was achieved by infiltration of the recombinant *Agrobacteria* to the underside of a leaf using a syringe without a needle. Tobacco plants were then kept for 2-3 days under the following conditions: Photoperiod 16h, light intensity 400 uE m^{-2} s^{-1}, 25°C and 70-90% humidity.

II.2.2.3 Particle bombardment

The biolistic gold particles were prepared as follow:

1ml ethanol was added to 50 mg of microcarrier (1 micron) gold in a 1.5 ml Eppendorf tube and mixed end-over-end for 1 h. The gold was pelleted by spinning at 2000 rpm for 2 mins, then resuspend in 100% ethanol. The washing was repeated 5 times with 100% ethanol and once with with dH_2O. Finally, the gold pellet was resuspended in 1 ml of 95% (v/) ethanol and stored at –20C.

For DNA transformation by particle bombardment, 10µl ethanol was added slowly over 4µl of highly concentrated plasmid DNA (0.8-1.2 ng/µl) (II.2.1.9). Then 22µl of the gold particles preparation was added and mixed by pipeting. 8-10µl of the mixture was used for three shots as described by Gal-On (1997) using 13-mm Plastic Swinney Filter holder, PALL Gelman Laboratory, Ann Arbor, MI.

II.2.2.4 Confocal microscopy

The infiltrated tobacco leaves (II.2.2.2) were checked for GFP, DsRed, or DAPI fluorescence using Leica TCS SP spectral confocal laser-scanning microscope. To locate nuclei precisely, leaf tissues were mounted in a buffer containing 49,69-diamidino-2-phenylindole (DAPI) and the same cells were excited at different wavelengths to visualize for GFP, DsRed, or DAPI fluorescence. GFP was excited at 488 nm and emissions collected at 500 to 515 nm. DsRed

was excited at 568 nm and emissions collected at 600 to 620 nm. For DAPI imaging, excitation at 405 nm and collection between 449 and 461 nm were used.

II.2.3 Expression and purification of recombinant proteins

II.2.3.1 Expression and purification of GST-AC1/AV1/AVNS fusion proteins from *E. coli*

GST-fusion proteins were expressed and purified according to a modified protocol based on (Smith, 1993). A freshly transformed single colony of *E. coli* strain BL21(λDE3) (Novagen) harbouring recombinant plasmid DNA was inoculated in 10 ml of 2YT medium containing 1% (w/v) glucose and 100 μg/ml ampicillin and cultivated o/n at 37°C with vigorous shaking. The following day 1L of fresh 2YT/0.1% (w/v) glucose media were inoculated with 10 ml overnight culture and grown at 30°C and 225 rpm to an OD 600 nm of 0.6-0.8. Expression of recombinant proteins was then induced by addition of IPTG to a final concentration of 0.4 mM. Cells were cultured o/n at 18-22°C. The cells were harvested by centrifugation (15 min/5000 g/4°C) and the supernatant was discarded. The pellet was resuspended in cold PBS buffer (20 ml/400 ml bacterial culture) and lysozyme was added to final concentration of 0.2mg/ml. After 30 minutes incubation at 4°C with gentle stirring, the cells were additionally broken by sonication on ice 4 times for 40 sec (150W with 30 sec intervals). Triton X-100 (20% (v/v) stock solution) was added to a final concentration of 1% (v/v). Cell debris and insoluble components were removed by centrifugation (20 min/15000g/4°C) and the supernatant subjected to glutathione affinity chromatography according to the manufacturers instructions (Amersham Pharmacia Biotech). The purified fractions were immediately stored at -20ºC until before use.

II.2.3.2 Expression and purification of MBP-AC1/AV1 fusion proteins from *E. coli*

A freshly transformed single colony of *E. coli* strain BL21(λDE3) (Novagen) harbouring recombinant plasmid DNA was inoculated in 10 ml of 2YT medium containing 1% (w/v) glucose and 100 μg/ml ampicillin and cultivated o/n at 37°C with vigorous shaking. The following day 1L of fresh 2YT/0.1% (w/v) glucose media were inoculated with 10 ml overnight culture and grown at 37°C and 225 rpm to an OD 600 nm of 0.5-0.8. Expression of recombinant proteins was then induced by addition of IPTG to a final concentration of 0.3

mM. Cells were cultured o/n at 30°C. The cells were then harvested by centrifugation (15 min/5000 g/4°C) and the supernatant was discarded. The pellet was resuspended in cold column buffer and frozen o/n at -20°C. The samples were thawed in cold water and sonicated in short pulses of 15-20 sec. The crude extract (supernatant) was obtained by centrifugation at 9000 x g for 30 minutes. 2ml amylose resin was added to the supernatant and the sample was incubated for 1h with a shaking at 4°C. The sample was then centrifuged at 5000 x g for 5 minutes, the supernatant was removed and the resin was washed twice with 25ml column buffer. To elute the bound proteins, 1ml 100mM-maltose containing column buffer was added to the resin and incubated 10 minutes at 4°C with gentle shaking. The eluted protein was collected by centrifugation 5 minute at 5000 x g and immediately stored at -20ºC till the usage time.

Column buffer B: 0.2 M NaCl; 0.02 M Tris HCl pH 7.4; 1 mM EDTA; 1 mM DTT

II.2.3.3 Large scale expression and purification of scFvs by IMAC

Recombinant pHEN and pIT2 plasmids were transformed into *E. coli* strain HB2151 (TG1 in case of scFv-SCRs-AV6). A single recombinant colony was inoculated in 5 ml of 2YT medium containing 1% (w/v) glucose and 100 μg/ml ampicillin (2YTGA) and cultivated o/n at 30°C with shaking (200 rpm). The o/n culture was diluted by 50 ml 2YTA 0.1% (w/v) glucose and grown for two hours at 30°C. The cells were then sediment by centrifugation at 5000 rpm for 20 minutes at 4°C and resuspended in 200 ml 2YTA containing 1mM IPTG and incubated o/n at 30°C with shaking (200 rpm). The culture was then centrifuged (4000 g/4°C/20 min) and the supernatant (S1) was kept on ice. The pelleted bacteria were resuspended in 10 ml ice-cold PBS buffer containing 1mM EDTA. The suspension was incubated at 4°C for 15-30 min followed by centrifugation at (4000rpm/4°C/40 min). The supernatant (S2) was mixed with the first supernatant (S1) and the proteins were precipitated by adding solid ammonium sulphate (30g/100ml supernatant) and stirring for a few minutes. The precipitated proteins (periplasmic extract) were collected by centrifugation at (4000rpm/4°C/40 min) and the pellet was resuspended in 1-2ml ice-cold PBS and dialysed against PBS prior to IMAC affinity purification.

Ni-NTA-agarose was added in a disposable column and equilibrated with 10-20 volumes of PBS. Imidazol was added into the dialyzed periplasmic extract to a final concentration of 10 mM. The sample was incubated with the washed Ni-NTA-agarose for 1h at 4°C with gentle rotation. Bound proteins were collected by centrifugation at (3000rpm/4°C/5 min) and

washed three times with 20mM-containing PBS. The bound protein was eluted with 200mM-containing PBS. The collected fraction was loaded into PBS pre-equilibrated PD-10 desalting column and the scFv was eluted with 10xs 500µl PBS. The OD A_{280} of the collected fractions was measured with spectrophotometer to estimate the protein concentration. The positive fractions were combined and freeze-dried.

II.2.4 Protein analysis

II.2.4.1 Protein quantification

Visual comparison with a purified protein of known concentration in Coomassie-stained gel (II.2.4.2) and/or Western blot (WB) **(**II.2.4.3) was used to estimate the concentration of purified proteins. Additionally, the OD A_{280} of the purified proteins was measured with spectrophotometer to estimate the protein concentration.

II.2.4.2 SDS-PAA gel electrophoresis and Coomassie brillant blue staining

Discontinuous SDS-polyacrylamide gels (for the stacking gel: T = 4%, C = 2.6%, pH 6.8; for the separating gel: T = 12%, C = 2.6%, pH 8.8) (Ausubel et al., 1995) were used for separation of protein samples. Before loading onto the gel, protein samples were denaturated in the presence of SDS and β-mercaptoethanol. The proteins were separated electrophoretically with 20V/cm for 1 hour. Protein bands were revealed by staining with Coomassie brilliant blue or transferred to nitrocellulose membrane for immunoblot analysis(Ausubel et al., 1995). Proteins were detected after incubating the gel for 1h in Coomassie staining solution at RT under constant rocking. Coomassie dye was removed by adding destaining solution until the protein bands were clearly visible.

SDS-PAGE running buffer (pH 8.3):

Tris	125 mM (w/v)
Glycine	960 mM (v/v)
SDS	0.5% (w/v)

Coomassie staining solution:

Coomassie brillant blue G-250	0.25% (w/v)
Methanol	50% (v/v)
Glacial acetic acid	9% (v/v)

Coomassie destaining solution:

Methanol	10% (v/v)
Glacial acetic acid	10% (v/v)

II.2.4.3 Immunoblot analysis (WB)

Separated proteins (II.2.4.2) were transferred from an SDS-PAA gel to PVDF or HybondTM-C nitrocellulose membrane (0.45 µm). After blotting, the membrane was blocked with PBS buffer containing 3-4% (w/v) skimmed milk powder (MPBS). As primary antibody either anti c-myc, anti-His, anti GST, or anti MBP was used in a dilution recommended by the manufacturer in 1xPBS. Attachment of the primary antibody was detected by addition of the secondary polyclonal antibody coupled to alkaline phosphatase (AP). The target protein was finally revealed by addition of substrate BCIP/NBT.

PBS buffer (pH 7.3):

NaCl	137 mM
KCl	2.7 mM
$Na_2HPO_4x2H_2O$	8.1 mM
KH_2PO4	1.5 mM

Transfer buffer (pH 8.3):

Tris-HCl, pH 8.3	25 mM
Glycine	92 mM
Methanol	20% (v/v)

II.2.5 Immunization of mice

The treatment and maintenance of laboratory animals was approved by the 'Regierungspräsidium des Landes NRW' (RP-Nr.: 23.203.2 AC 12, 21/95). Two female mice (Balb/c) each were immunized with 100 µg of GST-AC1 and GST-AV1 fusion proteins mixed with 50µl GEBRU's adjuvant. Three further 50µg injections into the tail vein were given at biweekly intervals with 20µl GEBRU's adjuvant per mouse. The final boost was performed 4 days prior to sacrifice with concentration of 30µg. In case of GST-AV1, another boost of 30µg was given to reach a good titer (II.2.5.1). After the final boost, blood was taken from the tail vein using a 26 gauge needle (1-2 µl) and a capillary for picking up blood. The blood was diluted up to 1000-fold with PBS for determination of antibody titers.

II.2.5.1 Determination of antisera titers by ELISA

The determination of polyclonal antibody titers from sera of mice was performed by direct ELISA (II.2.7.1) using GST-AC1, GST-AV1 and GST proteins as antigens/controls. To determine the titer of AC1/AV1-specific antibodies, 10µg/ml of GST-AC1/GST-AV1 fusion proteins were coated onto ELISA plates. GST (10 µg/ml) was included as a control. Antigens

were coated at 37°C for 2 hours and blocked with 4% (w/v) skimmed milk in 1xPBS. Serial dilutions of sera (1:500-1:256000) in 1xPBS were added to the coated plates and incubated at 37°C for 2 hours. After three washes with PBS-T, bound antibodies were detected by addition of 1:5000 diluted GAM^{AP} polyclonal antibodies in blocking buffer and p-nitrophenyl phosphate (pNPP) as substrate (Sigma). ELISA plates were incubated at 37°C for 20-60 minutes followed by measurement of the OD at 405 nm.

Additionally, the GST binders were adsorbed by incubation of the undiluted antisera with high concentration of GST protein (~20µg GST/ml 1:1000 diluted antisera) for 1 hour at room temperature. The pre-adsorbed antisera were then used in ELISA.

II.2.5.2 Isolation of mouse spleen and spleen cell preparation

The immunized (II.2.5.1) mouse was sacrificed with Isofluran, sterilized with 70% (v/v) ethanol and then dried with some paper towels. The isolation of the spleen was performed after cutting the pelt at the abdominal wall .

II.2.6 ScFv phage libraries

II.2.6.1 Isolation of total RNA from spleen cells

Spleens from immunised mice (II.2.5) were removed and dissected. Spleenocytes were prepared by disrupting the spleen through a meshing net and washed twice with RPMI 1640. The cells were homogenised in 1xPBS, aliquoted to $1x10^8$ cells and subjected to total RNA isolation using the 'RNeasy Midi kit' followed by a TRIZOL extraction (GibcoBRL) to obtain high yield and pure RNA.

II.2.6.2 First strand cDNA synthesis

First-strand cDNA was synthesised from 5 µg of total RNA using 'Superscript™ preamplification system kit' (GibcoBRL) and Oligo dT primers. cDNA synthesis was performed according to the manufacturers instructions.

II.2.6.3 Construction of scFv libraries

Mouse heavy and light chain variable regions were amplified by PCR using SOE phage display primers (II.1.8). For each forward primer separate PCR reactions were performed whereas backward primers were used as a cocktail. PCR reactions were carried out in a total volume of 50 µl by adding 10 pmol of each primer(s) and ~1/10 of the first strand cDNA

reaction under the following conditions: initial denaturation at 95°C for 5 minutes followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final elongation for 10 min at 72°C.

Heavy (V_H) and light (V_L) chain fragments were gel purified using "QIAquick gel extraction kit" (Qiagen) (II.2.1.10) and joined together by SOE reaction. The joined H & L chains (scFvs) were amplified by PCR. The following program was used for performing the SOE and the PCR:

For SOE reaction the following reaction mixture (RI) was prepared:

Components	Volume	Final concentration
10X PCR buffer	2.5 µl	1X
50 mM $MgCl_2$	1.25 µl	2.5 mM
10 mM dNTPs	1 µl	0.2 mM each
Amplified V_H	3 µl	
Amplified V_L	3 µl	
Taq DNA polymerase (5U/µl)	0.25 µl	1.25 units
dd H_2O	To 25 µl	

SOE reaction was carried out under the following conditions:

10 min 95°C

1 min 95°C
1.5 min 60°C } x 7
1.5 min 72°C

Pause till the reaction mixture RII is added 60°C.

The following mixture (RII) was immediately added:

Components	Volume	Final concentration
10X PCR buffer	2.5 µl	1X
50 mM $MgCl_2$	1.25 µl	2.5 mM
10 mM dNTPs	1 µl	0.2 mM each
mV_H 1-12 SFi Back primers mixture	2 µl	
m$V_{K/\lambda}$1-4 Not For primers mixture	2 µl	
Taq DNA polymerase (5U/µl)	0.25 µl	1.25 units
dd H_2O	To 25 µl	

The annealed VH and VL (scFvs) were amplified under the following conditions:

1 min 95°C
1.5 min 61°C } x 35
1.5 min 72°C
10 min 72°C

The amplified scFvs and the pHEN4II phagemid DNA were digested with *Sfi*I and *Not*I enzymes and gel purified. 200 ng of the purified vector were ligated with five fold molar excess of purified scFvs. Ligation products were electroporated into electrocompetent *E. coli* XL1-blue cells to create the scFv library.

II.2.6.4 *Bst*NI fingerprinting

The variability of the generated scFv libraries was tested by restriction analysis with *Bst*NI. PCR-amplified scFv fragments from 20 randomly selected *E. coli* colonies were digested with 5 U *Bst*NI at 60^{o}C for 2 h. This method was first described in phage display protocols by (Marks et al., 1991) and Hoogenboom et al. (1996). Digested fragments were separated in a 2% (w/v) agarose gel (II.2.1.10) and the variability of the library was estimated.

II.2.7 Phage display

II.2.7.1 Helper phage preparation

Home-made helper phage was used for screening of Tomlinson I library. The helper phage was obtained as follow:

A single colony of the *E. coli* strain TG1 was inoculated in 5ml of 2xYT medium and cultured o/n at 37°C. 200µl of fresh o/n culture was transferred into 5 ml of 2xYT. The cells were cultured at 37°C for 2-3 hours until the mid-log phase (OD_{600nm} = 0.5-0.8). 5µl helper phage was added to 1ml cell culture and kept 30 min for infection at 37°C without shaking. Then the infected cells were diluted in 100ml 2xYT containing 70µg/ml kanamycin and cultivated o/n at 37°C. The culture was centrifuged (4000g/30 min) and the cells were discarded. 20ml of PEG/NaCl (20M PEG6000, 2.5M NaCl) was added to the supernatant and incubated 1h on ice to precipitate the phages. The phages were then harvested by centrifugation (4000g/30 min) and the pellet was resuspended in 1ml dH_2O. The precipitation step was repeated using 200µl PEG/NaCl and 20 min incubation on ice. The pellet was

resuspended this time in PBS and spun down at high speed to remove any remaining cell debris. Phage titers were determined by addition of phage dilutions to exponentially growing *E. coli* TG1.

II.2.7.2 Phage displayed scFv selection

Phage particles were rescued from the scFv-libraries by super infection with helper phage M13KO7 (Clackson et al., 1991). Phage titers were determined by addition of phage dilutions to exponentially growing *E. coli* TG1/XL1-blue. For isolation of phages exposing antigen specific antibodies, panning procedures were performed. GST/MBP-AC1/AV1 fusion proteins (~10µg/ml) were immobilized o/n to immunotubes. After 2h blocking with PBS, 4% (w/v) skimmed milk, the phage solution was added to the antigen-coated immunotubes and incubated for 1.5h under rotation on an under-and-over turntable and then 2 h without rotation. Phages that showed no or low affinity for the immobilized antigen were washed away by PBS containing 0.05% (w/v) Tween20 followed by PBS. Each washing step was performed 20 times by filling the tube and decanting immediately. Phages with affinity to the antigen were eluted from the tube by addition of 1 ml of 100 mM triethylamine (freshly prepared) with rotation on an under and over turntable for 8-10 min followed by neutralisation with 1 M Tris-HCl pH 7.4. Nine ml of log phase *E. coli* TG1/XL1-blue cells were infected with eluted phages and plated on 2x YT agar plates containing 1% (w/v) glucose and 100 µg/ml ampicillin (2x YTGA-agar). The plates were incubated o/n at 37^{o}C. Cells were scrapped off the agar by adding 5 ml of 2x TY medium containing 15% (v/v) glycerol and stored at -80^{o}C for a new round of selection.

The total eluted phage titer indicating the successful binding and elution of phages, was determined after each round of panning by addition of dilutions to exponentially growing *E. coli* TG1/XL1-blue. An increasing titer of eluted phages in subsequent round of panning indicated the enrichment for clones, which bind most strongly to the target antigen.

II.2.8 Cloning of scFv against AV1 from hybridoma cells

II.2.8.1 Hybridoma fusion

Two ml myeloma cells were diluted in 10ml fresh medium. Three dilutions were performed in three days (once a day) prior to fusion to maintain the cells in their logarithmic growth phase.

The spleen of the immunized mouse was cut into small pieces with a sterile scissor and minced with the pistil of a 5ml syringe through a fine mesh into a sterile petri dish with 30ml of RPMI medium. The cells were pipetted carefully up and down and transferred to a 50 ml Falcon tube. After 5 min centrifugation at 300 x g, the spleen cells and the myeloma cells were resuspended each in 30ml RPMI medium and centrifuged again.

The spleen cells were mixed with the myeloma cells at a ratio of 4:1, (1×10^8 with 3×10^7) and the suspension was centrifuged at 300 x g for 5min. The resulting pellet was broken up by tapping and 1ml prewarmed PEG 1500 (Roche) was carefully added drop by drop over 60sec to the cells while rocking the tube. The cells were incubated for 90sec in a 37°C water bath and then 1ml of equilibrated RPMI medium was added over 60sec. 3ml medium was added over 3min and finally, 10 ml medium was added over 3min to the cells. All previous steps were performed while rocking the tube. The cells were then incubated for 5min in the incubator, centrifuged at 300 x g for 5min, resuspended in 2ml HAT-medium, transferred to 60ml HAT-medium, and plated on 96well tissue culture plates with 120µl/well.

R10 (Complete medium)

RPMI 1640 (Cytogen)	500ml
FCS (Cytogen)	10%
L-Glutamin (Gibco)	2mM
β-Mercaptoethanol	50µM
Penicillin/Streptomycin	100µg/ml

HAT/HT Medium

Hypoxanthin (Sigma)	100µM
Aminopterin (Sigma)	10µM
Thymidin (Sigma)	16µM

Complete Medium

II.2.8.2 Selection of hybridoma clones secreting specific MAbs

7 days after fusion (II.2.8.1) the plates were fed by aspirating most of the culture medium from the wells and replaced with 200µl/well of fresh HAT-medium. The clones were screened for selection of positive hybridomas two weeks after fusion, using direct ELISA (II.2.9.1). Selection and isolation of single cells secreting Mabs against AV1 from the positive hybridomas was carried out by limiting dilution. This was done as follow:

- a 96 well plate was prepared with 100µl HT-medium/well, (LD plates)

- 50µl of the ELISA positive hybridoma cells from the fusion plates were mixed with 100µl of HT-medium from the first well of the first column of the LD plate
- 50µl out of the first well was mixed with 100µl of HT-medium from the second well of the first row and so on (dilution of 1:2 in each well)
- Then 100µl of HT medium was added to each well of the first column with a multichannel pipette, mixed and 100µl of cell suspension was mixed with the HT-medium from wells of the next row. The same procedure was done for the third column
- With this method four clones can be diluted on one 96well plate.
- The plate was incubated at 37^{o}C in 5% CO_2 for 4-7 days without changing the medium. The growth of isolated clones of hybridoma cells was checked by light microscopy.
- The supernatants from all wells that contain one hybridoma clone/well were assayed for production of the desired antibody specificity by direct ELISA (II.2.10.1). Positive clones were then transferred into larger volumes of medium (24-well plates, 25 cm^2 flasks, etc.).
- A second cloning, (third, fourth) by limiting dilution of putative clones was performed to ensure clonality.

II.2.8.3 Purification of mouse mAb from the hybridoma supernatant

ELISA positive hybridoma clones were grown in 500 ml tissue culture flasks. The culture supernatant was harvested when the medium became acidic. The culture supernatant was collected over a period of 2 weeks. The storage bottle for collection of the cell supernatant was always handled in sterile conditions to avoid any contamination.

The mouse Mab was purified from the hybridoma supernatant via Protein A chromatography. 1.6 ml of Protein A matrix was packed in a column and equilibrated with 5 column volumes (CV) of PBS (pH 7.4). The hybridoma supernatant was centrifuged (10000 rpm/10 min/4°C) and filtered through Whatman 3M paper before applying it onto the column. The supernatant was passed through the column at a flow rate of 5 ml/min. Non-specifically bound proteins were washed away with PBS containing 100 mM NaCl (pH 7.4). The bound antibody was eluted from the column with 4 ml of elution buffer (100 mM glycine, pH 3) under gravity flow. The pH of the eluate was immediately adjusted to pH 7 by adding unbuffered 1 M Tris

pH 11. 0.01% (w/v) of sodium azide and 10% glycerol (v/v) were added to the eluate. The eluate was aliquoted and frozen at –20°C. The Protein A matrix was regenerated by washing the column with 2 column volumes of 1.5 M HCl and stored in 20% (v/v) ethanol at 4°C. 1-10 microlitres of the eluate were used for ELISA (II.2.9.1), WB (II.2.4.3) and SDS-PAGE analysis (II.2.4.2).

II.2.8.4 Construction of scFv mini library

Starting from the hybridoma cells, a scFv mini library was constructed using the same strategy used for scFv phage library (II.2.6).

II.2.9 Characterisation of scFv fragments

II.2.9.1 Soluble expressions of scFv fragments and direct ELISA

Screening of scFv-fragment libraries was performed after the third/fourth round of panning by small scale induction of scFv expression from pHEN4II or pIT2 phagemid vectors in ELISA plates.

120 recombinant clones of *E. coli* strain TG1/XL1-blue were randomly selected and inoculated in 100µl of 2xTY, 100µg/ml ampicillin, 1% (w/v) glucose in microtiter plates. The plates (master plates) were grown at 37^{o}C overnight. The next day, cells were transferred from the master plate to a second plate containing 125 µl 2x TY, 100 µg/ml ampicillin, 0.1% (w/v) glucose. Bacteria were grown at 37^{o}C for 2-3h. Soluble scFv fragment expression was induced at 30^{o}C for 16-24 h by addition of IPTG to a final concentration of 1 mM. The cells were removed by centrifugation and the supernatant was used for soluble ELISA. A high binding ELISA plate was coated with antigen (10µg/ml) o/n at 4^{o}C. After blocking with 2% (w/v) MPBS the supernatant was applied to the plate and incubated for 2 h at RT. Bound scFv fragments were detected using 1:5000 diluted anti c-myc 9E10 and/or 1:5000 diluted anti-His monoclonal antibodies as primary antibodies and 1:5000 diluted GAM^{AP} polyclonal antibody. ELISA readings were performed at OD405 nm after incubation at 37^{o}C with pNPP for 1 h.

II.2.9.2 Characterisation of Mab and scFv fragments by BIACORE

Biomolecular interaction analyses of binding of the purified AV1-specific Mab/scFvs to MBP-AV1 antigen were done by surface plasmon resonance (SPR) on a BIAlite™

(BIACORE). All injected samples were dialysed and diluted in HBS buffer (10 mM HEPES, 150mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Surfactant P20) and subjected to centrifugation prior to injection to remove insoluble components.

The ligands were immobilized on a CM3 sensor chip using the amine coupling kit (BIACORE). The immobilization of proteins on the chip was performed at a flow rate of 15µl/min. The carboxyl groups on the sensor surface were activated with an injection of a solution containing 100 mM EDC/NHS (N-Ethyl-N'-(Dimethylaminopropyl) - Carbodimide-Hydrochloride, 400 mM N-Hydroxyl-Succinimide). After each binding experiment the surface was regenerated with 15-20µl of 1.2 M Guanidine HCl, pH: 1.5-2. The data were analysed using the BIAevaluation (4.1) software.

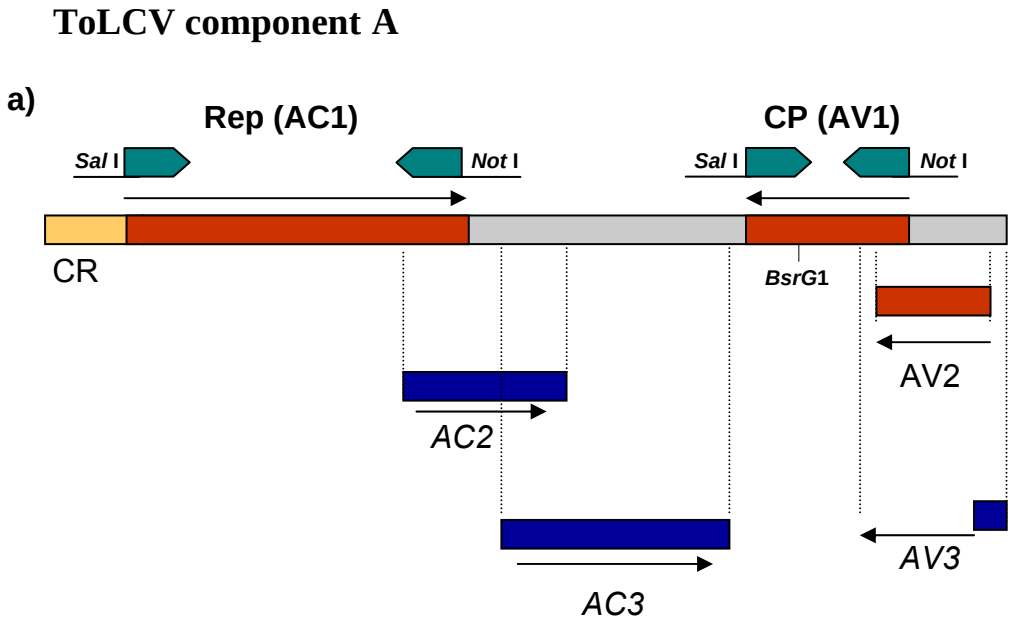
III Results

III.1 Cloning and expression of the viral genes

III.1.1 Cloning and expression of the viral genes as GST fusions

The AC1 and AV1 viral genes were amplified from a full length cDNA clone (Fig. 4; a) of ToLCV-India-A {kindly provided by Dr. Zehr, Maharashtra Hybrid Seed Company (MAHYCO), India}. Primers (II.1.8) were designed to amplify AC1 and AV1 genes to introduce the restriction sites *Sal*I and *Not*I at the 5` and 3` ends of the amplified genes for subcloning, respectively. The AC1 (~1.1 kb) and AV1 (~0.8 kb) PCR products (Fig. 4; b) were fused via *Sal*I/*Not*I cloning to the C-terminus of GST gene in pGEX5x-3 vector (Amersham/Pharmacia). The resulting constructs pGEX-AC1 and pGEX-AV1, were confirmed by RE-digesting and sequencing.

Additionally, the N-terminal part of AV1 (54aa) that carries the nuclear localization signal (NLS) was cloned as fusion with GST by removing the C-terminal part of AV1 in pGEX-AV1. This was achieved by *BsrG*1/*Not*I digestion, generating blunt-ends using exonuclease and subsequent ligation. The resulting plasmid was named pGEX-AVNS.



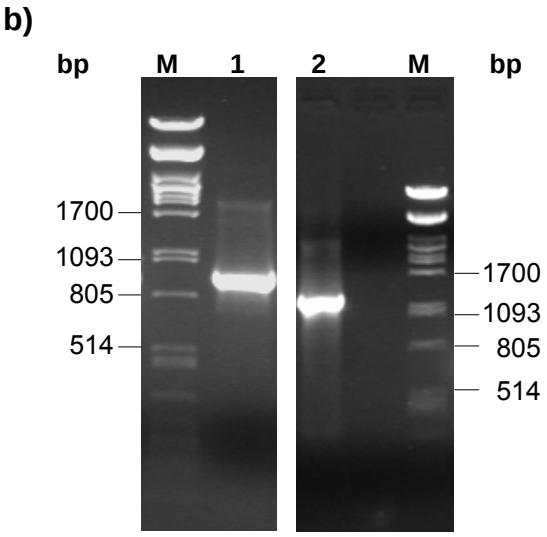


Fig. 4. Cloning the viral genes as fusions with GST. a: linear representation of the component A of ToLCV-India genome depicting the genes of interest and the primers used for cloning into the pGEX expression system. The Viral genes and their transcription direction are shown. The primers with the cloning sites are shown above the two genes of interest (AC1 and AV1). b: Agarose-gel electrophoresis (II.2.1.10) of the PCR-product showing the AC1 (~1100bp) (1) and AV1 (~800bp) (2) PCR products. M: λPst DNA marker.

The expression / purification protocol (II.2.3.1) of the GST-fusion proteins was carried out according to the manufacturers' instructions with the following modifications to increase the solubility and the yield. The growth and induction temperatures were lowered to 28°C and 18-20°C, respectively. The IPTG concentration was reduced to 0.4-0.5 mM with 14-16 h induction time. The yield was around 1-1.5mg per litre culture medium for both GST-AC1 and GST-AV1 gene products and up to 5mg/L for GST-AVNS (Fig. 5). GST-AC1 was further purified by size exclusion chromatography to remove the impurities. No size exclusion chromatography could be performed with GST-AV1 as it precipitated during the chromatography process even under optimized buffer conditions.

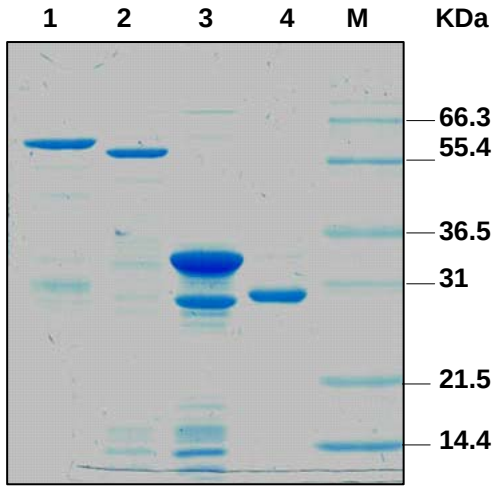


Fig. 5: SDS-PAGE analysis of affinity purified GST fusion proteins. Purified proteins were separated on 12% (w/v) SDS-PAA gels and stained with Coomassie brilliant blue (II.2.4.2).

1: GST-AC1 (~69 kDa), 2: GST-AV1 (~58 kDa), 3: GST-AVNS (~34 kDa), 4: GST (~28 kDa), M: Molecular weight marker.

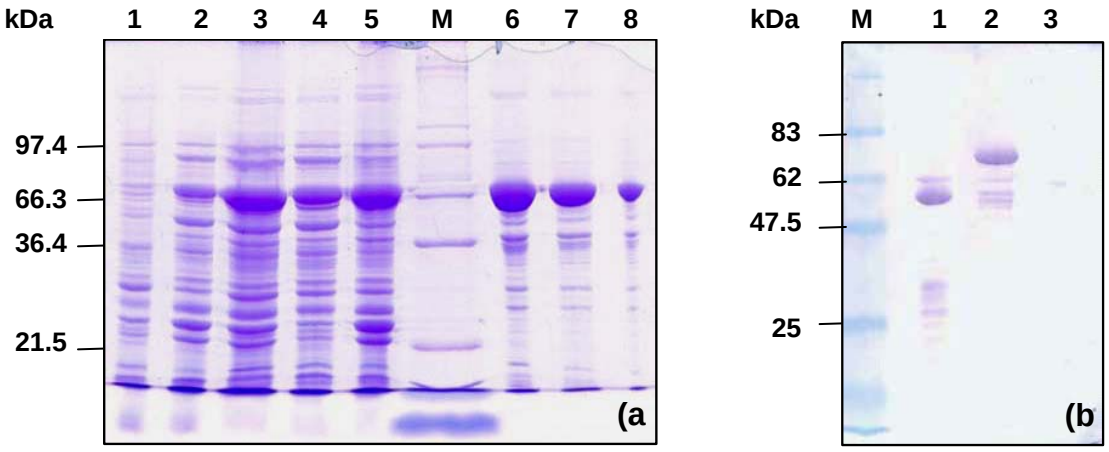
The purified proteins GST-AC1 and GST-AV1 were used for immunization of mice (II.2.5), phage display panning, and screening of the scFv libraries for specific binders. GST-AVNS was used for the analysis of the AV1-specific scFvs in direct ELISA.

III.1.2 Cloning and expression of viral genes as fusion proteins with MBP

In this system, the cloned gene of interest was inserted in frame downstream from the malE gene of *E. coli*, which encodes maltose-binding protein (MBP), resulting in the expression of an MBP fusion protein. The AC1 and AV1 genes were cloned into the vector pMAL-c2x (New England Biolabs) in frame with the MBP coding sequence. Primers (5´- AC1 *Bam*HI and 5´- AC1 *Hind*III) (II.1.8) were designed to amplify AC1 gene and introduce the restriction sites *Bam*HI/*Sal*I and *Not*I/*Hind*III at the 5` and 3` ends of the amplified gene for subcloning, respectively.

The AC1 PCR product was fused via *Bam*HI/*Hind*III to the C-terminus of MBP gene in pMAL-c2x vector. The resulting clone, pMAC1, was confirmed by RE-digestion and sequencing. The plasmid pMAV1 was obtained by replacing AC1 in pMAC1 with AV1 from pGEX-AV1 (III.1.1) via *Sal*I/*Not*I digestion. pMAC1 and pMAV1 were transformed and expressed in *E. coli* strain BL21 (II.1.4). The expression and purification were based on the manufacturer protocols (II.2.3.2). The induction time and temperature (II.2.3.2) were optimized to decrease the level of inclusion bodies formation (data not shown).

Although more than 50% of the fusion proteins were lost by precipitation, inclusion body formation, limited capacity of the column, not binding to the maltose matrix, and/or washing (Fig. 6). High amounts (up to 4mg/L culture) of soluble fraction could be obtained.



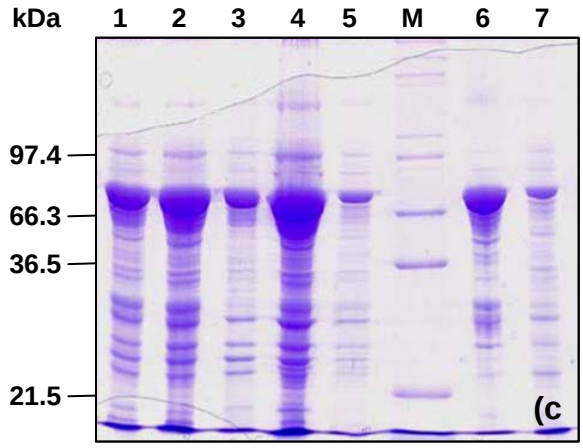


Fig. 6: SDS-PAGE analysis of affinity purified MBP fusion proteins. Purified MBP-AV1 (a) and MBP-AC1 (c) fusion proteins (II.2.3.2) were separated on 12% (w/v) SDS-PAA gels (II.2.4.2) then stained (a & c) with Coomassie brilliant blue (II.2.4.2). b: purified MBP-AV1 (~74kDa), MBP-AC1 (~85kDa) as negative control, and GST-AV1 (~58kDa) as positive control were separated by SDS-PAGE (II.2.4.2) and blotted onto a nitrocellulose membrane (II.2.4.3). Immunodetection was carried out with anti-ACMV CP polyclonal antibody (II.1.3) as primary antibody in dilution 1:2000, followed by GAM^{AP} antibody in dilution 1:5000. Detection was performed with NBT/BCIP for 1 min at RT.

a) 1: non-induced cells, 2: induced cells, 3: cell lysate, 4: crude extract, 5: pellet, 6-8: elution fractions, M: Mark 12 protein size marker. b) M: prestained protein size marker, 1, 2, and 3: elution fractions of GST-AV1, MBP-AV1, and MBP-AC1, respectively. c) 1: cell lysate, 2: crude extract, 3: pellet, 4: flow through, 5: wash fraction, 6-7: elution fractions, M: Mark 12 protein size marker.

The MBP-AC1 and MB-AV1 purified proteins were used for phage display panning (II.2.7.2) and screening the scFv libraries for specific binders (II.2.9.1).

III.1.3 Cloning and transient expression in tobacco leaves of the AV1 gene as fusion with DsRed

DsRed is a red-emitting fluorescent protein isolated from reef corals (*Discosoma sp.*) possessing an excitation peak wavelength (553 nm) just above the excitation peak of chlorophyll (Matz et al., 1999). It emits at wavelength (600-620nm) different from that of GFP (500-530nm). The different emission colour makes it valuable for *in vivo* multi labelling experiments, allowing co-monitoring and co-expression of different fusion proteins (Rodrigues et al., 2001).

Using the primers 5'AV1 (*Nco*I) and 3'AV1 (*Nco*I) (II.1.8), *Nco*I sites were introduced at the 5'and 3'of AV1 gene by PCR. Then the AV1 coding gene was introduced upstream the DsRed coding gene in the vector pGJ1425 (35S-dsRed) (Jach et al., 2001) (kindly provided by Dr. Guido Jach, MPIZ, Cologne, Germany). The correct cloning was verified by test digestion and sequencing. Then the AV1-DsRed gene fusion was introduced into the plant expression vector pTRA-kc (II.1.7) via *EcoR*I- *Bam*HI and the obtained plasmid was called pT-AV1-DsRed (Fig. 7). The plasmid pT-DsRed that expresses DsRed only was obtained by removing AV1 from pT-AV1-DsRed by *NcoI* digestion (Fig. 7).

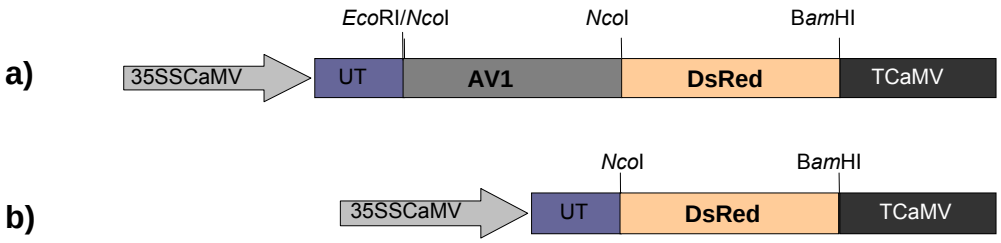


Fig. 7. Schematic presentation of the constructs used for AV1-DsRed (a) and DsRed (b) expression in plant. 35SSCaMV: 35S promoter from Cauliflower Mosaic Virus (CaMV) with duplicated 35S enhancer, UT: 5` UTR of chalconsynthase, TCaMV: termination sequence of CaMV. The cloning sites are shown.

The constructs pT-DsRed and pT-AV1-DsRed were transformed into *Agrobacterium* by electroporation (II.2.1.6). Ten independent recombinant colonies from each transformation were screened for the presence of insert by colony PCR (II.2.1.12). Recombinant *Agrobacterium* cultures were prepared from single colonies of each construct (II.2.1.8) and used for transient expression (II.2.2.2). Alternatively, the construct pT-AV1-DsRed was directly transformed into plant cells by particle bombardment (II.2.2.3). The Leica TCS SP spectral confocal laser scanning microscope (CLSM) was used to monitor the intracellular localization of the AV1-DsRed and DsRed.

AV1 carries an NLS (Noris et al., 1998) to target the fusion protein, AV1-DsRed to the nucleus. To locate nuclei precisely, leaf tissues were mounted in a buffer containing 49,69-diamidino-2-phenylindole (DAPI) and the same cells were excited at different wavelengths to visualize DsRed and DAPI fluorescence (Fig. 8; a1).

The construct pT-AC1-DsRed was cloned following the same strategy as pTAV1-DsRed. The expression of AC1-DsRed worked only once but could not be confirmed in repeating experiments.

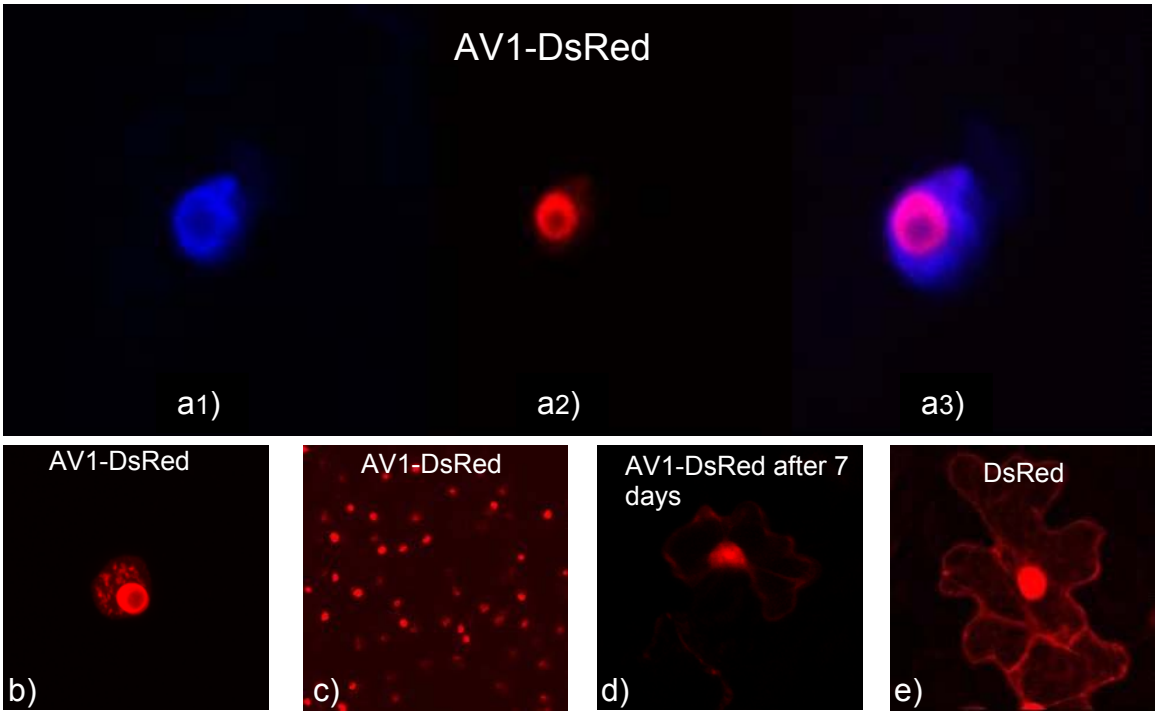


Fig. 8: Transient expression of AV1-DsRed and DsRed fluorescent proteins in *N. benthamiana* leaves. Tobacco leaves were infiltrated (III.2.2.2) with recombinant *Agrobacteria* harbouring the constructs pT-DsRed and pT-AV1-DsRed. Alternatively, the constructs pT-AV1-DsRed was directly transformed into plant cells by particle bombardment (II.2.2.3). After 3 days (7 days in case of d) incubation, AV1- DsRed and DsRed expression and localization were analyzed using a Leica TCS SP spectral confocal laser-scanning microscope. a1- 3) represent the same cell expressing AV1-DsRed where a1 shows the DAPI imaging, a2 shows the DsRed imaging, and A3 shows the overlay image. b and c represent a single cell and low magnification showing many cells expressing AV1-DsRed, respectively. d represents AV1-DsRed expression 7 days after agroinfiltration. e shows a single cell expressing DsRed. DsRed was excited at 568 nm and emissions collected at 600 to 620 nm. For DAPI imaging, excitation at 405 nm and collection between 449 and 461 nm were used.

Three days post-infiltration the red fluorescence of AV1-DsRed was present mainly in nuclei (Fig. 8; a, b, & c), preferentially targeting nucleoli and forming a circle around it (Fig. 9; a & b). After 7 days, however, some fluorescence was observed in the cytosol (Fig. 8; d). This probably indicates the activity of the AV1 NES and its ability to retarget the whole fusion (AV1-DsRed) out of the nucleus. In contrast, free DsRed expressed from the pT-DsRed plasmid was clearly visible in the cytoplasm and nucleoplasm (Fig. 8; e).

III.2 Immunization of mice with recombinant AC1 and AV1 proteins

Mice were immunized using subcutaneous administration of the recombinant proteins (GST-AC1 and GST-AV1) (III.1.1). Four boosts in case of GST-AC1 and 6 in case of GST-AV1 were given in two week intervals until the desired titer (II.2.5.1) was reached. Three days

after the last boost, the final antibody titer was determined by blood sampling from the tail vein and subsequent ELISA-testing (II.2.5.1) of the resulting polyclonal antibodies against the administered antigens. In each case, purified GST and the other GST fusion were used as negative controls (GST-AC1 as a control for AV1 binders and *vice versa*). Another ELISA test was performed after the GST binders were pre-absorbed with high concentration of purified GST protein (Fig. 9). MBP-AC1 and MBP-AV1 purified proteins were not available at that time.

The titer of polyclonal mouse antibody specific for AC1 and AV1 was over 1:150,000. The antibody titer refers to the highest dilution at which antigen-specific binding was detectable above background binding to the negative controls.

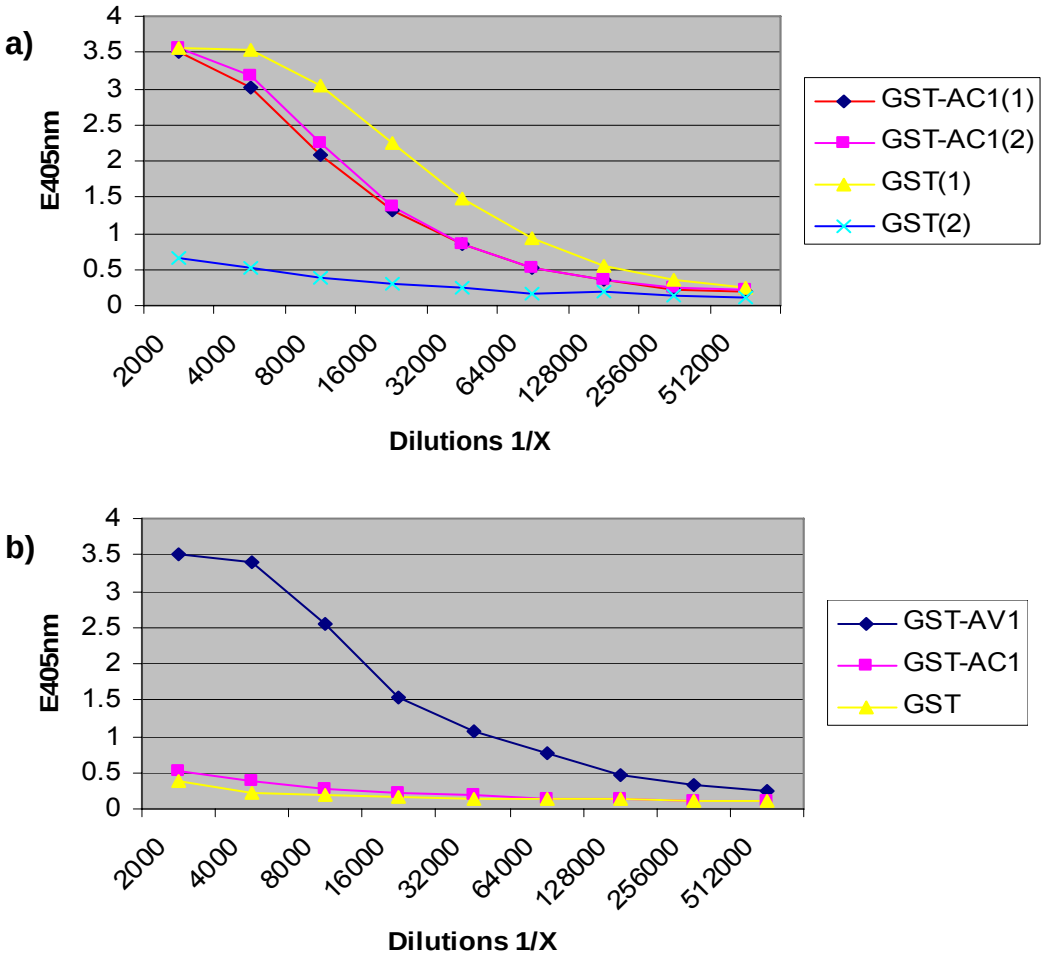


Fig. 9: Determination of polyclonal antibody titers from mouse antiserum by direct ELISA. The purified proteins GST-AC1, GST-AV1 (III.2.3.1), and GST (2.5µg/ml each) were coated to ELISA plates (II.2.5.1). Serial dilutions of sera were added to the coated plates and incubated for 2 hours. Bound antibodies were detected by addition of GAM^{AP} polyclonal antibody (1:5000). ELISA readings were performed at OD405 nm after 30 min incubation with pNPP substrate at 37°C. a: Antiserum from mice immunized with GST-AC1 where (1) refers to the sera without any treatment and (2) refers to the sera in which the GST binders were pre-adsorbed by incubation with high concentration of GST (500µl sera + 20µg GST, 1h at room temperature). b: Only GST-pre-adsorbed antiserum from mice

immunized with GST-AV1 was used. X indicates the antiserum dilutions. Antigens used in ELISA for titer determination are indicated.

Cross reactivity of both polyclonal antibodies with GST was observed. This cross reactivity was drastically lowered when the antisera were pre-adsorbed with GST indicating that both GST-AC1 and GST-AV1 proteins have triggered good immune response to AC1 and AV1 proteins, respectively.

III.3 Construction of phage display library

III.3.1 The cloning strategy

Total RNA isolated from the spleen cells (II.2.6.1) of the GST-AC1 immunized mouse was reverse transcribed. The obtained cDNA was used as a template to amplify the variable region of the V_H and the V_L chains. Amplified V_H and V_L were cloned in the pHENHI vector for the generation of a scFv library as depicted in Fig. 10.

III.3.2 Isolation of the total RNA from mouse spleen cells

Total RNA was isolated from spleen cells of mice immunized with GST-AC1 protein (II.2.5). The concentration and the purity of isolated total RNA was determined by spectrophotometry. The yield of total RNA isolated from an individual mouse was around 1mg. The integrity of the RNA was checked on a 1.2% (w/v) agarose gel (data not shown).

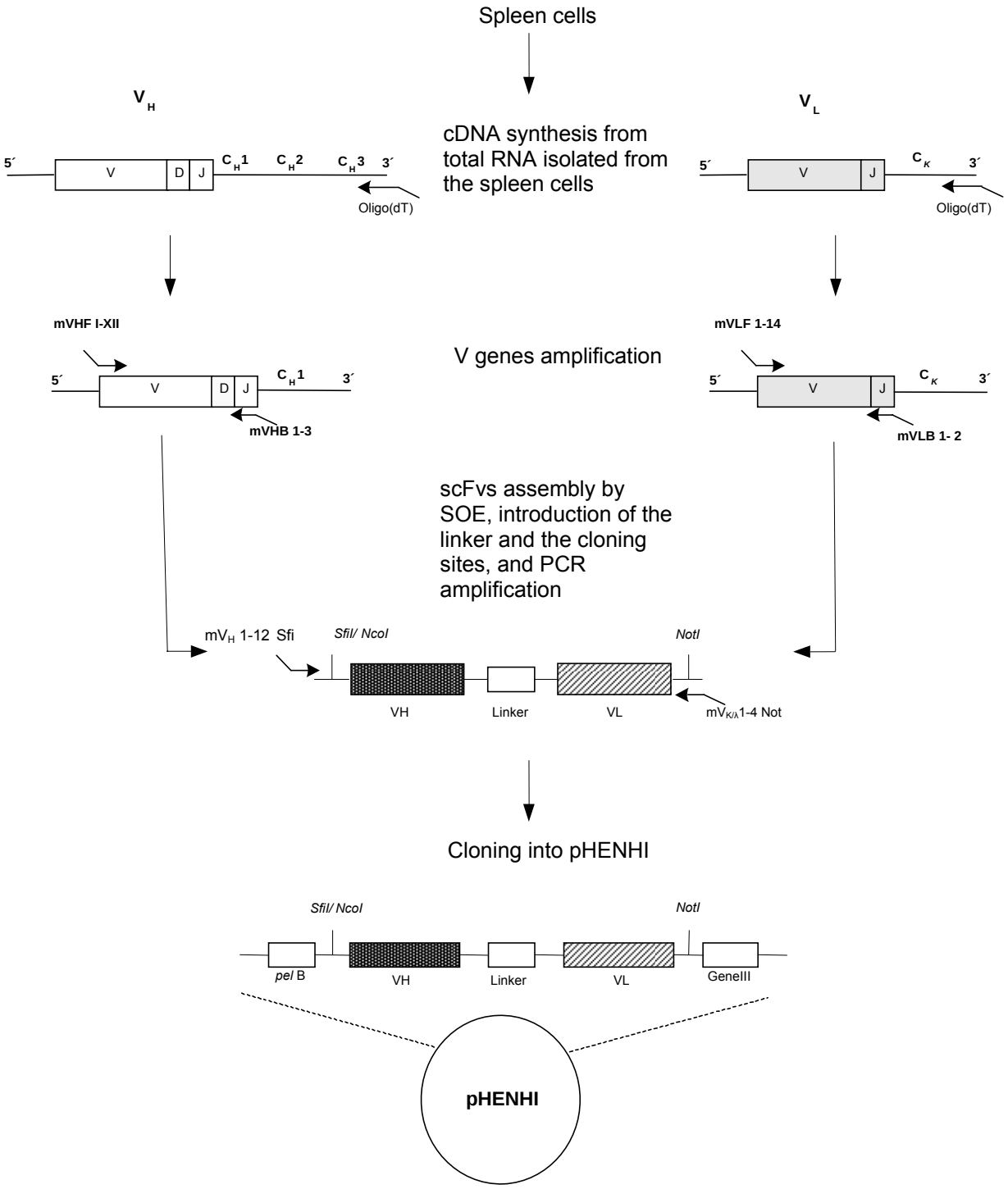


Fig. 10: Schematic representation of the strategy used for cloning a scFv library from the spleen cells of the GST-AC1 immunized mice. VH: variable domain of antibody heavy chain; VL: variable domain of antibody light chain; pHENHI vector: phagemid vector used for cloning the variable antibody domains; mVHF, MVLF, and mVHSfi: forward primers for murine heavy and light chain amplification respectively; mVHB, mVHL, and mVK/ λ Not: back primers for amplification of murine heavy and light chains respectively; Oligo (dT): primers for cDNA synthesis. For primer sequences see section II.1.8.

III.3.3 cDNA synthesis and PCR amplification of variable heavy and light chain fragments

mRNA (III.3.2) was reverse transcribed using an oligo dT primer from the Invitrogen Superscript first strand synthesis kit (II.2.6.2). Mouse IgG heavy chain variable region and variable fragments from κ and λ light chains were amplified with primer combinations listed in Materials and Methods (II.1.8). Two rounds of 26 individual PCR reactions were performed. The first round was to amplify V_H and V_L fragments whereas the second round was to introduce the cloning sites and linker to the amplified fragments. 11 out of 12 primer combinations amplified a product for the heavy chain and 13 out of 14 primer combinations for the κ light chain (Fig. 11). No visible bands were detected when λ primers were used (data not shown). All PCR products had the expected size of 400 to 450 bp.

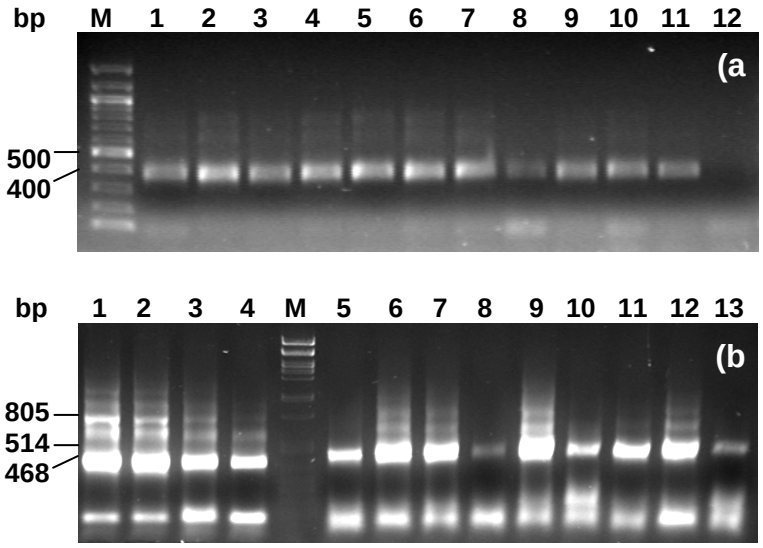


Fig. 11: PCR-based amplification (II.2.1.12) of variable heavy and light chain fragments from GST-AC1 protein immunized mice. Nucleic acids were separated on a 1.2% (w/v) agarose gel (II.2.1.10). M: 100 bp DNA ladder (A) and λPst DNA marker (b). Lanes 1-12 (a) and 1-13 (b) indicate the different mVH forward (a) and MVk forward (b) primers (II.1.8) used for the 2nd round of amplification of the heavy and light chain fragments.

III.3.4 Construction of the scFv phage library

The amplified V_H and V_L PCR products were combined in an SOE-PCR reaction mixture (II.2.6.3) (Fig. 12; a). The resulting scFvs genes were cloned into the vector pHENHI (II.1.7) via *Sfi*I and *Not*I and transformed into electrocompetent *E.coli* XL1-Blue cells (II.I.4). After electroporation, cells were plated on 2xYT medium containing 1% (w/v) glucose and 100 mg/ml ampicillin) and incubated overnight at 30°C. All grown colonies (SAC1 scFv library) were scraped off the plates in 5mL 2×YT medium with 25% (v/v) glycerol and subsequently stored at -80°C. To check the cloning efficiency in the resulting SAC1 library, 14 individual colonies were randomly selected and tested by colony PCR (II.2.1.12) using pHEN specific

primers (II.1.8) (Fig. 12; b). The percentage of positive clones, based on the last test results, in SAC1 library carrying a full size scFv fragment was 100%. The library size was $2x10^7$.

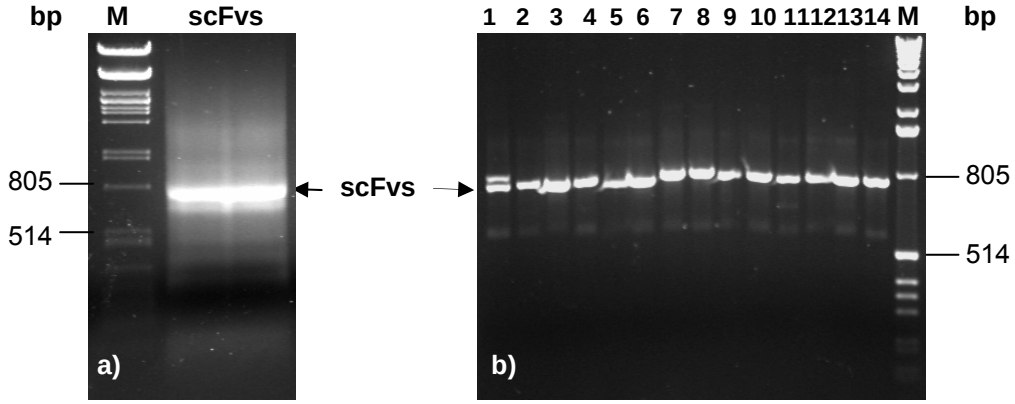


Fig. 12: Analysis of the VH and VL combination by SOE PCR (a) and colony PCR test of scFv SAC1 library (b). After the SOE reaction, a major band with the expected size of scFv is visible (a) indicating the correct combination of VH and VL. In (b) 14 randomly selected colonies from murine scFv SAC1 were tested for the cloning efficiency by colony PCR. Nucleic acids were separated on a 1.2% (w/v) agarose gel. M: λPst DNA size marker; 1-14: selected colonies-PCR product of amplified scFvs genes.

III.4 Selection of AC1 and AV1 specific scFvs

III.4.1 Solid phase panning of SAC1 and Tomlinson I scFv libraries against recombinant fusion proteins GST-AC1, GST-AV1, MBP-AC1, and MBP-AV1

The Tomlinson I scFv library (MRC Laboratory of Molecular Biology and the MRC Centre for protein engineering, Cambridge, UK) (kindly provided by Dr. Angelika Ziegler, SCRI, Dundee, UK) comprises over 100 million different scFv fragments. It was cloned in phagemid (pIT2) and transformed into TG1 *E. coli* cells.

SAC1 and Tomlinson I scFv libraries were subjected to 3 and 4 rounds of panning, respectively, against 10-25 µg/ml purified antigens immobilized on the surface of the immuno tubes (II.2.7).

The SAC1 library was screened for AC1 binders, GST-AC1 (III.1.1) was used as an immobilized antigen in the three panning rounds. The Tomlinson I library was screened for AC1 and AV1 binders using MBP-AC1/GST-AC1 and MBP-AV1/GST-AV1 as antigens, respectively. Four rounds of panning were performed for each case and, in order to remove the GST/MBP binders, MBP-AC1 and MBP-AV1 (III.1.2) were used in the first and the 4th

panning rounds whereas GST-AC1 and GST-AV1 (III.1.1) were used in the 2nd and the 3rd panning rounds. As shown in table 5, there was no clear enrichment of specific phages after the third round of panning of the SAC1 library. This could be due to a late-discovered problem associated with the SOE primers, which were used to create SAC1 library. These primers were shown proven to induce a frame shift in most of the scFvs due to low quality of synthesis. There was, on the other hand, good enrichment of specific phages against AC1 and AV1, respectively, after their 4th round of panning with the Tomlinson I library.

Table 5: Selection and enrichment of AC1 and AV1 binders through multiple rounds panning of scFv phage libraries.

scFv library	Antigen	Panning round	Input phage	Output phage
SAC1	**GST-AC1**	1	$1x10^{13}$	**$1.8x10^{4}$**
		2	$1x10^{13}$	**$1.5x10^{4}$**
		3	$2x10^{13}$	**$1x10^{5}$**
Tomlinson I	**MBP-AC1**	1	$5x10^{13}$	**$8x10^{8}$**
	GST-AC1	2	$4x10^{13}$	**$6x10^{7}$**
	GST-AC1	3	$\sim5x10^{13}$	**$2x10^{9}$**
	MBP-AC1	4	$\sim5x10^{13}$	**$5x10^{9}$**
Tomlinson I	**MBP-AV1**	1	$3x10^{13}$	**$1x10^{8}$**
	GST-AV1	2	$\sim5x10^{13}$	**$1x10^{7}$**
	GST-AV1	3	$\sim5x10^{13}$	**$3x10^{9}$**
	MBP-AV1	4	$\sim5x10^{13}$	**$3x10^{9}$**

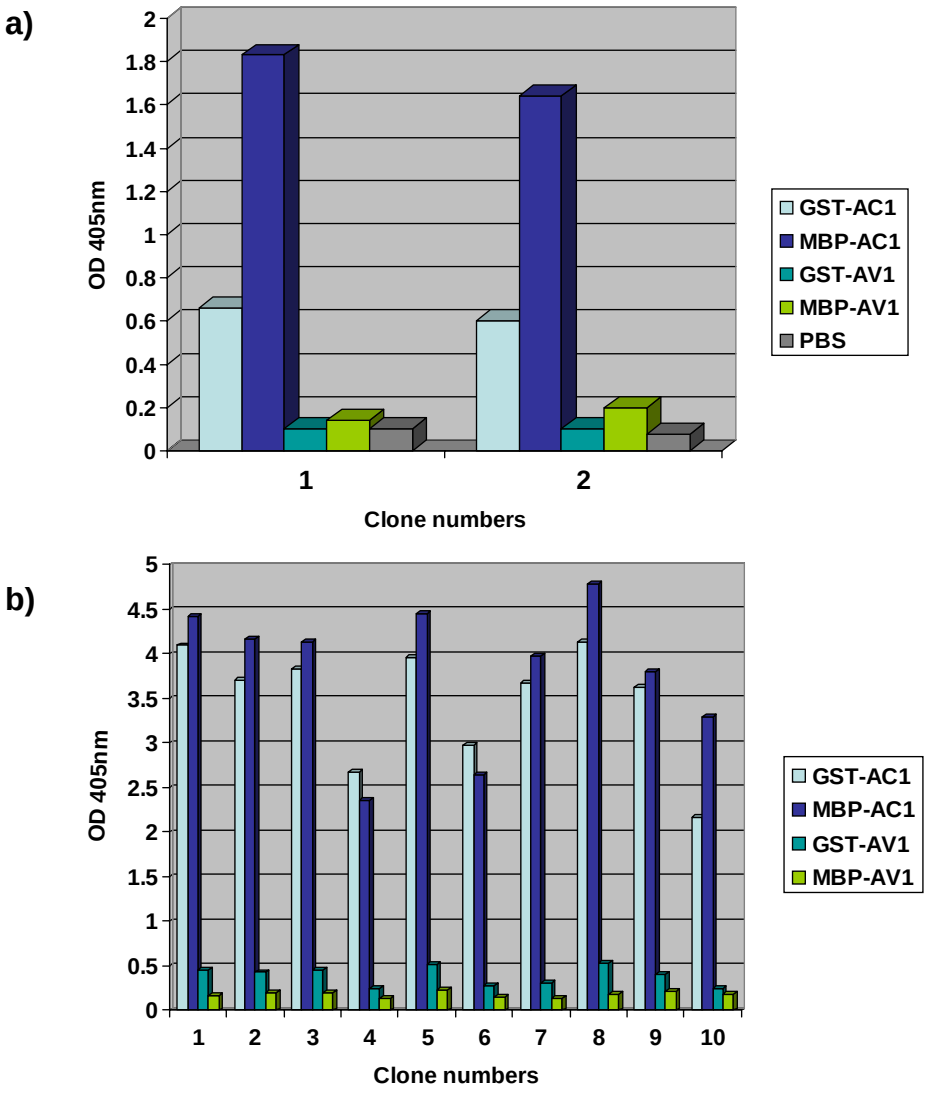
III.4.2 Screening and characterization of scFv-fragments against AC1 and AV1 viral proteins

After the panning rounds of phage display libraries, up to 96 colonies (700 in case of SAC1 library) were randomly selected and grown in microtiter plates. Expression of soluble scFv-fragments was induced by 1 mM IPTG. The specificity of scFv-fragments was tested in direct ELISA experiments against bacterially expressed GST-AC1, GST-AV1, MBP-AC1, and MBP-AV1 fusion proteins. In case of the clones originated from Tomlinson I library, around 30% of the analyzed clones showed a specific binding to the corresponding AC1 and AV1

antigens (only positive clones are shown in Fig, 13 b & c). No MBP or GST binders were found indicating the efficiency of using two different antigen fusions for excluding all the binders against the fusion partners.

On the other hand, only two clones (scFv-RW-AC1/2) originated from SAC1 library showed specificity against AC1 (Fig. 13; a). Some other clones, however, were specific against GST (data not shown).

To verify the presence of full size scFv-fragments, the DNA of 20 clones that showed a high reactivity to the corresponding antigen were isolated and sequenced using specific primers (data not shown). The selected AV1 specific clones were the ones with a high reactivity with GST-AV1 and MBP-AV1 and no cross reactivity with GST-AC1, GST, or MBP-AC1. The same strategy was used to select AC1 binders.



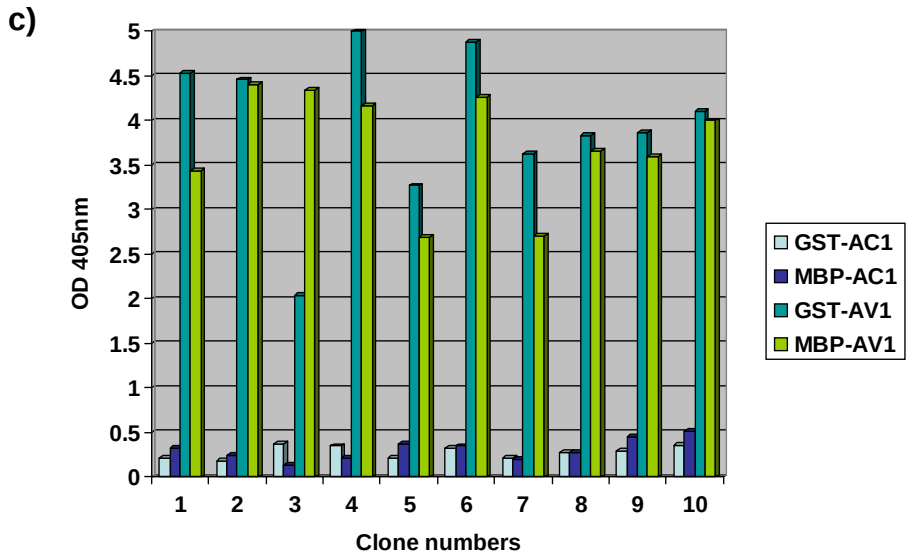


Fig. 13: Screening of selected clones from the scFv libraries SAC1 (a) and Tomlinson I (b & c) in soluble ELISA. Binding activities of randomly selected soluble murine scFvs to AC1 (a & b) and AV1 (c) viral proteins after panning rounds were revealed by direct ELISA (II.2.9.1). GST-AC1 and MBP-AC1 fusion proteins were used to select AC1 binders, GST-AV1, MBP-AV1, and/or PBS were used as controls. The same strategy was used to select the AV1 binders. Around 10µg/ml of fusion proteins were coated on microtiter plates. 100µl of bacterial supernatant was added and bound scFvs were detected by addition of anti-c-myc or anti-His monoclonal antibodies and GAM^{AP} polyclonal antibodies conjugated to alkaline phosphatase as secondary antibody. ELISA readings ($OD_{405\ nm}$) were performed after 15-30 min incubation with pNPP substrate at 37°C. a: soluble scFv-RW-AC1 and scFv-RW-AC2 (1 and 2) from SAC1 library were tested for AC1 binding. b & c: soluble scFvs from Tomlinson I library were tested for AC1 and AV1 binding, respectively, 10 clones each are represented. The fusion proteins used to coat the microtiter plates are indicated to the right of each figure.

The sequencing results showed that the two clones from SAC1 library (scFv-RW-AC1 and scFv-RW-AC2) were identical. This scFv was designated scFv-RW-AC and the plasmid was designated pHEN-scFv-RW-AC.

Moreover, 20 positive clones against AV1 were sequenced, from those:

- 12 were identical (called scFv-SCRs-AV3 in the plasmid pIT-scFv-SCRs-AV3). This scFv had a stop codon in the framework at aa position H105 (Fig. 14).
- Two other scFvs were identical (called scFv-SCRs-AV6 in the plasmid pIT-scFv-SCRs-AV6) with stop codon at the same position as scFv-SCRs-AV3 (Fig. 14).
- Two different scFvs (called scFv-SCR-AV1 and scFv-SCR-AV4 in the plasmids pIT-SCR-AV1 and pIT-SCR-AV4, respectively) without stop codons.
- All clones (4) containing more than one stop codon were discarded.

```
•  SC     MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSDIDNAGSYTDYADSVKGRFT
•  SV-1   MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTINDSGSGTSYADSVKGRFT
•  sV-3   MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSGISNDGSATSYADSVKGRFT
•  SV-4   MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIRAPGTRTWYADSVKGRFT
•  sV-6   MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTINDSGSGTSYADSVKGRFT

•  SC     ISRDNSKNTLYLQMNSLRAEDTAVYYCAKSSDSFDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSP
•  SV-1   ISRDNSKNTLYLQMNSLRAEDTAVYYCAKTGSYFDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSP
•  sV-3   ISRDNSKNTLYLQMNSLRAEDTAVYYCAKTITYFDYWG*GTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSP
•  SV-4   ISRDNSKNTLYLQMNSLRAEDTAVYYCAKNLLKFDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSP
•  sV-6   ISRDNSKNTLYLQMNSLRAEDTAVYYCAKTGSYFDYWG*GTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSP

•  SC     SSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYSASALQSGVPSRFSGSGSGTDFTLTISSLQ
•  SV-1   SSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYGASRLQSGVPSRFSGSGSGTDFTLTISSLQ
•  sV-3   SSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASTLQSGVPSRFSGSGSGTDFTLTISSLQ
•  SV-4   SSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYKASRLQSGVPSRFSGSGSGTDFTLTISSLQ
•  sV-6   SSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYGASSLQSGVPSRFSGSGSGTDFTLTISSLQ

•  SC     PEDFATYYCQQSDTSPTTFGQGT
•  SV-1   PEDFATYYCQQADTSPATFGQGT
•  sV-3   PEDFATYYCQQSSTSPTTFGQGT
•  SV-4   PEDFATYYCQQAMKIPSTFGQGT
•  sV-6   PEDFATYYCQQGDTSPATFGQGT
```

Fig. 14: Alignment of the aa sequences of the scFv fragments selected from Tomlinson I library with specificity for AC1 and AV1 proteins. The arrow indicates position H105 where scFvs-SCRs-AV3 and scFv-SCRs-AV6 have a stop codon and the other scFvs from the same library have the aa Glutamine (Q). The names were shortened where "S" represents SCR, "s" represents SCRs, "C" represents AC and "V" represents AV.

All sequenced clones against AC1 from Tomlinson I library were identical. This scFv was called scFv-SCR-AC and the plasmid pIT-scFv-SCR-AC.

Selected clones were tested again in ELISA against all available antigens including GST-AVNS (III.1.1) (Fig. 15).

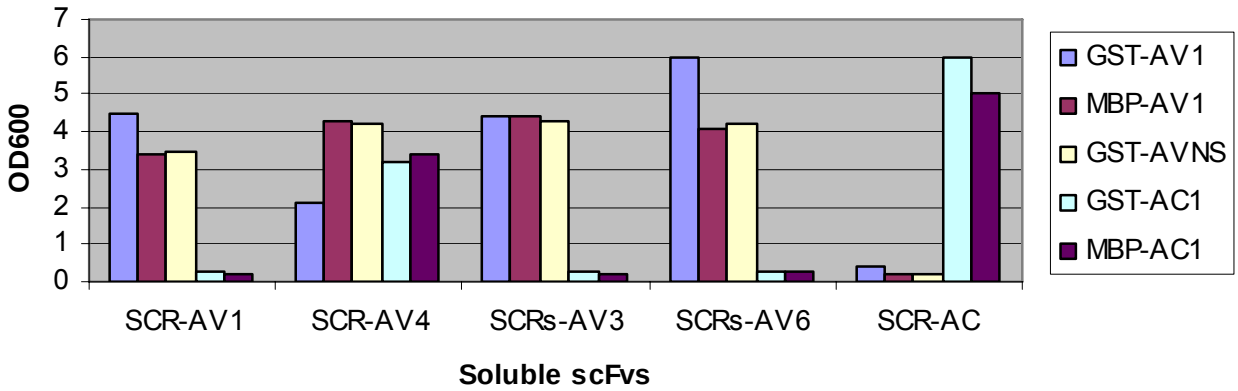


Fig. 15: Direct ELISA of the selected scFvs reactivity with different antigens. 100µl of ~ 2.5µg/ml of GST-AC1, GST-AV1, MBP-AV1, MBP-AC1, and GST-AVNS fusion proteins were coated on microtiter plates. 100µl of the o/n induced bacterial supernatant was added and bound scFvs were detected by addition of anti-His monoclonal antibody and GAM^{AP} polyclonal antibody conjugated to alkaline phosphatase as secondary antibody. ELISA readings (OD_{405nm}) were performed after 30 min incubation with pNPP substrate at 37°C.

The results show the specificity of scFvs SCR-AV1, SCRs-AV3, SCR-AV4, and SCRs-AV6 against AV1. These scFvs also bound to GST-AVNS indicating that their epitopes are located in the N-terminal part of AV1. On the other hand, SCR-AC showed high reactivity with AC1. All scFvs did not react with the negative controls except SCR-AV4 that showed reactivity with all used antigens.

III.4.2.1 Stop codon removal by *in vitro* mutagenesis.

The *E. coli* strain TG1 which was used with Tomlinson I library is a suppressor strain. The stop codon, TAG, is translated as Glutamate (E) by suppressor strains whereas we find Glutamine (Q) at the same position of the stop codon of scFv-SCRs-AV3/6 in all other scFvs originated from the same library, Tomlinson I library, (Fig. 14). So the stop codon TAG in scFv-SCRs-AV3 and scFv-SCRs-AV6 was back mutated by mutagenesis to CAG and GAG that encode the aa Q and E, respectively. The QuickChange II mutagenesis kit was used according to the manufacturer's protocol (II.2.1.15).

For the Q exchange with the stop codon the primers 5'aAV3 and 3'aAV3 (II.1.8) were used and the resulting plasmids were called pIT-scFv-SCR-AV3 and pIT-scFv-SCR-AV6. For the E exchange with the stop codon the primers 5'AV3-E and 3'aAV3E (II.1.8) were used and the resulting plasmids were called pIT-scFv-SCR-AV3E and pIT-scFv-SCR-AV6E. All resulting plasmids in addition to the original ones, pIT-scFv-SCRs-AV3 and pIT-scFv-SCRs-AV6 were each transformed and expressed in non-suppressor *E. coli* strain HB2151 (II.1.4). WB analysis (Fig. 16; a) showed good expression of scFv-SCR-AV3, scFv-SCR-AV6, scFv-SCR-AV3E, and scFv-SCR-AV6E indicating the successful exchange of the stop codon. No expression could be detected in the cells transformed with pIT-scFv-SCRs-AV3 and pIT-scFv-SCRs-AV6 (Fig. 16; a).

Direct ELISA (II.2.9.1) of the mutated scFvs demonstrated better binding activity of scFv-SCR-AV3E and scFv-SCR-AV6E than scFv-SCR-AV3 and scFv-SCR-AV6 (Fig 16b).

a)

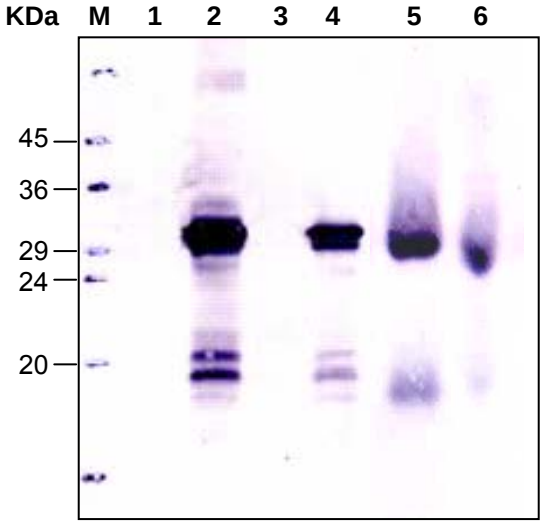
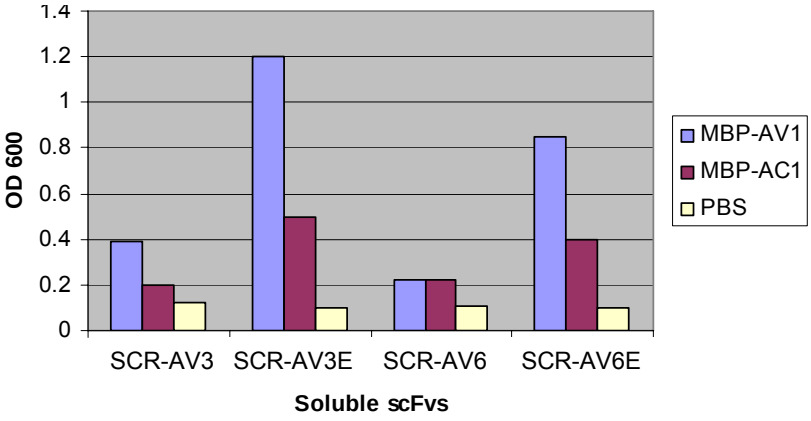


Fig. 16: WB (a) and ELISA (b) analysis of the scFv-SCRs-AV3 (1), scFv-SCR-AV3 (2), scFv-SCRs-AV6 (3), scFv-SCR-AV6 (4), scFv-SCR-AV3E (5), and scFv-SCR-AV6E (6) expression in non- suppressor *E. coli* strain HB101.

5µl of the o/n induced cells loaded into SDS-Page gel. Immunodetection was carried out with Mab anti-His followed by GAM^{AP}. Detection was performed with NBT/BCIP for 1-2 min at RT. M: protein size marker.

b)



Direct ELISA of soluble scFvs reactivity with AV1 protein. 100 µl of the bacterial culture supernatant induced by 1mM IPTG for expression of soluble scFvs was applied on a MBP-AC1, MBP-AV1, and PBS coated ELISA plate (II.2.9.1). Bound scFvs were revealed by addition of anti-c-myc tag monoclonal antibody (9E10) and GAM^{AP} antibody conjugated to alkaline phosphatase. ELISA readings (OD_{405nm}) were performed after 45 min incubation with pNPP substrate at 37°C. PBS was used as negative controls.

III.4.2.2 Large scale expression and IMAC purification of soluble scFvs from phage libraries

Upscaled expression of scFv-SCR-AV1, scFv-SCR-AV3, scFv-SCR-AV3E, scFv-SCR-AV4, scFv-SCR-AV6, scFv-SCR-AV6E, scFv-SCR-AC, and scFv-RW-AC was performed in *E. coli* strain HB2151. Additionally, and for comparison purpose, scFv-SCRs-AV6 was expressed in *E. coli* suppressor strain TG1 (II.1.4). The His6 tagged scFvs were purified by IMAC as described in material and methods (II.2.3.3). SDS-PAGE analysis of the affinity purified scFvs revealed the presence of a band of approximately 30 kDa for all scFvs (Fig. 17). Other bands include impurities, dimers, and multimers (Fig. 17).

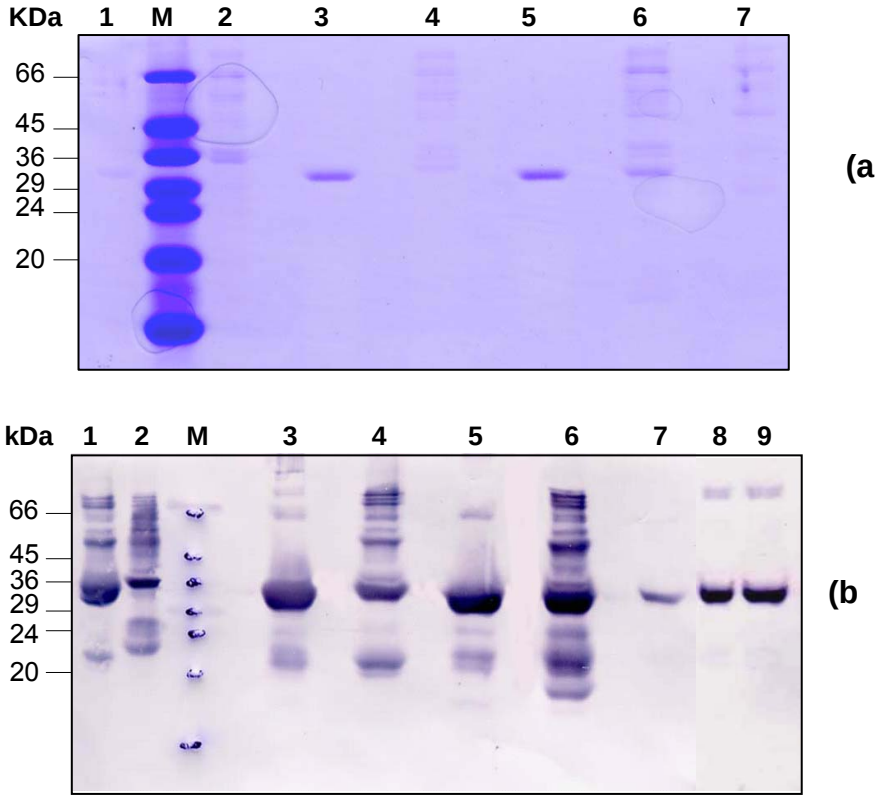


Fig. 17: SDS-PAGE analysis of affinity purified scFvs. scFv-SCR-AC (1), scFv-RW-AC(2), scFv-SCR-AV3 (3), scFv-SCR-AV4 (4), scFv-SCR-AV6(5), scFv-SCR-AV1(7), scFv-SCR-AV3E (8), and scFv-SCR-AV6E(9) were expressed in the *E.coli* strain HB2151 (II.1.4). ScFv-SCRs-AV6 (6) was also expressed in *E.coli* suppressor strain TG1 (II.1.4). The expressed scFvs were purified by IMAC (II.2.3.3). Proteins were separated on 12% (w/v) SDS-PAGE (II.2.4.2) and stained with Coomassie brilliant blue (II.2.4.2) (a). Immunodetection (II.2.4.3) (b) was carried out with Mab anti-His followed by GAM^{AP}. Detection was performed with NBT/BCIP for 1-2 min at RT. M: protein size marker.

III.5 Cloning of scFv against AV1 from a hybridoma fusion cell line

III.5.1 Production of hybridoma clones secreting monoclonal antibodies against AV1 protein

Spleen cells from the mouse immunized with GST-AV1 (III.2) were fused with a myeloma cell line to develop a hybridoma clone that secretes a specific antibody against AV1 protein (II.2.8.1). The hybridoma clones were screened in a direct ELISA (II.2.9.1) against GST-AV1 and MBP-AV1 fusion proteins to capture the clones that produce monoclonal antibodies against AV1. Fourteen wells with single colonies were identified after 4 rounds of limiting dilutions (II.2.8) under the microscope. All 14 clones showed similar binding activity in ELISA and similar levels of antibody secreted into the supernatant demonstrating the

homogeneity of the cell line. One such clone was selected and propagated for further work. The fusion cell line was named HAV and the monoclonal antibody Mab HAV.

III.5.2 Purification and analysis of mouse Mab HAV

The HAV hybridoma cell line was propagated in tissue culture flasks and the culture supernatant was collected for the purification of the secreted mouse Mab HAV. The hybridoma culture supernatant was centrifuged to remove the cells and cell debris before applying it to the equilibrated Protein A matrix (II.2.8.3). Approximately 9-10 mg of purified antibody was obtained from one litre of hybridoma culture supernatant. The SDS-PAGE analysis of the purified mouse Mab HAV showed two major bands of approximately 53 and 26 kDa corresponding to the mouse Mab HAV heavy and light chains, respectively (Fig. 18).

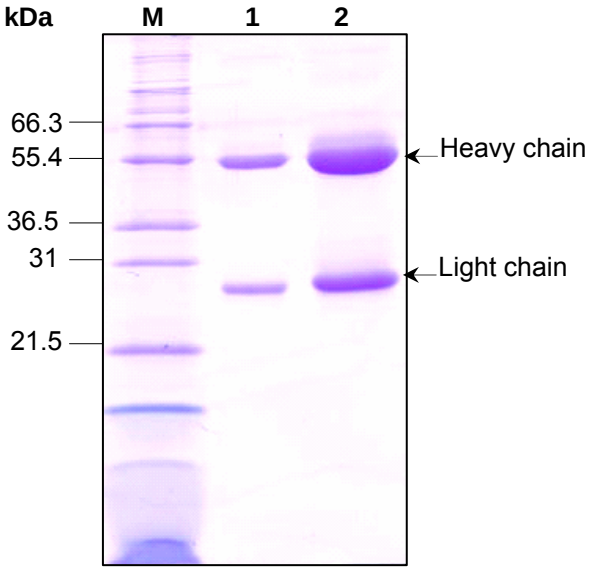


Fig. 18: SDS-PAGE analysis of the affinity purified mouse Mab HAV. The mouse Mab HAV purified from the hybridoma culture supernatant (II.2.8.3) was separated on a 12% (w/v) SDS-PAGE gel and stained with Coomassie brilliant blue (II.2.4.2). M: Molecular weight mark12; 1-2: 5 and 15 µg of Protein A-purified mouse Mab HAV, respectively.

Direct ELISA (II.2.9.1) with the purified antibody demonstrated binding activity of the antibody to GST-AV1 and MBP-AV1 but not to GST-AVNS that carries N-terminal part of AV1 (Fig. 19; a). Mab HAV recognized AV1 in WB assay indicating that it probably binds to a linear epitope (Fig. 19; b).

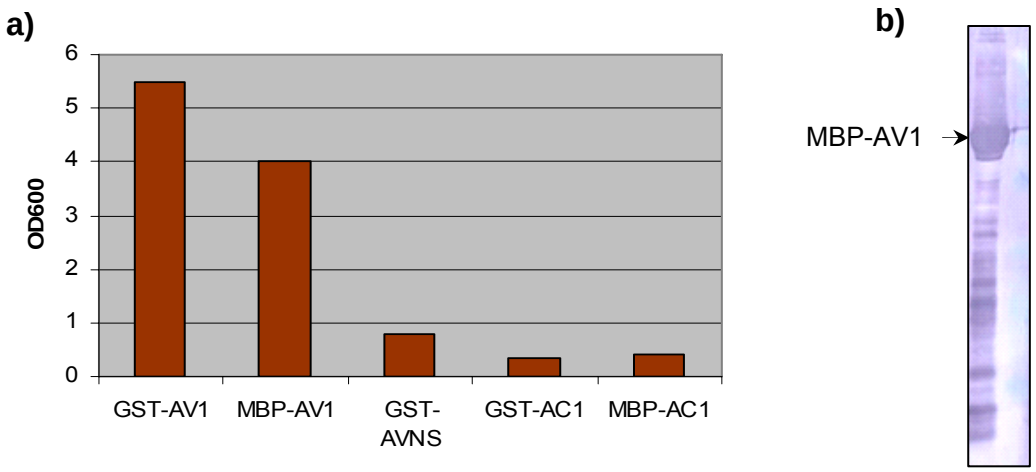


Fig. 19: Mab HAV binding activity test in ELISA and WB. a: Direct ELISA of Mab HAV reactivity with AV1 protein. 100 µl of 5µg/ml purified (II.2.8.3) Mab HAV were applied on a MBP-AC1, MBP-AV1, GST-AVNS, GST-AC1, and GST-AV1 coated ELISA plate (II.2.9.1). Bound Mab HAVs were revealed by monoclonal GAM^{AP} antibody conjugated to alkaline phosphatase. ELISA readings (OD405 nm) were performed after 30 min incubation with pNPP substrate at 37°C. b: WB assay of Mab HAV reactivity with AV1 protein. MBP-AV1 was loaded into SDS-Page gel and transferred onto nitrocellulose membrane as described in II.2.4.2 and II.2.4.3. Immunodetection was carried out with alkaline phosphatase conjugated GAM^{AP} antibody. Detection was performed with NBT/BCIP for 1 min at RT.

III.5.3 Amplification and cloning of the variable domains of Mab HAV

mRNA isolated from the HAV hybridoma cell line (II.8.2.4) was reverse transcribed utilizing an oligo dT primer using the Invitrogen Superscript first strand synthesis kit (II.2.6.2). The V_H and the V_L of the mouse Mab HAV were amplified from cDNA and cloned in the pHENHI vector for the generation of a mini-scFv library following the same strategy used for the construction of the SAC1 phage library (II.2.6.3). Fourteen clones were randomly picked and sequenced. Six clones contained only light chains and 8 clones had a full size scFv. The sequences of V_H and V_L of all clones were identical including the ones with light chain only. One of the clones with full size scFv was selected and a small-scale expression in *E. coli* strain XL1-blue was carried out as described in section II.2.9.1. Direct ELISA of the culture supernatant containing the secreted scFv showed that the selected clone expressed scFv fragment that bound to the MBP-AV1 and GST-AV1 fusion proteins (Fig. 20; a). The reactivity with GST-AV1 was far less than that with MBP-AV1 probably due to differences in the coating efficiency of the antigens. WB analysis of the culture supernatant confirmed the presence of a band of ~30kDa, the expected size for a scFv (Fig. 20; b). This scFv was named scFv-SAV and the plasmid pHEN-scFv-SAV.

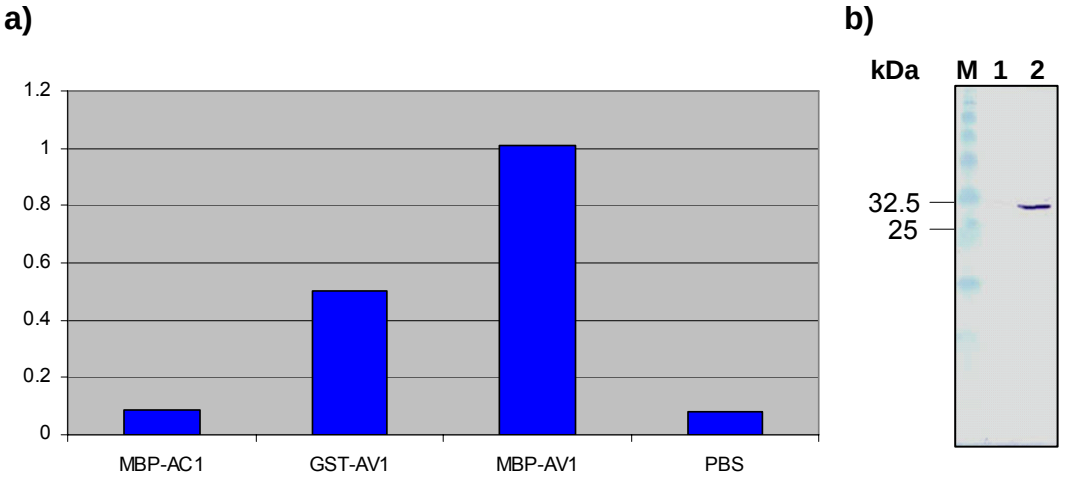


Fig. 20: ScFv-SAV analysis. (a): Direct ELISA of soluble scFv-SAV reactivity with AV1 protein. 100 µl of the bacterial culture supernatant induced by 1mM IPTG for expression of soluble scFv was applied on a MBP-AC1, MBP-AV1, GST-AV1, and PBS coated ELISA plate (II.2.9.1). Bound scFvs were revealed by anti-c-myc tag monoclonal antibody (9E10) and GAM^{AP} antibody conjugated to alkaline phosphatase. ELISA readings (OD_{405nm}) were performed after 30 min incubation with pNPP substrate at 37°C. MBP-AC1 and PBS were used as negative controls. (b): Immunoblot analysis of bacterially expressed soluble scFv-SAV. 15 µl of the bacterial culture supernatant containing soluble scFv was separated on a 12% (w/v) SDS-PAGE gel (II.2.4.2) and blotted onto nitrocellulose membrane (II.2.4.3). Blotted scFvs were revealed by (9E10) antibody (and GAM^{AP} polyclonal antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: non-induced culture; 2: soluble scFv; M: Molecular weight standard/ pre-stained protein marker.

III.5.3.1 Sequence analysis of scFv-SAV

The nucleotide sequence of scFv-SAV revealed a stop codon (TAG) 60 nucleotides downstream of the ATG start codon located in the *Nco*I site. An internal open reading frame, starts from a start codon at position 65, encodes a protein with aa sequence obeys to the KABAT rules for antibodies except the missing N-terminal 20 aa. Aligning the nucleotide sequence with other antibodies using BLAST (www.pubmed.com) showed a point mutation (deletion) at position 30 downstream the original ATG start codon in *Nco*I site. The nucleotide "A" was found at the same position in other scFvs. Insertion of the nucleotide "A" at the position of the point mutation restored the original frame and brings both ATG start codons to the same frame (Fig. 21) which fully agrees with the KABAT rules.

a)

CCATGGCCCAGGTGCAGCTTCTGCAGTCAGGGCTGCACTGGCAAAACCTGGGGCCTCAG
M A Q V Q L L Q S G L H W Q N L G P Q
A L A K P G A S

TGAAGATGTCCTGCGAGGCTT
* R C P A R L
V K M S C E A

b)

CCATGGCCCAGGTGCAGCTTCTGCAGTCAGGGACTGCACTGGCAAAACCTGGGGCCTCA
M A Q V Q L L Q S G T A L A K P G A S

GTGAAGATGTCCTGCGAGGCTTCT
V K M S C E A S

Fig. 21: Sequence analysis of scFv-SAV. The original sequence of scFv-SAV (a) and the successful frame correction (b) are shown. The frame was restored by inserting the nucleotide "A" (arrow) at position 30 downstream the original ATG start codon (underlined) located in the *Nco*I site. "A" insertion brings both ATG start codons to the same frame. The one-letter amino acid translation starting from the first start codon is shown under the DNA sequence and the asterisk indicates the stop codon. The first and the internal start codons are underlined.

III.5.3.2 Open reading frame correction in scFv-SAV

The missing nucleotide "A" in the N-terminal part of scFv (III.5.3.1) was inserted by *in vitro* site-directed mutagenesis using the QuickChange® II site-directed mutagenesis kit (Stratagene) according to the manufacturer protocol (II.2.1.15). using the primers "5` scFv1 forward/backward" (II.1.8). The correct insertion of the nucleotide "A" was confirmed by sequencing twice and the resulting scFv was named scFv-RW-AV and the plasmid pHEN-scFv-RW-AV.

Both scFv-RW-AV and scFv-SAV were expressed in *E. coli* strain HB2151 and IMAC purified as described in II.2.3.3. Due to the high impurities (Fig. 22) it was difficult to determine the exact concentration of the purified scFvs. The obtained amount of both scFv-SAV and scFv-RW-AV was roughly estimated as 100-200µg/200ml culture. The Coomassie stained gel and WB show clearly that the MW of scFv-RW-AV is slightly higher than scFv-SAV indicating the correct "A" insertion (Fig. 22).

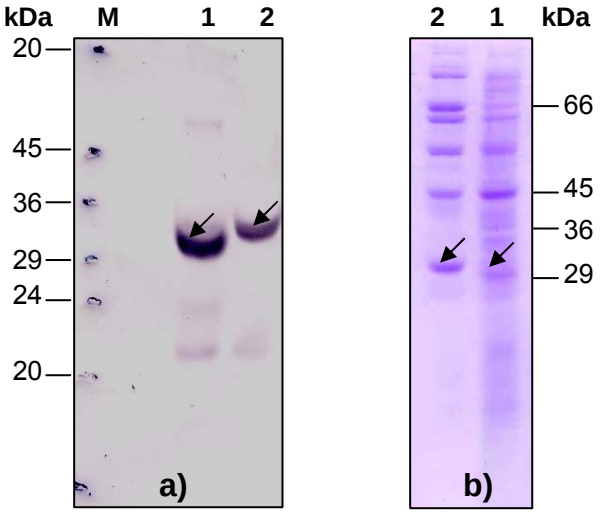


Fig. 22: Immunoblot (a) and Coomassie-stained (b) SDS-PAGE analysis of bacterially expressed and IMAC purified scFv-SAV and scFv-RW-AV. ~1 µg (rough estimation) of each of the purified scFvs was separated on a 12% (w/v) SDS-PAGE gel and Coomassie-stained (b). In WB analysis (a) the blotted scFvs were revealed by anti-His tag monoclonal antibody and GAM^{AP} antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. The MW of RW-AV is clearly higher than SAV (arrow heads). 1: scFv-SAV; 2: scFv-RW-AV; M: Molecular weight protein marker.

III.5.3.3 Comparative analysis of the binding activities of scFv-SAV and scFv-RW-AV by ELISA

Direct ELISA (II.2.9.1) using purified scFvs revealed the high specificity of scFv-RW-AV and scFv-SAV against AV1 (Fig. 23).

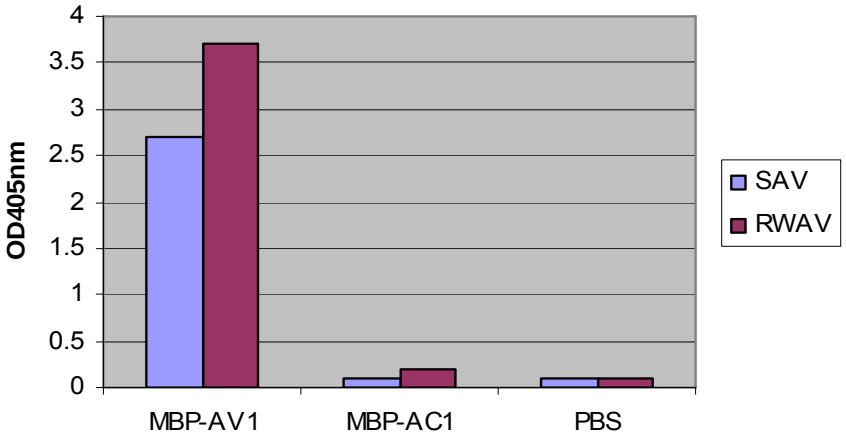


Fig. 23: Direct ELISA of scFv-SAV and scFv-RW-AV reactivity with AV1 protein. ~5µg of the purified scFvs were applied on a MBP-AC1, MBP-AV1, and PBS coated ELISA plate. Bound scFvs were revealed by anti-His tag monoclonal antibody and GAM^{AP} antibody conjugated to alkaline phosphatase. ELISA readings ($OD_{405\ nm}$) were performed after 1h incubation with pNPP substrate at 37°C. MBP-AC1 and PBS were used as negative controls.

The ELISA results also showed that the reactivity of scFv-RW-AV with GST-AV1 and MBP-AV1 was higher than that of scFv-SAV (Fig. 23). But because of the high impurities and the difficulties to determine the active concentration of both scFvs the ELISA was not highly reliable method to compare the two scFvs. Calculating the off rate using BIACORE

(II.2.9.2 & III.6.2) represents more appropriate way for comparing scFvs independently of their concentration (III.6.2).

III.6 Surface plasmon resonance analysis of the AV1 binders

III.6.1 Mab-HAV analysis by BIACORE

The binding properties of the Mab-HAV to MBP-AV1 were further characterized by surface plasmon resonance (SPR) assay on BIACORE (II.2.9.2). The MBP-AV1 antigen was immobilized to a BIACORE CM3 sensor chip in the flow cell 2 (FC2). As a negative control, BSA was immobilized on FC1. Five different concentrations of Mab-HAV were tested (17, 35, 70, 140, and 280nM) and every concentration was repeated 3 times. The obtained data were analysed using the BIAevaluation (4.1) software (Fig. 24 & Table 6).

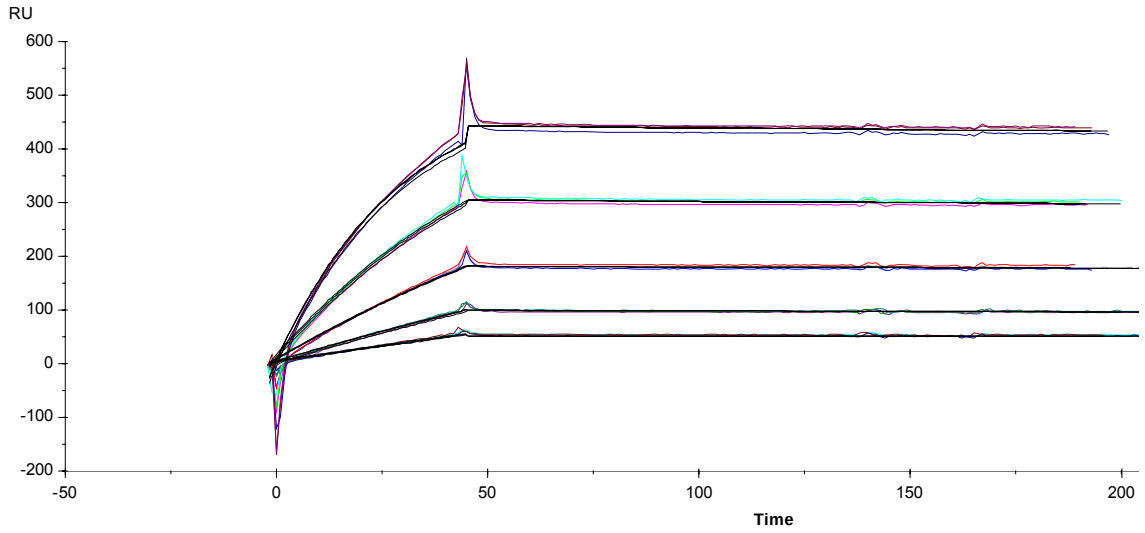


Fig. 24: Biomolecular interaction analysis of binding of Mab-HAV to MBP-AV1 by BIACORE (II.2.9.1). The graphs represent the FC2-FC1 curves. The MBP-AV1 antigen was immobilized on a BIACORE CM3 sensor chip on FC2 and BSA was immobilized on FC1. Increasing concentrations (17, 35, 70, 140, and 280nM) of Mab-HAV were injected over both FCs and the obtained data were analysed using the BIAevaluation (4.1). The kinetic values are shown in table 6.

Table 6: The kinetic analysis of binding of Mab-HAV (analyte) to MBP-AV1 (ligand) by BIACORE. ka and kd: the association and the dissociation rate constants, respectively. KA and KD: equilibrium binding and dissociation constants, respectively. Chi^2: standard statistical measure of the closeness of fit. T (ka-kd): the statistical T-values of ka and kd. Rmax: maximum analyte binding capacity.

	ka ($M^{-1}s^{-1}$)	(kd s^{-1})	KA (M^{-1})	KD (M)	Chi^2	T (ka-kd)	RU(max)
Mab-HAV	$1.21x10^5$	$1.48\ x10^{-4}$	$8.15\ x10^8$	$1.23\ x10^{-9}$	12.2	496-14.7	559

III.6.2 AV1-specific scFvs analysis by BIACORE

The scFvs impurity, dimerization, and multimerization made it difficult to measure the active concentration of each scFv. Surface plasmon resonance (SPR) provides a concentration-independent method to compare different scFvs by comparing their dissociation rate constants (kd). The binding activities of scFv-SAV and scFv-RW-AV to MBP-AV1 were compared using assay on BIACORE (II.2.9.2). The MBP-AV1 antigen was immobilized to a BIACORE CM3 sensor chip on the FC2. As a negative control, BSA was immobilized on FC1.

Visual comparison of the FC2-FC1 graphs (Fig. 25) as well as the kd values (Table 8) showed clearly that scFv-RW-AV (kd=7.51x10^{3}**s^{-1}**) had better binding activity than scFv-SAV (kd=1.35x10^{-2}**s^{-1}**) indicating that the frame correction and restoring the N-terminus of scFv-SAV has significant impact on the binding properties.

On the other hand, when scFvs SCRs-AV6, SCR-AV6, and SCR-AV6E (identical sequence except for position 105) are compared we find that SCR-AV6E (kd=1.07x10^{-2}s^{-1}) with E at position 105 has better binding properties than SCR-AV6 (kd=4.91 x10^{-1}s^{-1}) that has Q at that position. ScFv-SCRs-AV6 (kd=8.78x10^{-3}s^{-1}) that has a stop codon at position 105 and expressed in suppressor strain has better binding affinity than both scFvs SCR-AV6 and SCR-AV6E (Fig. 26 and Table 8). scFv-SCR-AV3E (kd=7.00x10^{-3}s^{-1}), which also has E at position 105, has a kd value close to that of SCRs-AV6 but this value was obtained from one injection and has to be confirmed by repeated injections.

ScFv-SCR-AV4 has a kd value (kd=7.62x10^{-3}s^{-1}) close to that of scFv-SCRs-AV6 and scFv-RW-AV whereas scFv-SCR-AV1 (kd=1.62x10^{-2}s^{-1}) has less binding affinity and its kd value is closer to those of scFvs SCR-AV6E and SCR-SAV (Fig. 25 and Table 7).

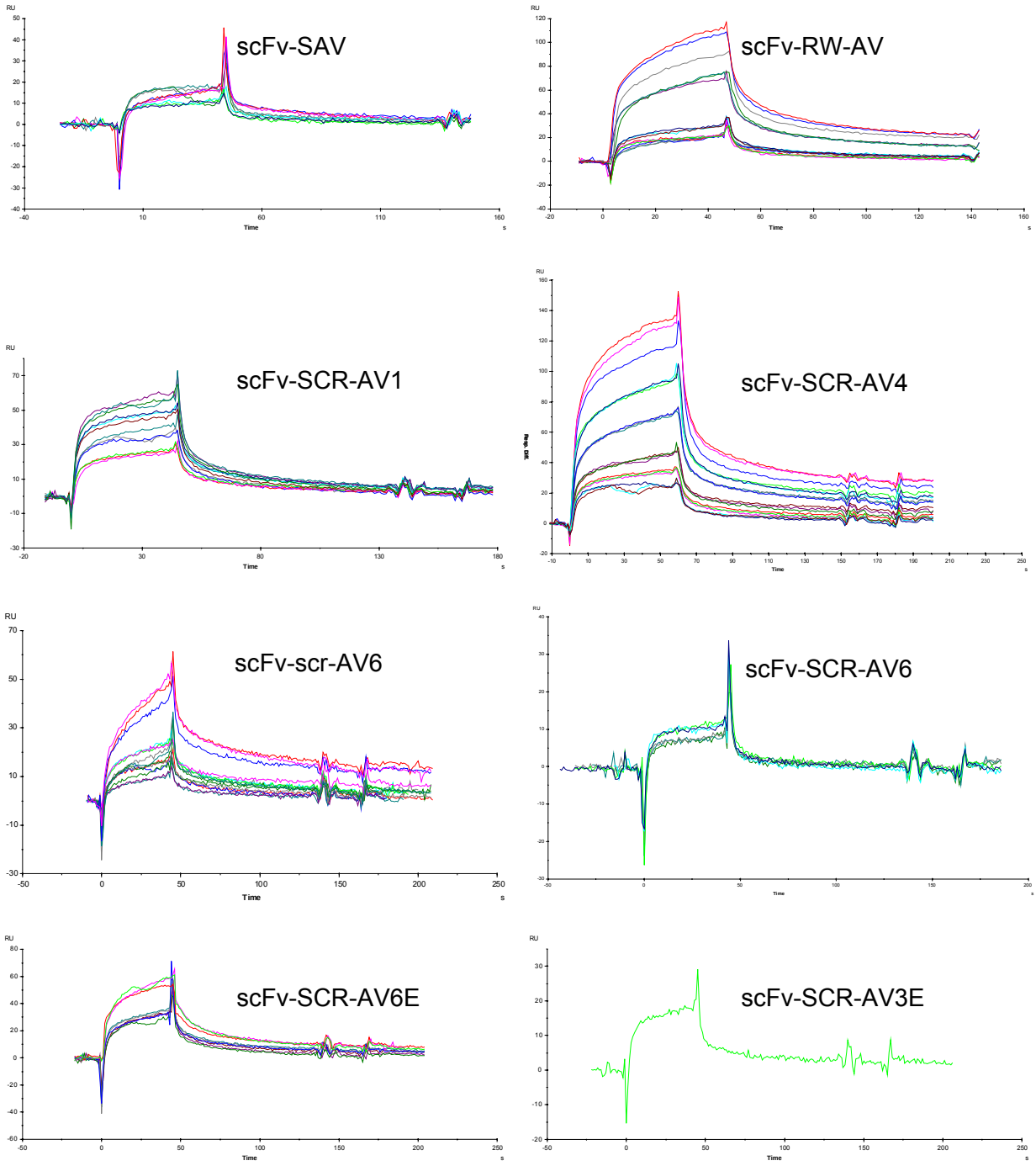


Fig. 25: Biomolecular interaction analysis of binding of AV1-specific scFvs to MBP-AV1 by BIACORE (II.2.9.1). The graphs represent the FC2-FC1 curves. The MBP-AV1 antigen was immobilized on a BIACORE CM3 sensor chip in FC2 and BSA was immobilized on FC1. Increasing concentrations of scFvs were injected over both FCs and the obtained data were analysed using the BIAevaluation (4.1).

Table 7: Off rate comparison between the AV1-specific scFvs. kd: the dissociation rate constants, respectively. Chi^2: standard statistical measure of the closeness of fit. T(kd): the statistical T-values of kd.

	kd (s^{-1})	Chi^2	T(kd)
scFv-SCR-AV1	$1.62x10^{-2}$	1.06	16.8
scFv-SCR-AV4	$7.62x10^{-3}$	0.58	28.7
scFv-SCR-AV3E	$7.00x10^{-3}$	0.81	11.1
scFv-SCRs-AV6	$8.78x10^{-3}$	0.45	20.6
scFv-SCR-AV6	$4.91x10^{-1}$	0.64	16.4
scFv-SCR-AV6E	$1.07x10^{-2}$	2.13	16.4
scFv-RW-AV	$7.51x10^{-3}$	0.77	26.4
scFv-SAV	$1.35x10^{-2}$	0.22	15.0

III.7 Cloning and transient expression of scFvs in tobacco leaves

III.7.1 ScFv cloning as N-terminal fusions with GFP

pTRA-GFP is a plant expression vector derived from pTRAkc (II.1.7) by insertion of the green fluorescent protein (GFP) coding sequence. For scFvs expression and localization in the cytosol as N-terminal fusion with GFP, scFv-SCR-AV3 was amplified by PCR from pIT-scFv-SCR-AV3 (III.4.2.1) using the primers Cyt-f and NLS-b (II.1.8). The amplified scFv was double digested with *Nco*I/*Afl*III and cloned upstream the GFP coding sequence in pTRA-GFP digested with *NcoI*. The correct cloning in the resulting plasmid pT-scFv-SCR-AV3-GFP (Fig. 26) was verified by test digestion and sequencing. The other scFvs SCR-AV1, SCR-AV4, SCR-AV6, RW-AV, SCR-AC, and RW-AC were each exchanged with scFv-SCR-AV3 in pT-scFv-SCR-AV3-GFP via *Nco*I/*Not*I digestion. The resulting plasmids were called pT-scFv-SCR-AV1-GFP, pT-scFv-SCR-AV4-GFP, pT-scFv-SCR-AV6-GFP, pT-scFv-RW-AV-GFP, pT-scFv-SCR-AC-GFP, and pT-scFv-RW-AC-GFP, respectively. The plasmids pT-scFv-SCR-AV3E-GFP and pT-scFv-SCR-AV6E-GFP, which have E instead of Q on position 105, were obtained by mutagenesis using the primers 5'AV3-E and 3'aAV3E (II.2.1.15) and the plasmids pT-scFv-SCR-AV3-GFP and pT-scFv-SCR-AV6-GFP as templates, respectively. Additionally, two scFvs (SCR-AC and SCR-AV3) were targeted as fusion with GFP to the nucleus by introducing the "SV40 T antigen" nuclear localization

signal (NLS) at the N-terminus of the scFvs. To do so, the same strategy as the one used for the cytosolic expression was used except for using the primer NLS-For (II.1.8) that carries NLS coding sequence as forward primer. The resulting plasmids were pT-NLS-scFv-SCR-AC-GFP and pT- NLS-scFv-SCR-AV3-GFP.

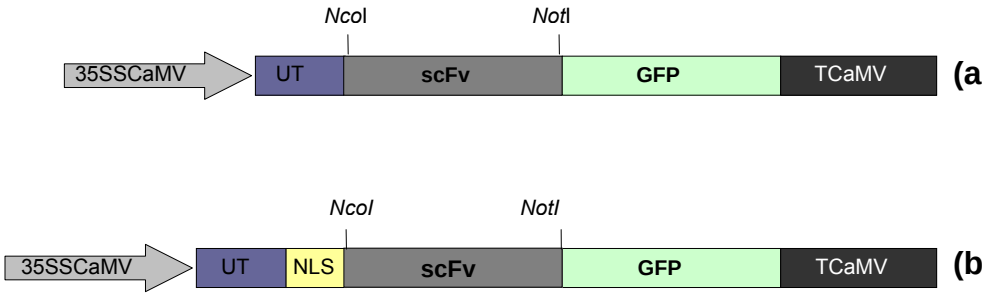


Fig. 26: Schematic presentation of the constructs used for scFvs expression in plant. The vector pTRAkc was used for expression in tobacco leaves. a: the constructs used for expression in the cytosol, b: the constructs used for expression in the nucleus. 35SSCaMV: 35S promoter from Cauliflower Mosaic Virus (CaMV) with duplicated enhancer, UT: 5` untranslated region of Chalconsynthase, TCaMV: termination sequence of CaMV, NLS: SV40 T antigen nuclear localization signal. The cloning sites are shown.

III.7.2 ScFv transient expression in tobacco leaves

Transient expression of scFv-GFP constructs was achieved either by injection of the recombinant *Agrobacterium* to the underside of a *N. benthamiana* leaf using a syringe without a needle as described in (II.2.2.2) and/or by particle bombardment of the recombinant plasmid (II.2.2.3). Both transformation methods were tested with most constructs and no significant difference was observed concerning the localization and level of expression of the fusion proteins (data not shown). All scFvs were successfully expressed except scFvs SCR-AV1, SCR-AV4, and RW-AC. The latest was detected only once and could not be successfully repeated. Confocal laser scanning microscopy (CLSM) (II.2.2.4) was used to monitor the intracellular localization of each construct. All scFv-GFP fusions without NLS were localized in the cytosol and the nucleus (Fig. 27). The level of expression, based on visual observations, was to some extent correlated with the expression level in *E. coli* without considering the scFvs which were not expressed successfully in plant. ScFvs SCR-AV3 and SCR-AV6 had the highest expression level and SCR-AC and RW-AV had the lowest expression level (Fig. 27). ScFvs with NLS (scFv-NSCR-AV3-GFP and scFv-NSCR-AC-GFP) were exclusively localized in the nucleus (Fig. 28).

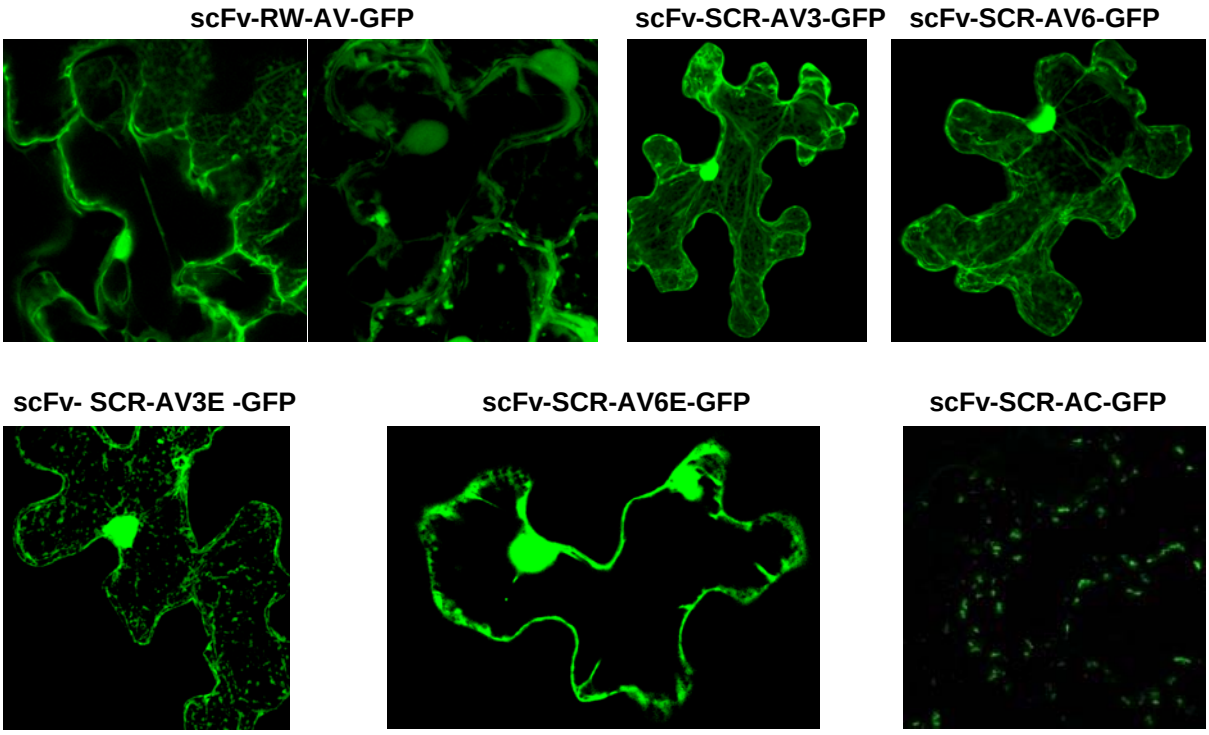


Fig. 27: scFvs Expression as fusion with GFP in *N. benthamiana* leaves. Tobacco leaves were transformed with plasmids (II.2.1.9) carrying the scFvs-GFP fusions either by agroinfiltration (II.2.2.2) (RW-AV-GFP and SCR-AC-GFP) or by particle bombardment (II.2.2.3) (SCR-AV3/AV6/AV3E/AV6E-GFP). ScFvs-GFP fusions expression and localization were analyzed using a Leica TCS SP spectral confocal laser-scanning microscope (II.2.2.4). GFP was excited at 488 nm and emissions collected at 500 to 515 nm.

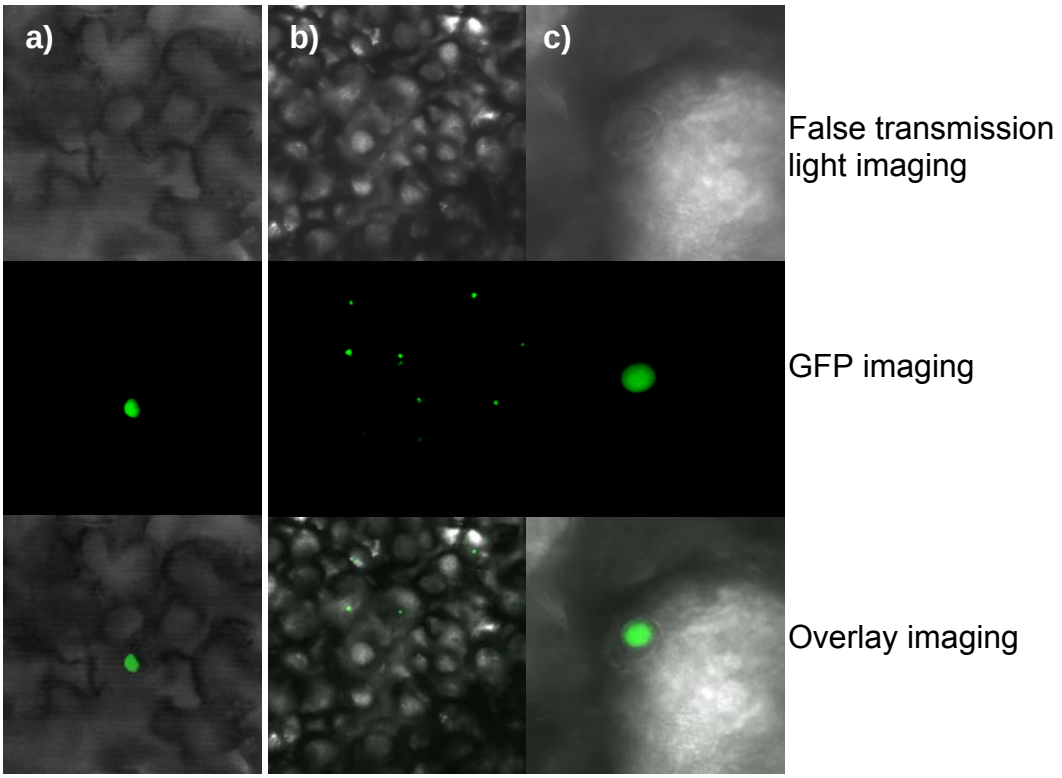


Fig. 28: Targeting the scFvs-GFP fusions into the nucleus. scFv-SCR-AV3 (a) and scFv-SCR-AC (b & C) were targeted to the nucleus as fusion with GFP. pT-scFv-NSCR-AV3-GFP was transiently expressed in tobacco leaves using particle bombardment (II.2.2.3) whereas agroinfiltration (II.2.2.2) (b) and particle bombardment (II.2.2.3) (c) were used to transform pT-scFv-NSCR-AC-GFP. To show the cell periphery false transmission light image was overlaid with the green emission image to produce the overlay image.

III.8 AV1-specific scFvs activity in vivo

III.8.1 ScFv-RW-AV binding activity

To test the scFv-RW-AV binding to AV1 *in vivo* scFv-RW-AV-GFP (III.7.1) and AV1-DsRed (III.1.3) were transiently co-expressed in tobacco leaves using agroinfiltration (II.2.2.2). The DsRed and GFP fluorescence was monitored by confocal microscopy. Interestingly, scFv-RW-AV-GFP and AV1-DsRed were co-localized in the nucleus (Fig. 29; a) clearly indicating that both fusions had bound to each other and driven to the nucleus by the NLS of AV1. ScFv-RW-AV-GFP was also visible in the cytoplasm and the nucleus when co-expressed with DsRed (Fig. 29; b). The same cytosolic localization was observed in case of scFv-SCR-AC-GFP when co-expressed with AV1-DsRed (Fig. 29; c). The latter two results proved clearly that the co-localization of scFv-RW-AV-GFP and AV1-DsRed was a

result of scFv-RW-AV and AV1 binding. The nuclear localization was further confirmed by DAPI staining (II.2.2.4) (Fig. 29).

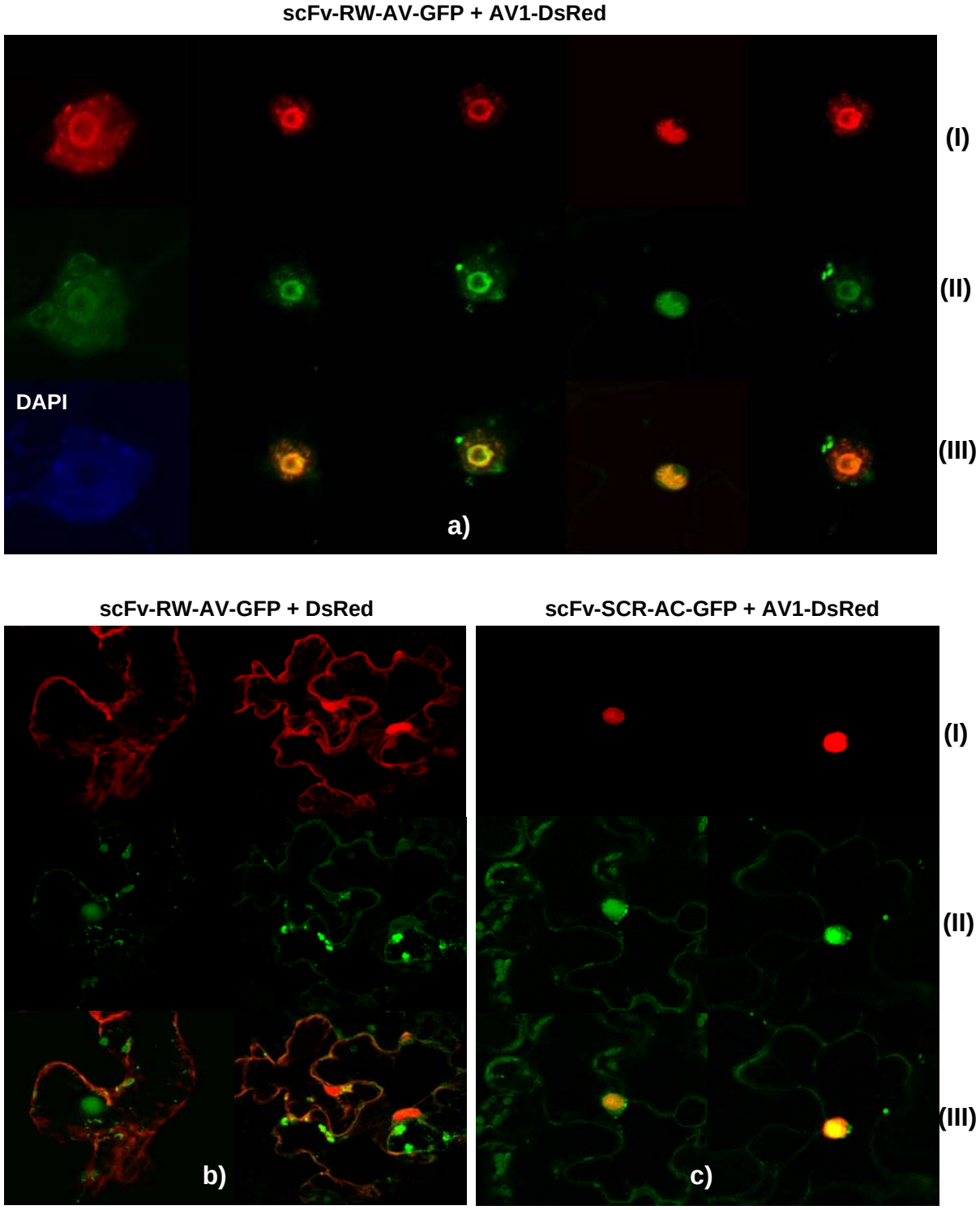


Fig. 29: scFv-RW-AV binding activity *in vivo*. AV1-specific scFv-RW-AV-GFP was co-expressed in tobacco leaves (II.2.2.2) with AV1-DsRed (a) and DsRed (b). AV1-DsRed was also co-expressed (II.2.2.2) with the AC1-specific scFv-SCR-AC (c). Fusion proteins expression and localization were analyzed using a Leica TCS SP spectral confocal laser-scanning microscope (II.2.2.4). Rows I and II show the DsRed imaging and GFP imaging, respectively. Row III shows the overlaid images except

for the left panel in (a) which shows the DAPI imaging. DsRed was excited at 568 nm and emissions collected at 600 to 620 nm. GFP was excited at 488 nm and emissions collected at 500 to 515 nm. For DAPI imaging, excitation at 405 nm and collection between 449 and 461 nm were used.

III.8.2 ScFv-SCR-AV3/6 and scFv-SCR-AV3/6E binding activity

SCR-AV6-GFP, SCR-AV3E-GFP, and SCR-AV6E-GFP were each co-expressed with AV1-DsRed in tobacco leaves using agroinfiltration (II.2.2.2). Opposite to scFv-RW-AV, none of the other AV1-specific scFvs showed clear binding activity *in vivo*. All scFv-GFP fusions were visible in the cytoplasm as well as the nucleus (Fig. 30). Some co-localized red and green spots could be seen but they are not enough to prove a possible binding.

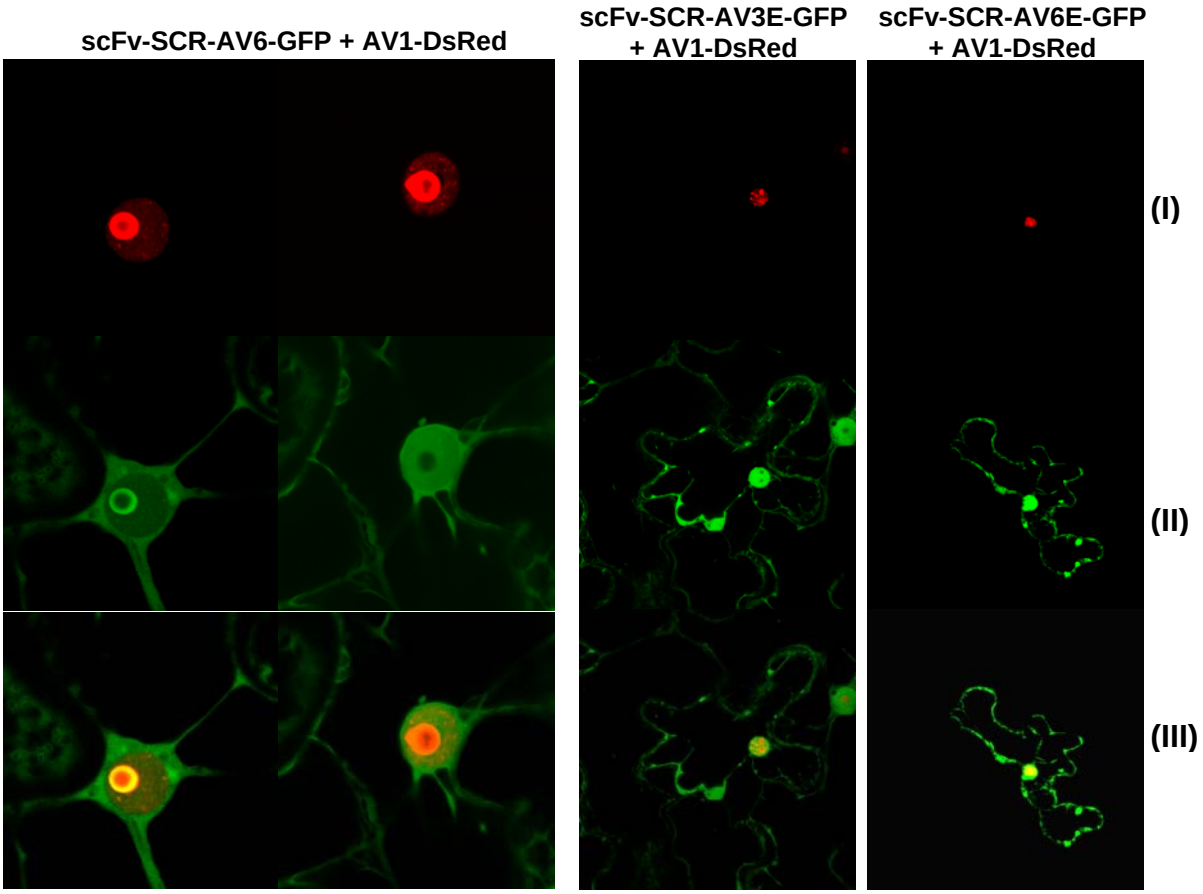


Fig. 30: Other AV1-specific scFv binding activity *in vivo*. ScFvs SCR-AV6-GFP, SCR-AV3E-GFP, and SCR-AV6E-GFP were each co-expressed with AV1-DsRed in tobacco leaves using agroinfiltration (II.2.2.2). The fusion proteins expression and localization were analyzed using a Leica TCS SP spectral confocal laser-scanning microscope (II.2.2.4). The rows I and II show the DsRed imaging and GFP imaging, respectively. The row III shows the overlaid images. DsRed was excited at 568 nm and emissions collected at 600 to 620 nm. GFP was excited at 488 nm and emissions collected at 500 to 515 nm.

IV Discussion

Viral diseases are a major issue in agriculture, and natural sources of resistance are limited. Therefore, great attention has been given to the different forms of engineered resistance. Transgenic expression of recombinant antibody fragments holds the promise to be one of those strategies.

The objective of this study was to generate and characterize specific scFv fragments against the AC1 (replication initiator protein) and AV1 (coat protein) of ToLCV-India geminivirus using hybridoma fusion and phage display technologies. Further analyses included transient expression/co-expression in tobacco leaves of the scFvs and the viral proteins as fusion with GFP and DsRed, respectively. Described in this study is the generation of two AC1-specific and five AV1-specific scFvs.

The AC1 and AV1 genes were amplified from a full length cDNA, cloned and expressed in *E. coli* as C-terminal fusions with GST (III.1.1) and MBP (III.1.2). The N-terminal domain of AV1 (56 aa) was also expressed as C-terminal fusion with GST (III.1.1). Additionally, the AC1 and AV1 genes were cloned and expressed in tobacco leaves as N-terminal fusions with DsRed.

SAC1 scFv library (III.3) and HAV hybridoma fusion (III.5.1) were constructed from spleen mRNA isolated from mice immunised with GST-AC1 and GST-AV1 fusion proteins (III.2), respectively. A Mab against AV1 (Mab HAV) was purified from the HAV hybridoma supernatant (III.5.2) and the scFv of this Mab (scFv-RW-AV) was cloned (III.5). Using the phage display technology one scFv against AC1 and four against AV1 were isolated from the naïve Tomlinson I library (III.4.1) and another scFv against AC1 was isolated from the SAC1 library generated in this study.

All scFvs were cloned and expressed in tobacco leaves as N-terminal fusion with GFP. The AV1-specific scFvs were analysed for their binding activity *in vivo* by co-expressing the scFv-GFP fusions with AV1-DsRed in tobacco leaves and monitoring their localization using confocal microscopy. Two scFv-GFP fusion proteins were also targeted to the nucleus by introducing N-terminal “SV40 large T antigen” NLS by PCR.

IV.1 Generation and characterization of scFvs specific against AC1 and AV1

IV.1.1 Bacterial expression and purification of recombinant viral antigens

Obtaining sufficient amounts of the viral antigens was necessary to immunize mice, to pan the scFvs phage libraries, and to characterize the selected scFvs. The AC1 and AV1 cDNAs were cloned (following the same strategy for both genes) downstream the GST gene in pGEX-5X-3 vector and expressed as C-terminal fusions with GST (GST-AC1 and GST-AV1) using the GST expression vector. Similarly, the AC1 and AV1 genes were cloned and expressed as C-terminal fusion with MBP in pMAL-c2X. Using GST and MBP as fusion partner allows one-step affinity purification of the fusion protein under non-denaturing conditions. Additionally these fusion partners may enhance the solubility of AC1 and AV1 as reported for GST (Frangioni and Neel, 1993) and shown in our results for MBP. Overexpression of AC1 and AV1 fusions in both systems resulted in formation of inclusion bodies. To overcome this problem and obtain enough soluble and correctly folded protein the induction condition had to be optimized. This was achieved by lowering the induction temperature to 18-22ºC and increasing the induction time to 12-16h. By doing so, the driving force for protein self-association is decreased and the fusion proteins have extra time to fold correctly. The relatively low temperature has limited effect on the protein folding whereas the level of transcription and translation is drastically decreased (Goldenberg et al., 1996; Farewell and Neidhardt, 1998; Hunke and Betton, 2003; Schumann and Ferreira, 2004). Another problem was the partial protein precipitation (especially in case of the AV1 fusions) during and after the purification steps. This problem persisted in all tested buffers and purification conditions (data not shown). The loss in the purified proteins due to the precipitation was decreased by performing the purification steps as fast as possible and freezing the purified fractions immediately after purification. The bacterially expressed coat proteins of different geminiviruses were recovered from SDS-gels and freeze-dried until immunization (Abouzid et al., 2002).

Although the optimized conditions did not completely prevented formation of inclusion bodies and despite the precipitation problem the obtained amounts of the soluble fusion proteins were sufficient to carry out all necessary assays. 1-1.5mg and up to 4mg/L culture of GST and MBP fusion proteins were obtained, respectively.

The N-terminal domain of AV1 (56 aa) which contains the NLS was also cloned and expressed as fusion with GST. This domain was chosen because it has been reported that it contains functional NLS in both mono- and bipartite geminiviruses (Kunik et al., 1998; Qin et al., 1998; Liu et al., 1999; Kotlizky et al., 2000; Unseld et al., 2001; Unseld et al., 2004). This fusion protein was used for the analysis of the AV1-specific scFvs in ELISA. Around 5mg of soluble protein was obtained from 1L culture.

IV.1.2 Mice immunization

Mice were immunized with GST-AC1 and GST-AV1 fusion proteins to elicit strong immune response against AC1 and AV1 viral antigens by repeated injection of small doses of the antigens. Serum titers were over 1:150,000 with high cross reactivity of both polyclonal antisera with the fusion partner GST. To confirm the presence of antibodies against the viral antigens, the GST binders were pre-adsorbed by high concentration of purified GST. The cross reactivity with free GST was drastically lowered and high reactivity with the antigen fusion was observed when the pre-adsorbed antisera were used in ELISA indicating that both GST-AC1 and GST-AV1 proteins have triggered good immune response to AC1 and AV1 proteins, respectively. The high immunogenicity of begomoviruses coat proteins has been previously reported. Antibodies to bacterially expressed coat proteins of different geminivirus coat proteins were obtained by rabbit immunization (Abouzid et al., 2002; Harrison et al., 2002).

IV.1.3 Phage display

A scFv library (SAC1) was constructed from the spleen cells of the mouse immunized with GST-AC1 fusion protein using a set of specific SOE primers (II.1.8) making use of the sequence conservation in the frameworks of V_H and V_L. The forward primers for both V_H and V_L regions amplification were designed so that they bind to the 1st framework region while the back primers anneal to the 4th framework region of the variable domains of the antibodies. To amplify most of the mRNA of the mouse immunoglobulin repertoire the primers contained degeneracies at certain positions. The overhang region of the primers introduced the linker and restriction sites regularly not found in the mouse variable antibody domains to enable cloning of the scFvs to the phagemid vector pHENHI. The *Sfi*I and *Nco*I sites were incorporated at the 5` end of the $V_H$ while *Not*I site was introduced at the 3` end of V_L. The V_H and V_L genes were joined by SOE reaction and PCR-amplified (Fig. 12; a). The size of

the resulting SAC1 library was $2x10^7$ and the percentage of the positive clones carrying full size scFvs in the randomly selected clones was 100% as shown by the colony PCR test (Fig. 12; b).

Phage display technique was used to screen the SAC1 scFvs library for specific binders, GST-AC1 fusion protein was used in three panning rounds. There was no clear enrichment of specific phages after the third round of panning of SAC1 library. This could be due to a problem associated with the SOE primers, which were used to create this library. Obviously these primers induced a frame shift in most of the scFvs due to low quality of oligonucleotide synthesis. After the third selection round more than 700 clones were tested for AC1 specificity by soluble ELISA. Few clones showed reactivity with GST and only two identical clones expressed scFv (scFv-RW-AC) against AC1.

After the limited success with the constructed scFvs library the naïve library Tomlinson I was screened by phage display for scFvs against AC1 and AV1 viral proteins. Many naïve libraries have been successfully used to derive highly specific antibody reagents to a wide range of different proteins, peptides or small molecule compounds without the need for animal immunization. Selection and affinity maturation were performed by immobilizing the antigens on immunotubes. For both antigens four rounds of selection were carried out using two different fusion proteins for each antigen. GST-AC1 and MBP-AC1 were used to select AC1 binders. Similarly, GST-AV1 and MBP-AV1 were used to select AV1 binders. The GST fusions were used in the first and the fourth selection rounds whereas the MBP fusions were used in the second and the third rounds. The idea behind using different fusions of the same antigen is to exclude the binders raised against the fusion partners (GST and MBP). After the fourth selection round, approximately 30% of the analyzed clones showed a specific reactivity with the corresponding AC1 and AV1 antigens (Fig. 14). No MBP/GST binders were found indicating the efficiency of using two different antigen fusions for excluding potential binders against the fusion partners (GST/MBP). All sequenced scFvs against AC1 were identical resulting in one scFv-SCR-AC whereas four different scFvs (SCR-AV1, SCR-AV3, SCR-AV4, and SCR-AV6) against AV1 were identified. The selected AV1 specific clones were the ones with **a:** high reactivity with GST-AV1 and MBP-AV1. **b:** no cross reactivity with GST-AC1, GST, and MBP-AC1. The same strategy was used to select AC1 binders. The reaction of a scFv with its antigen in two different fusions confirms its specificity and probably indicates that the antigen (AC1 or AV1) folding was not affected by

the fusion partner. Additionally, the fact that the cloning strategy and the vector used for cloning of both GST fusions were identical excluded the possibility that the scFv was raised against the linker.

All four scFvs against AV1 reacted in ELISA assay with GST-AVNS (Fig. 15) indicating that their epitopes are located in the N-terminus of AV1. Therefore, each of the four scFvs is a potential candidate to interfere with AV1 NLS function and other functions assigned to the N-terminal domain like DNA binding and multimerisation (Liu et al., 1997; Qin et al., 1998; Hallan and Gafni, 2001; Harrison et al., 2002).

Two scFvs, SCRs-AV3 and SCRs-AV6 carried the stop codon "TAG" at position H105, and since the TG1 strain used in phage display is a suppressor strain, which translates the "TAG" codon to E, it was possible to select such scFvs. Comparing the aa sequences of these scFvs with other scFvs originated from the same library and other scFvs from the database, belonging to the same murine subgroup, showed that position H105 is always occupied by the aa Q. The difference between "TAG" (stop codon) and "CAG" (encoding Q) and "GAG" (encoding E) is only one nucleotide, so the stop codon is a result of a point mutation.

Replacing the stop codon with "CAG" (coding for the aa Q) by mutagenesis (scFv-SCR-AV3/6) resulted in significant loss in the binding activity compared to the original un-mutated scFv-SCRs-AV6, expressed and purified from suppressor strain TG1, as shown by ELISA and BIACORE results (Fig. 16; b & 25). This replacement has led, however, to huge increase in the level of expression compared to other scFvs including the original scFv-SCRs-AV6. Around 4-5mg were purified from 200ml culture. When the stop codon was exchanged for "GAG" (coding for the aa E) (scFv-SCR-AV3E/6E), clear improvement in the binding activity was notable in ELISA and BIACORE (Fig. 16; b & 25). However, the improvement in the binding activity was accompanied with a decrease in the level of expression. Regarding the significance of position H105, located in framework 4 of V_H 6 residues away from CDRH3, there was no indication in the literature that this position might be critical for bacterially expressed scFvs. On the other hand, the importance of framework residues has been shown in many studies. Framework residues are usually involved in supporting the conformation of the CDR loops. So a change in the framework residues might change the CDR conformation, and therefore the binding activity (Langedijk et al., 1998; Khalifa et al., 2000; Jung et al., 2001; Ewert et al., 2003). Adjacent residues were found to affect the CDR conformation and to fine-tune antigen recognition (Foote and Winter, 1992; Honegger and

Plückthun, 2001). It has been shown that binding interactions are not limited to the CDRs, but also some framework residues may directly contact antigens (Ewert et al., 2004). A number of mutations have been identified in different framework positions such as H6, H7, H9, H66, H101, L8, L82, and L89 to have effect on the stability and/or functionality of scFvs (Morea et al., 1998; Wörn and Plückthun, 2001; Ewert et al., 2004). Q is the most frequent aa at this position in an alignment of homologous scFvs and thus should be favourable for the stability of the scFv (Ewert et al., 2003). The effect of Q→E substitution was shown for other framework residues rather than H105. A loss of binding affinity when Q was replaced with E at position H6 in framework 1 has been reported (Kipriyanov et al., 1997; De Haard et al., 1998), while in other cases the binding was not affected (Langedijk et al., 1998; Khalifa et al., 2000; Jung et al., 2001). Kipriyanov et al. (1997) found that substitution of E at position H6 in framework 1 by Q led to a more than 30-fold increase in the production of a very poorly soluble scFv. Forsberg et al. (1997) showed that Fv framework substitutions significantly enhanced the yield of Fab-fusion protein in *E. coli*. It was suggested that placing a negatively charged E residue into the core of the V_H domain would always have a destabilizing effect, and that this is the reason for the effect of the substitution on production levels, stability, and functionality of the antibodies (Honegger and Plückthun, 2001).

IV.1.4 Cloning a scFv specific for AV1 from a hybridoma clone

IV.1.4.1 Hybridoma fusion

Derivatives of BALB/c myelomas have become the most commonly used partners for fusions. The other fusion partners are the cells isolated from immunised animals. These cells carry the rearranged immunoglobulin genes that specify the desired antibody. Because of the difficulties in purifying cells that can serve as appropriate partners, fusions are normally performed with a mixed population of cells isolated from a lymphoid organ of the immunised animal. Although a number of studies have helped to characterize the nature of this B-cell-derived partner, the exact state of differentiation of this cell is still unclear (Köhler and Milstein, 2005).

Hybridomas can be prepared by fusing myelomas and antibody-secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used (Littlefield, 1964; Köhler and Milstein, 2005). Spleen cells from the mouse immunized with GST-AV1 were fused with a myeloma cell line to develop a

hybridoma clone that secretes a specific antibody against AV1 protein. Such clones were isolated from a mixed population of cells (including non-secreting hybridomas, cells secreting antibodies of specificities other than the one desired, other cell types that survive in HAT-medium, etc.) by four rounds of limiting dilution with ELISA tests against GST-AV1 and MBP-AV1 after each round. The limiting dilution is based on the assumption that if a suspension of cells is diluted with sufficient culture medium, a concentration of cells will be produced such that an accurately measured volume of the diluted suspension will contain 1 cell (Fazekas de St, 1982). Fourteen positive clones producing Mabs against AV1 were identified after the 4th round of limiting dilution. All isolated clones showed similar binding activity in ELISA and BIACORE and similar levels of antibody secreted into the supernatant demonstrating the homogeneity of the cell line (data not shown). One of these clones (HAV) was selected and propagated for purification of the Mab-HAV and cloning the scFv of this Mab.

IV.1.4.2 Purification and characterization of the mouse Mab-HAV

Mab-HAV was purified from HAV hybridoma cell line using protein A matrix. Around 10 mg of purified antibody was obtained from one litre of hybridoma culture supernatant. SDS-PAGE analysis of the purified Mab HAV showed two major bands of approximately 53 and 26 kDa corresponding to the mouse H and L chains of Mab HAV, respectively (Fig. 18). The purified Mab HAV showed high specificity and reactivity with AV1 in ELISA, WB, and BIACORE. Mab HAV reacted with MBP-AV1 and GST-AV1 in ELISA but it did not recognize GST-AVNS (Fig. 19; a) which may indicate that its epitope, or at least part of it, is not in the N-terminus. That is why Mab HAV is a potential binder against a broad spectrum of geminivirus coat proteins. This hypothesis is based on the fact that most of the variability in aa sequences of begomoviruses coat proteins is limited to the N-terminal 60-70 aa residues, the rest of the CP structure is highly conserved (Abouzid et al., 2002; Harrison et al., 2002). This conclusion was supported by Mab HAV reactivity with bacterially expressed and purified TYLCV-Iran CP in ELISA (data not shown). The epitope is likely of linear nature as Mab HAV has bound to denatured MBP-AV1 fusion protein in WB assay (Fig. 19; b). Rabbit antiserum raised against purified TYLCV-CP could detect begomoviruses in denatured and undenatured states in WB and ELISA (Abouzid et al., 2002). The bimolecular interaction between Mab HAV and its antigen AV1 was further monitored by surface plasmon resonance assay on BIACORE. In recent years, biosensor instruments based on

surface plasmon resonance have been increasingly used for measuring the binding characteristic of biomolecules. Of those instruments, BIACORE has been used in about 90% of the studies (Rich and Myszka, 2001 a; Rich and Myszka, 2001 b; Choulier et al., 2002). A major advantage of the technique is that interactions are measured in real time which allows reliable kinetic rate constants to be determined (Day et al., 2002).

MBP-AV1 (ligand) was immobilized on the surface of FC2 whereas the surface of the reference cell (FC1) was coated with unrelated protein, BSA. Immobilizing an unrelated protein on the reference cell counteracts the non-specific binding between the analyte (Mab HAV) and the dextran surface and/or the sensor chip (Gonzales et al., 2002). The difference in the magnitude of the signals generated by the binding of Mab HAV to the surfaces of FC 2 and 1 (Fig. 24) provides real time kinetics of specific binding of Mab HAV to the immobilized MBP-AV1. The high affinity of Mab HAV was reflected by obtained kinetic data (Table 7) with equilibrium disassociation constant of $1.23x10^{-9}$M and association and disassociation constant rates of $1.21x10^{5}$ $M^{-1}s^{-1}$ and $1.48x10^{-4}s^{-1}$, respectively.

IV.1.4.2 Cloning the variable domains of Mab HAV

The V_H and the V_L of Mab HAV were amplified from cDNA and cloned in the pHENHI vector for the generation of a mini-scFv library following the same strategy used for the construction of the SAC1 phage library (IV.1.3). Screening and sequencing some colonies from the mini-scFv library revealed its homogeneity and all sequenced clones were identical (scFv-SAV). Soluble expression and WB analysis of scFv-SAV revealed a band with the expected size for a scFv (Fig. 20; b). ScFv-SAV showed weak reactivity with MBP-AV1 and GST-AV1 in direct ELISA (Fig. 20; a). The nucleotide sequence analysis of scFv-SAV revealed one open reading frame (ORF) translated from an internal start codon 65 nucleotides downstream the ATG start codon in the *Nco*I site where the scFvs translation was supposed to start from. The original ORF was disrupted by a stop codon (TAG) 60 nucleotides downstream the start codon. The aa sequence of scFv-SAV obeyed to the KABAT rules for antibodies except for the missing N-terminus. Alignment of the nucleotide sequence of scFv-SAV with other scFvs from the database using BLAST showed a possible point mutation (deletion) at position 30 downstream the original ATG start codon in the *Nco*I site. The mutation might have been introduced during the PCR amplification of the scFv gene and it is located in framework 1 of V_H. Different clones derived from the same amplification reaction, even starting from cDNA derived from one particular hybridoma clone, may show

considerable sequence variability in the first few residues at the N-terminus of either domain, since different molecules in the degenerate primer set may hybridize productively (Honegger and Plückthun, 2001). The nucleotide "A" was found at the same position in other scFvs from the database. Insertion of the nucleotide "A" at the position of the point mutation brought both ATG start codons to the same frame and resulted in new scFv-RW-AV whose sequence is identical to scFv-SAV in addition to the missing 21 aa at the N-terminus (Fig. 21). The new scFv-RW-AV obeyed fully to the KABAT rules for antibodies.

ScFv-SAV and scFv-RW-AV were compared using SDS-PAGE, ELISA, and BIACORE assays. The Coomassie stained gel and WB analysis confirmed the correct "A" insertion by showing the difference in MW between both scFvs due to the additional 21 aas at the N-terminus of scFv-RW-AV (Fig. 22). In ELISA, scFv-RW-AV showed higher reactivity with AV1 than scFv-SAV (Fig. 23). But because of the high level of impurities and the difficulties to determine the active concentration of both scFvs the ELISA is not highly reliable method to compare the two scFvs. Alternatively, their dissociation constant rates (off rates) were measured and compared using BIACORE. This can be done independently of the concentration using the BIAevaluation (4.1) software. In many cases, the dissociation constant rate is the crucial parameter controlling the presence of a useful function (Choulier et al., 2002). In cases of viruses, the capacity of an antibody to neutralize virus infectivity tends to be correlated with the antibody off rate rather than the equilibrium constant rates (VanCott *et al.*, 1994; Van Regenmortel, 2001; Zeder-Lutz *et al.*, 2001). As shown in Fig. 25, scFv-SAV showed very weak binding even when the highest available concentration was used. ScFv-RW-AV showed, on the other hand, better binding activity as can be concluded from the curves in Fig. 25 and the calculated off rates (scFv-SAV=$1.35x10^{-2}s^{-1}$; scFv-RW-AV=$7.51x10^{-3}s^{-1}$). The weak binding of scFv-SAV compared to scFv-RW-AV is probably due to the missing 19 aa from its V_H framework 1 which is known to have high impact on scFvs functionality, conformational structure, stability, and production yield (De Haard *et al.*, 1998; Langedijk *et al.*, 1998; Lo-Man *et al.*, 1998; Honegger and Plückthun, 2001; Kala *et al.*, 2002). Framework 1 of the V_H4-34 gene segment is essential for the interaction between antibodies to cold agglutinin and the I antigen (Li et al., 1996). The aa at positions H6, H7, and H10 define the distinct conformations of framework 1 of V_H (Jung 2001). Particularly H6, a buried glutamine or glutamate residue, has been found to have a strong effect on antibody functionality (Langedijk *et al.*, 1998; Lo-Man *et al.*, 1998; Honegger and Plückthun,

2001). Substitutions of residue H6 can have drastic effect on stability and potentially on antigen affinity (Ewert et al., 2004). (Khalifa et al., 2000) have shown that binding kinetics are affected by framework residues remote from the binding site. In many cases, PCR-induced sequence changes in the frameworks resulted in impaired antigen-binding, poor production yield, and decreased thermodynamic stability although in some other cases the resulting scFvs were fully functional (De Haard *et al.*, 1998; Honegger and Plückthun, 2001). The ability of scFv-SAV to bind to AV1 despite the missing 19aa is surprising and no similar incident has been reported to date. Recombinant antibodies smaller than "normal" scFv like mouse single variable domains (Ward et al., 1989; Holliger and Hudson, 2005) and camelids and cartilaginous fish single V-like domains (Muyldermans and Lauwereys, 1999; Holliger and Hudson, 2005) were shown to be functional. Heavy-chain antibodies can also occur in human and mouse myeloma mutants (Seligmann, 1979; Seligmann et al., 1979; Muyldermans and Lauwereys, 1999). Cloning of V_Hs from an immunized mice proved that it is feasible to isolate antigen binders (Ward et al., 1989). But since the antigen binding site is matured during immunization as a combination of V_H and V_L, the isolated V_Hs had a lower affinity for antigen compared to the original, matured antibody (Muyldermans and Lauwereys, 1999) . Clarifying weather scFv-SAV binds to AV1 via its V_H and V_L or V_L alone needs further investigation.

IV.2 Expression of the AV1 gene in tobacco leaves

The AV1 gene was cloned into the plant expression vector pTRAkc as N-terminal fusion with DsRed and expressed in tobacco leaves. The N-terminal cloning of AV1 guarantees that all the fluorescence will be a result of the fusion expression and not the DsRed alone, agroinfiltration and microprojectile bombardment were used as means of transformation. No clear difference between the two methods was noted except for the number of transformed cells. Only few cells surrounding the bombarding point were transformed whereas most of the cells were transformed with *Agrobacteria*. The intracellular localization of the AV1-DsRed was monitored under confocal laser scanning microscope. Three days post-infiltration AV1-DsRed was present exclusively in nuclei (Fig. 9; a, b, & c), preferentially targeting nucleoli and forming a circle around it (Fig. 9; a & b). The AV1-DsRed molecular weight is 55.4 kDa is above the size for efficient passive nuclear diffusion (20-40 kDa) (Gorlich and

Mattaj, 1996; Grebenok et al., 1997; Kotlizky et al., 2000; Ribbeck and Gorlich, 2002). This localization is consistent with the presence of NLS in the coat protein of geminiviruses (Kunik et al., 1998; Qin et al., 1998; Liu et al., 1999; Kotlizky et al., 2000; Unseld et al., 2001; Unseld et al., 2004). Similarly, the MSV and ACMV CP-GFP fusions were exclusively localized in the nucleus (Kotlizky et al., 2000; Unseld et al., 2001). As a control, free DsRed was localized in the cytoplasm and the nucleus. Seven days post-infiltration, some AV1-DsRed fluorescence was observed in the cytosol (Fig. 9; d). This retargeting is probably a result of the AV1 NES activity. This observation is contrary to the bipartite MSV CP-GFP fusion that was localized in the nucleus and did not leave it again (Kotlizky et al., 2000). The central domain of the bipartite ACMV CP100-127 was able to direct GFP out of the nucleus which is thought to be mediated by three leucine residues (positions: 102, 118, and 123) (Unseld et al., 2001). Only the first two leucine residues were found at the same position as ACMV CP in the ToLCV-India AV1, which was used in this study, the third position is occupied by methionine residue (data not shown). Free DsRed was distributed evenly in the nucleus including the nucleolus whereas AV1-DsRed fusion formed a circle around (probably inside) the nucleoli and spots (aggregates) in nucleoplasm (Fig. 9; a & b). Similar observation was previously reported, the ACMV CP-GFP fusion was localized exclusively in the nucleus of maize and tobacco (Kotlizky et al., 2000) and the Potato leafroll Virus CP-GFP localized in the nucleus, preferentially targeting the nucleolus (Haupt et al., 2005). A number of viruses (including DNA viruses) interact with the nucleolus and its proteins. Certain viral proteins co-localize with, recognize and re-distribute nucleolar antigens such as nucleolin. It has been suggested that viruses may target the nucleolus and its components to favour viral transcription, translation, and perhaps to alter cell growth and the cell cycle to promote virus replication (Kim et al., 2004).

IV.3 Expression of scFvs in tobacco leaves

All selected scFvs against AV1 and AC1 were transiently expressed in tobacco leaves as N-terminal fusion with GFP using either agroinfiltration and/or particle bombardment as means of transformation. No significant difference was observed between the two methods concerning the localization and level of expression of the fusion proteins. The rational behind using different transformation methods is the additional confirmation of the obtained results

and proving that the failure in some cases is not due to the transformation method. Fusion of scFvs with GFP has been shown not to alter the binding properties of the scFvs (Hink et al., 2000; Lu et al., 2005). The scFvs were cloned as N-terminal fusions and this guarantees that all observed green fluorescence resulted from the scFvs-GFP fusion and not from free GFP. The scFvs were tested for their intracellular distribution in the cytosol and nucleus. All scFvs were successfully expressed as GFP fusions in the cytosol except scFvs SCR-AV1, SCR-AV4, and RW-AC. ScFv-RW-AV that originated from hybridoma clone in addition to scFv-SCR-AV3, scFv-SCR-AV3E, scFv-SCR-AV6, scFv-SCR-AV6E, and scFv-SCR-AC, selected by phage display, showed clear localization in the cytosol. Different recombinant antibodies were successfully expressed in transgenic plants (De Jaeger et al., 2000) and were accumulated successfully in different plant cell compartments (Conrad and Fiedler, 1998; Conrad and Manteuffel, 2001; Jobling et al., 2003). Expression of scFvs in the cytoplasm of plants has long been troublesome (Prins et al., 2005) and only in few cases, individual antibody fragments not specifically selected for intracellular expression could be produced as functional cytoplasmic proteins (Hyland et al., 2003). A scFv could be expressed in a functional form in the cytoplasm of *E. coli* (Lombardi et al., 2005). High expression levels in the plant cytoplasm of phage-display derived anti-viral scFvs were reported (Boonrod et al., 2004; Prins et al., 2005). For stability and functional activity, antibodies are usually dependent on the formation of intradomain disulfide bonds within the variable regions of antibody H and L chains (Schouten et al., 1997; Ewert et al., 2003). The disulfide bonds are correctly assembled in the ER but not in the reducing environment in the cytosol (Proba et al., 1997; Cattaneo and Biocca, 1999; Hyland et al., 2003; Prins et al., 2005). The level of expression, based on visual fluorescence observations, was to some extent correlated with the expression level in *E. coli* (Fig. 27). ScFvs SCR-AV3 and SCR-AV6 (both selected from Tomlinson I library by phage display) had the highest expression level whereas SCR-AC and RW-AV (originated from hybridoma clone) had the lowest expression level (Fig. 27). It was previously shown that phage display-derived scFvs possess enhanced stability and high level of cytosolic expression, probably due to phage mediated selection procedure (Ohage et al., 1999; De Jaeger et al., 2000; Wörn and Plückthun, 2001; Prins et al., 2005).

To test the possibility of targeting a scFv-GFP fusion to the nucleus, the SV40 large T antigen NLS (Kalderon et al., 1984) was introduced by PCR to the N-terminus of two scFvs-GFP fusions (scFv-NSCR-AV3-GFP and scFv-NSCR-AC-GFP). Targeting the specific scFvs

to the nucleus may interfere with viral functions which take place in the nucleus, such as replication and particle assembly. Both fusions, when transiently expressed in plant, were exclusively localized in the nucleus (Fig. 28). Mhashilkar et al., 1995 demonstrated that scFvs can be localized and function in the nucleus of eukaryotic cells. It was shown recently that a scFv against the CP of *Plum pox virus* was localized to the nucleus and led to high reduction in viral infection (Gil et al., 2005). However there is no indication in the literature for targeting of a scFv as fusion protein in the nucleus to date.

IV.4 Functional activity of AV1-specific scFvs in vivo

The *in vivo* binding activity of the AV1-specific scFvs was tested by monitoring their effect on the nuclear localization of AV1-DsRed. ScFv-SCR-AV3-GFP, scFv-SCR-AV3-E-GFP, scFv-SCR-AV6-GFP, scFv-SCR-AV6-E-GFP, and scFv-RW-AV-GFP were each co-infiltrated with AV1-DsRed fusion in tobacco leaves. We speculated that a specific scFv may interfere with AV1 function in the cytosol and probably prevents AV1 from entering the nucleus where the geminiviruses replication takes place (Gafni and Epel, 2002) and thus interfering with the viral infection. The intracellular expression of specific antibody (intrabodies) fragments holds the promise of being potential strategy to prevent viral infection (Boonrod et al., 2004; Prins et al., 2005). Several studies have shown that specific intrabodies could result in incomplete resistance against ACMV (Tavladoraki et al., 1993), delay and suppression of *Beet necrotic yellow vein virus* symptoms (Fecker et al., 1996) and TMV (Schillberg et al., 2001), and complete resistance against *Tomato bushy stunt virus* (Boonrod et al., 2004). High level of resistance could be achieved by cytosolic expression of scFvs raised against *Tomato spotted wilt virus* (Prins et al., 2005). None of the scFvs showed clear effect on the nuclear localization of AV1-DsRed. However the co-expression of scFv-RW-AV-GFP and AV1-DsRed resulted in nuclear co-localization of both fusion proteins (Fig. 29). This result probably indicates that scFv-RW-AV bound AV1 in the cytosol and then the whole protein complex was driven to the nucleus by the AV1-NLS. This hypothesis is supported by the observation that scFv-RW-AV had no effect on the DsRed localization and *vice versa* and that scFv-SCR-AC localization was not altered when co-expressed with AV1-DsRed (Fig. 29). The inability of scFv-RW-AV to interfere with nuclear localization of AV1 is probably because its epitope is not located in the N-terminus of AV1 where the main

NLS is located. The monoclonal antibody Mab HAV, which scFv-RW-AV was derived from, did not bind to the GST fusion protein GST-AVNS, which covers the main NLS present at the N-terminal part of AV1, in ELISA assay (III.5.2). The scFv-RW-AV failure to prevent AV1-DsRed fusion from entering the nucleus does not exclude the possibility to inhibit the entry of the complete viral particles into the nucleus when covered with many scFvs. If not, scFv-RW-AV is still a potential candidate to interfere with other biological functions of AV1. The central and the C-terminal domains of AV1 are involved in viral transmission (Hofer et al., 1997; Noris et al., 1998; Hohnle et al., 2001), multimerisation (Hallan and Gafni, 2001), nuclear export, and DNA binding (Unseld et al., 2001; Unseld et al., 2004). The relatively low cytosolic expression level of scFv-RW-AV (Fig. 27) is probably the main drawback of this scFv. Positive correlation between the concentration of scFv and the susceptibility to viral infection has been reported (Boonrod et al., 2004). In the early stages of infection, however, the viral particles concentration is relatively low and might be neutralized by low concentration of the scFvs, further analysis (like viral challenging) are needed to asses the scFv effect on viral infection.

The other scFvs (SCR-AV3 and SCR-AV6 and their modified forms SCR-AV3E and SCR-AV6E) did not show clear binding with AV1 (Fig. 30). This could be either due to the weak binding properties as shown in ELISA (Fig. 16; b) and BIACORE (Fig. 25) analysis or the high expression level of these scFvs in the plant cytosol. Probably some binding has occurred in the cytosol and then the protein complex was driven to the nucleus as happened in the case of scFv-RW-AV, but because of the high expression level the majority of the scFvs remained unbound in the cytosol. This idea is supported by the observation of some co-localized spots in the cytosol (Fig. 30).

V Summary

In this study, scFvs against key proteins of the ToLCV-India geminivirus were generated using phage display and/or hybridoma technologies. Purified AV1 and AC1 viral proteins were used for mice immunization and screening of phage display libraries and hybridoma clones. The binding properties of the isolated scFvs were determined by WB, ELISA and BIACORE.

The sequence analysis of the hybridoma derived scFv-SAV against AV1 revealed a missing nucleotide at position 30 downstream the original ATG start codon, which led to frame disruption and 19 aa truncation at the N-terminus. The frame was restored by *in vitro* site directed mutagenesis and the resulting scFv (scFv-RW-AV) showed better binding properties than scFv-SAV.

Four scFvs against AV1 were derived by solid phase panning of the naïve Tomlinson I phage display library. Two of these scFvs carried a stop codon at position H105. The stop codon was mutated *in vitro* to either code for Q, found at the same position of other scFvs in the database, or code for E, as translated by suppressor strains. The Q-mutated scFvs showed higher expression level but less binding activity than the E-mutated scFvs. Additionally, two scFvs against AC1 were selected, one from the Tomlinson I library and another one from a phage library constructed from spleen cells of immunized mouse.

All scFvs were cloned into plant expression vector as N-terminal fusion with GFP for cytosolic expression in tobacco leaves. The expression ranged from undetectable to very high. Additionally, the ability to target scFvs as fusion with GFP to the nucleus was tested. Two scFv-GFP fusions (scFv-NSCR-AV3-GFP and scFv-NSCR-AC-GFP) were targeted to the nucleus by an NLS introduced by PCR to the N-terminus of the scFvs. Both scFv-GFP fusion proteins were exclusively localized in the nucleus.

The AV1 protein was also expressed in tobacco leaves as N-terminal fusion with DsRed. The AV1-DsRed fusion was completely localized in the nucleus as a result of the well-characterized AV1-NLS.

The binding activity of the AV1-specific scFvs was tested by the co-expression of the scFvs-GFP fusion and AV1-DsRed. Only the hybridoma derived scFv-RW-AV showed clear binding and the formed protein complex was targeted to the nucleus by the AV1 NLS.

The data presented in this study demonstrate the expression of specific and functional scFvs against the viral proteins in the reducing environment of the plant cytosol. Such a scFv is a potential candidate to interfere with the viral life cycle and thus abolishing or delaying the viral infection.

As for the future work, creation of stable transgenic plants expressing scFv-RW-AV would be the first step to challenge these plants with the ToLCV-India geminivirus and analyse any possible resistance phenomena. The scFv level of expression might be a crucial factor for good resistance, so improving the expression level by CDR grafting to more stable and efficient folding frameworks and structure-based framework engineering, (reviewed by Ewert et al., 2004) might be advantageous. The same applied on the other scFvs (SCR-AV1, SCR-AV4, SCR-AC and RW-AC) that showed good binding properties *in vitro* (when expressed in *E. coli*) but undetectable or very low expression in tobacco leaves.

Finally, cloning, expression, and purification of different domains of AC1 and AV1 may help for epitope mapping of the AV1. Creation of transgenic plants producing more than one scFv against different epitopes or binding to multiple viral targets may strengthen the virus resistance.

VI References

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VII Appendices

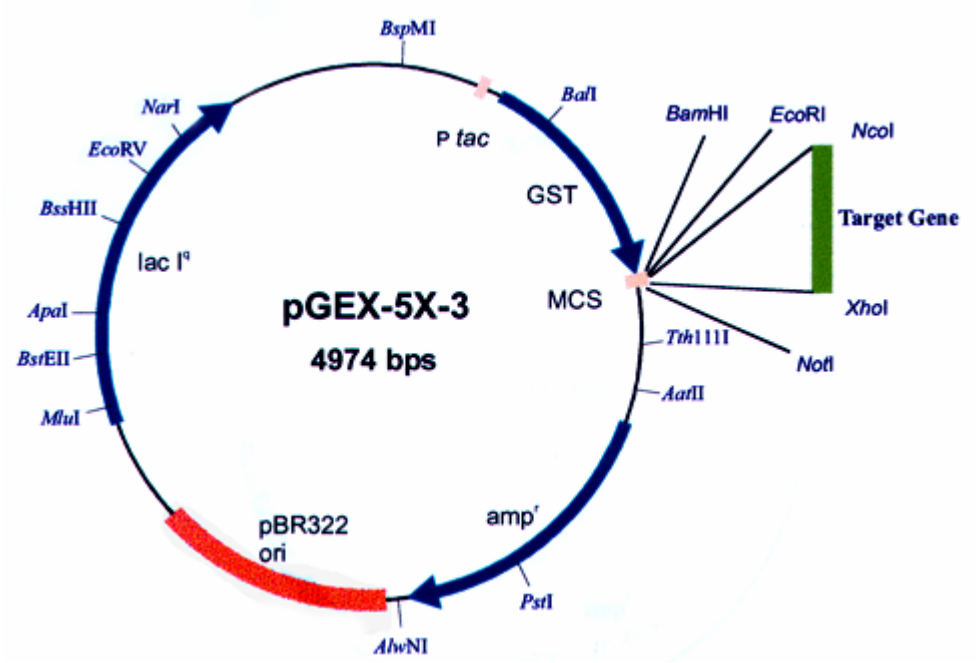
VII.1 List of abbreviations

°C	degree Celsius
%	percentage
A	adenine
aa	amino acid(s)
Ab	antibody
Amp	ampicillin
amp^r	ampicillin resistance
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CaMV	cauliflower mosaic virus
Cb	carbenicillin
CB	coating buffer
CDR	complementarity determining region
C_H	constant region of heavy chain
C_L	constant region of light chain
CHS	chalcone synthase
Da	Daltons, g/mol
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FC	flow cell

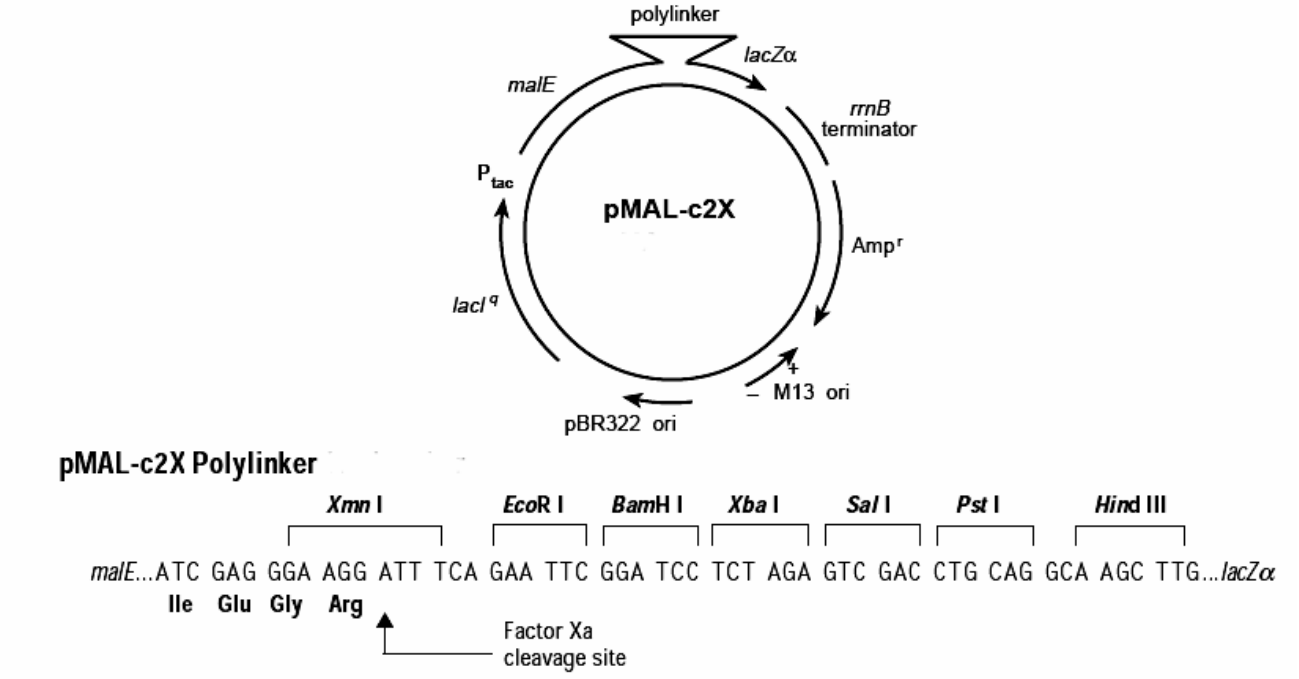
Fc	fragment crystallizable
G	glycine
GAM	goat-anti-mouse (antibodies)
gm	gram
h	hour(s)
HBS	Hepes buffered saline
His	histidine
Ig	immunoglobulin
K_A	equilibrium association constant
kDa	kilodalton
kg	kilogram
Km	kanamycin
l	liter(s)
LBA	Luria broth with ampicillin
M	molarity (mol/L)
mAb	monoclonal antibody
min	minute(s)
mRNA	messenger ribonucleic acid
MW	molecular weight
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% (v/v) Tween20
PCR	polymerase chain reaction
PNPP	*para*-nitrophenylphosphate
PTGS	posttranscriptional gene silencing
rAb	recombinant antibody
RE	restriction enzyme
Rif	rifampicin
rpm	rotations per minute
RT	room temperature

scFv	single chain Fragment variable
SDS	sodium dodecylsulfate
SOE	splicing by overlap extension
T	thymin
Taq	*Thermus aquaticus*
T-DNA	transfer DNA
Tris	trishydroxymethylaminomethane
U	unit
UTR	untranslated region
UV	ultraviolet
V	Volt
V_H	variable region of heavy chain
V_L	variable region of light chain
VIGS	virus induced gene silencing
v/v	volume per volume
w/v	weight per volume

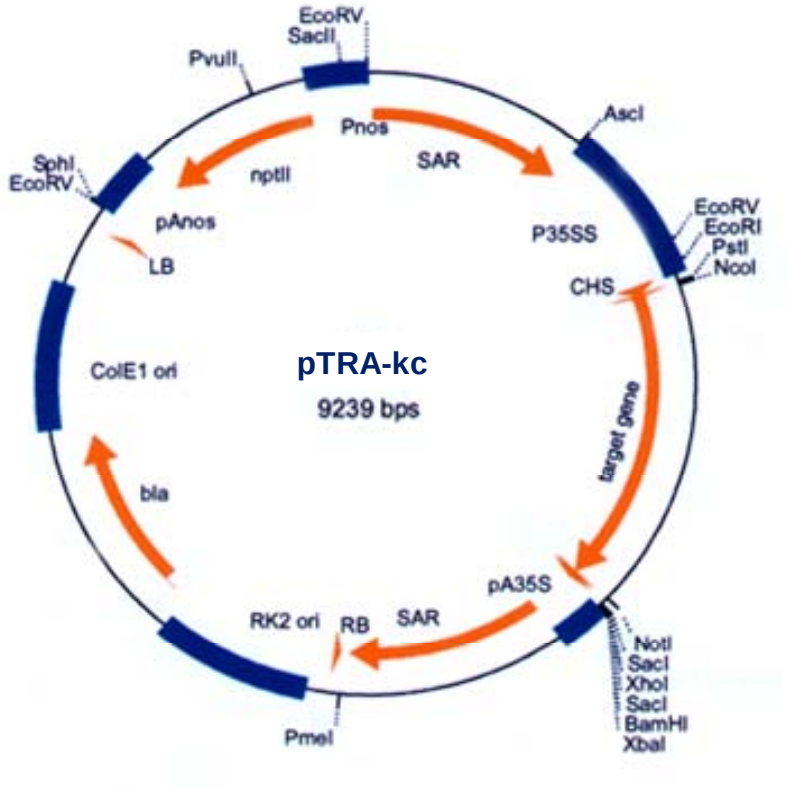
VII.2 Vector maps



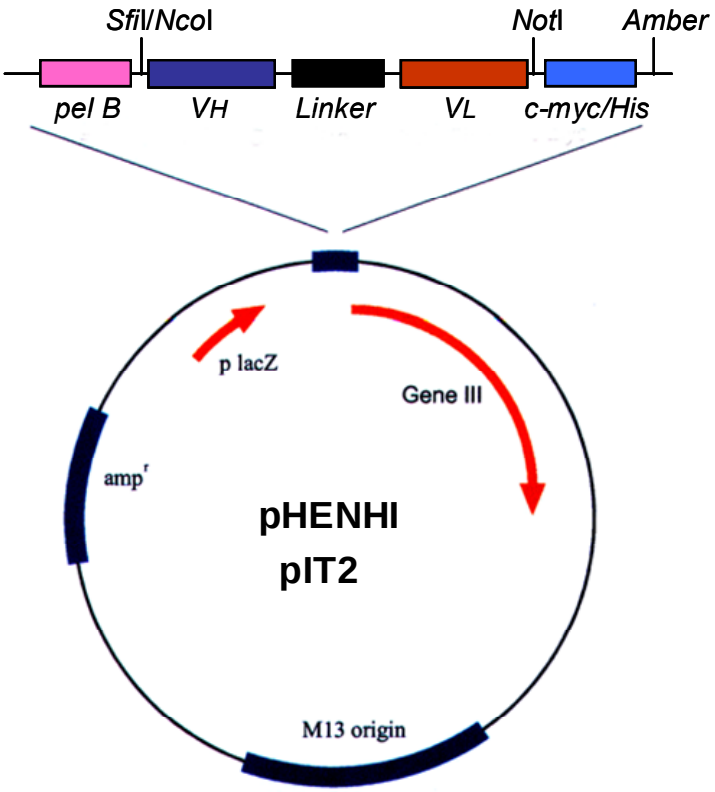
A) Map of the GST fusion vector (pGEX-5X-3) showing the reading frames and main features. MCS: multiple cloning sites, amp^r: ß-lactamase ampicillin resistant gene, p *tac:* promoter induced by IPTG, *lac* I^q: repressor protein coding region, pBR322ori: plasmid replication origin.



Map of the pMAL-c2X vector (6648 base pairs) with its main features. *lac* I^q: repressor protein coding region, malE: the maltose binding protein coding gene, pBR322 ori: plasmid replication origin fused to the lacZα gene. Unique restriction sites are indicated. Arrows indicate the direction of transcription of the reading frames.



Map of the plant expression vector pTRA-kc. p35S: 35S promoter from CaMV with duplicated 35S enhancer, CHS: 5' UTR of chalconsynthase, RB: right border, ColE1 ori: replication origin for *E. coli*, PK2 ori: replication origin for *A. tumefaciens*, bla: Ampicillin resistance for *E. coli* / Carbenicillin resistance for *A. tumefaciens*.



Map of the pHENHI/pIT2 phagemid vectors. *Pel* B: leader peptide (pectate lyase gene) that targets the expressed protein into the periplasmic space, Amber: TAG stop codon.

Fig. 31: Schematic presentation of the vectors used in this study.

VII.3 Figures

VII.4 Tables

VIII Acknowledgments

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Declaration / Erklärung

Herewith I declare that I have written this PhD thesis myself, using only the referenced literature.

Hiermit versichere ich, dass ich die vorliegende Doktorarbeit selbstständig verfasst und keine anderen als die angebenen Hilfsmittel und Quellen verwendet habe.

Aachen, 2006