Development of recombinant antibody-mediated resistance against Tomato yellow leaf curl virus

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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Tag der mündlichen Prüfung: 16.01.2008

Institute for Biology VII (Molecular Biotechnology)
RHEINISCH **W**ESTFÄLISCHE **T**ECHNISCHE **H**OCHSCHULE **A**ACHEN

PhD Thesis

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Aachen, January 2008

ZUSAMMENFASSUNG

In dieser Studie wird die Expression spezifischer scFv-Fragmente in pflanzlichen Zellen für die Unterdrückung der Krankheitssymptome durch TYLCV-Infektion genutzt.
Die für Rep, CP und MP kodierenden Gene C1, V1 und V2 wurden mit spezifischen Primern mit dem kompletten klonierten TYLCV Genom als Template amplifiziert. Die PCR-Produkte wurden zunächst in den TOPO Vektor und später in die Expressionsvektoren pGEX-5x3-und pMALc2x kloniert. Die rekombinanten Proteine wurden in E. coli als C-terminale Fusion mit GST oder MBP exprimiert und durch Affinitätschromatographie aufgereinigt. Darüber hinaus wurde auch der aminoterminale Teil des CP und des Rep Proteins als Fusionsprotein mit GST und MBP exprimiert.

Mit Hilfe der Phagen-Display-Technologie wurde ein scFv-Fragment gegen Rep (scFv-ScRep1) aus der naiven Tomlinson Phagen-Bibliothek isoliert. Darüber hinaus wurde eine Rep-Phagen-Display-Bibliothek aus Gesamt-RNA der Milz einer präimmunisierten Maus hergestellt und ein weiteres scFv-Fragment (scFv-ScRep2) identifiziert und charakterisiert. Die scFv-Fragmente wurden in den bakteriellen pHEN-HI Expressionsvektor kloniert und mittels Immobilisiertes-Metall-Affinitätschromatographie aufgereinigt. ELISA-und Western-Blot-Analysen wurden genutzt, um die Bindungsaktivitäten der bakteriell exprimierten scFv zu analysieren. Die beobachteten hohen Bindungaktivitäten von scFv-ScRep1 sowohl mit dem Volllängen- als auch dem C-terminalen verkürzten Rep-Protein weisen auf eine spezifische Bindung hin. ScFv-ScRep2 interagiert dagegen ausschließlich mit dem intakten Rep-Protein. Vier weitere scFv-Fragmente wurden aus Gesamt-RNA von Hybridomazellen, welche spezifische MAbs gegen TYLCV-Virionen produzieren, gewonnen.
Der Pflanzenexpressionsvektor pTRAkt wurde zur Klonierung der generierten scFv-Fragmente einzeln und als amino-terminale Fusion mit GFP verwendet. Darüber hinaus wurde die nukleare Lokalisationssignalsequenz von Simian Virus 40 für den Kerntransport der scFv-ScRep1 und scFv-ScRep1-GFP eingesetzt. Alle Konstrukte wurden in einer transienten Expression durch Agrobakterien-vermittelter Infiltration von Tabakpflanzen getestet und auf Funktionalität überprüft. Blotting-Analysen zeigten nachweisbare Mengen von scFv-ScRep1, scFv-ScRep2 und NLS-scFv-ScRep1 in rohem Blattextrakt aus agroinfiltrierten Pflanzen. Weitere Analysen bewiesen auch die Bindungsfähigkeit der aus den Blättern extrahierten scFvs gegen rekombinantes Rep-Protein. Die Ergebnisse der Fluoreszenz-Mikroskopie bestätigten die Lokalisierung von scFv-ScRep1-GFP-, scFv-ScRep2 GFP- und scFv-NLS-ScRep1-GFP-Fusionprotein im Zellkern bzw. dem Zytoplasma.

Ausgesuchte Konstrukte wurden für die stabile Transformation von *N. benthamiana* Pflanzen durch die Blattscheibenmethode verwendet. Nachfolgende Resistenzuntersuchungen wurden mit einem infektiösen Volllängenklon (pBIN19-2TYLCV Ir) und Agrobakterien-vermittelter Infektion im 5-8 Blattstadium durchgeführt. Frühe Symptome wie Blattrollen und Wachstumsreduktion der Blätter wurden auf nicht transgenen und sensitiven transgenen Pflanzen 3-4 Wochen nach der Inokulation beobachtet. Die Akkumulation der viralen DNA- und die Anwesenheit des Virushüllproteins in den Pflanzen wurden durch PCR-Analyse, Southern Blotting und TAS-ELISA-Test nachgewiesen. Die Ergebnisse der PCR-Analyse bestätigten die Anwendbarkeit dieser Technik für die Erkennung von Virus-DNA im rohen Blattextrakt von inokulierten Tabakpflanzen. Die TAS-ELISA-Ergebnisse zeigen, dass dieser Test nur nützlich für die Erkennung von infizierten Pflanzen mit schweren Symptomen und gleichzeitiger hoher Viruskonzentration ist. Man ist mit diesem ELISA nicht in der Lage zwischen gesunden Pflanzen und denen mit milden Symptomen und niedrigem Virustiter zu unterscheiden.

Zusätzliche Hybridisierungsanalysen wurden für die Erkennung der verschiedenen replikativen viralen DNA-Konformationen wie z.B. offener Kreis, linearisiert und supercoiled dsDNA sowie ssDNA herangezogen. Southern-Blotanalysen bestätigten die Reduktion bzw. vollständige Unterdrückung der viralen DNA-Replikation in symptomlosen Pflanzen. Die Bewertung des Resistenzgrads bei den T0 Pflanzen wurde 5 Wochen nach der Inokulation durchgeführt. Resistenztests mit den T0 Nachkommen zeigten, dass alle NSR-, HSC2-, HSC3- und HSC4-Linien sowie Wildtyp-Pflanzen anfällig für TYLCV sind. Die SRG-, SR- und RW-Linien zeigten ein unterschiedliches Ausmaß der Resistenz im Bereich von 8-28 Prozent.

Unabhängige T1-transgene Pflanzen wurden zur Resistenztestung im 5-8 Blattstadium agroinfiziert. Inokulierte Pflanzen wurden bezüglich der Entwicklung von Krankheitssymptomen als auch mit Hilfe von DNA-Hybridisierung analysiert. Frühe Symptome erschienen bei nicht transgenen und sensitiven Pflanzen bereits 2-3 wpi und entwickelten sich in den darauf folgenden Wochen weiter, Resistenzausprägung wurde bei 4-5 wpi beobachtet. Diese Ergebnisse zeigen, dass alle T1 Nachkommen der RW14, RW22 und SR27 Pflanzen typische TYLCV-Symptome ausbilden, während bei SRG T1-Pflanzen, die das ScRep1-GFP rekombinante Fusionsprotein exprimieren, ein Spektrum von Symptomen von schweren zu milden und auch das vollständige Fehlen jeglicher Symptome zu beobachten war. Der Resistenzphänotyp zeichnet sich durch Abwesenheit oder eindeutige Verringerung der Krankheitssymptome und eine gleichzeitige deutliche Reduktion bzw. vollständige

Unterdrückung der viralen DNA-Replikation aus. Die T1 Pflanzen aus den SRG28 und SRG18 Linien zeigten die höchsten Resistenzwerte. Vergleichende Q-PCR- und GFP-Fluoreszenz-Intensitätsanalysen wiesen darauf hin, dass Pflanzen mit höheren Transkriptkonzentrationen auch ein höheres Maß an Virusresistenz besitzen. Durch die hohe Ähnlichkeit der Rep-Proteine unter den Begomoviren, ist eine breitere Resistenzausprägung auch gegenüber verwandten Viren, wie z.B. ACMV möglich. Aufgrund der hohen Empfindlichkeit von *N. benthamiana* gegen TYLCV und dem hohen Inokulumsdruck bei der Agroinfektion von Pflanzen kann die schützende Fähigkeit der rekombinanten scFvs eventuell leichter überwunden werden. Daher sollten die Infektiösitätsassays als Kontrolle mit entsprechenden transgenen Tomatenpflanzen und mit Hilfe des natürlichen Übertragungsvektors *B. tabaci* überprüft werden. Da die viralen Hüllproteine von entscheidender Bedeutung für die Übertragung durch Insekten sind, ist die Expression der in dieser Arbeit beschriebenen Viruspartikel-spezifischen scFvs in transgenen Tomatenpflanzen besonders vielversprechend.

Summary

In this study, we exploited the expression of specific scFv fragment in plant cells for suppression of disease symptoms caused by TYLCV infection.

The C1, V1 and V2 genes encoding Rep, CP and MP, respectively, were amplified with specific primers using the full length TYLCV genome construct pBIN19-2TYLCV-Ir as template. The PCR products were first cloned into the TOPO vector and subsequently into pGEX-5x3 and pMALc2x expression vectors. Recombinant proteins were expressed in *E. coli* as C-terminal fusion with GST or MBP and purified proteins obtained by affinity chromatography method. In addition, the amino terminal part of CP and Rep proteins were also cloned and expressed as fusion proteins with GST and MBP.

Using phage display technology one scFv fragments against Rep (scFv-ScRep1) was isolated through panning of naïve Tomlinson I scFv phage library. In addition, an ARep phage display library constructed from total spleen RNA of a mouse immunized with MBP-Rep was analyzed by panning and another scFv fragment (scFv-ScRep2) was selected and characterized. The scFv fragments were expressed in pHENHI bacterial expression vector and purified by immobilized metal affinity chromatography. ELISA and Western blot analyses were used to analyze binding activities of bacterially expressed scFv. The observed high binding activity of bacterially expressed scFv-ScRep1 to both full length and C- terminal truncated of Rep protein indicated specific binding to the amino terminal end of Rep while scFv-ScRep2 interacted exclusively to intact Rep protein. Four more scFv fragments were developed from total RNA of murine hybridoma cells secreting specific MAbs against TYLCV virions.

The pTRAkt plant expression vector was used to clone the generated scFv fragment genes individually and/or as an amino terminal fusion to GFP. In addition, the nuclear localization signal of Simian virus 40 was used for nuclear targeting of scFv-ScRep1 and scFv-ScRep1-GFP inside the cells. All constructs were used for transient transformation of tobacco plants via agrobacterium infiltration. Expression of scFv fragment constructs and their functionality within transiently transformed plant cells were analyzed. Blotting analyses showed detectable amounts of scFv-ScRep1, scFv-ScRep2, and NLS-scFv-ScRep1 presented in crude leaf extract of transformed plants. Further analyses proved binding ability of these scFv extracted from leaves against recombinant Rep. Fluorescence microscopy results confirmed expression and localization of scFv-ScRep1-GFP, scFv-ScRep2-GFP and scFv-NLS-ScRep1-GFP fusion protein within the cytoplasm and nucleus.

Selected constructs were also used to generate stable transformations in entire *N. benthamiana* plants through leaf disc transformation. To determine the protection ability of transgenically expressed proteins, independent T0 progenies expressing different scFvs were challenged with the agroinfectious clone harbouring the pBIN19-2TYLCV-Ir construct. Early symptoms including leaf curling and size reduction of newly emerged leaves were observed on non-transgenic and sensitive transgenic plants 3-4 weeks after inoculation. The accumulation of viral DNA and presence of virus particles in the inoculated plants were analyzed by PCR, Southern blotting and TAS-ELISA. The PCR results confirmed its feasibility for detection of viral DNA within inoculated tobacco plants using either crude leaf extract or purified total DNA. The TAS-ELISA results showed that this assay is only useful for detection of infected plants with severe symptoms and concomitant high virus concentration, it failed to discriminate healthy plants from those with mild symptoms and low virus titres. The hybridization analyses showed its great potential for detection of different viral replicative DNA conformations including open circular, linearized and supercoiled dsDNA as well as ssDNA structure within the infected plants. Southern hybridization analyses confirmed reduction or complete suppression of viral DNA replication in the symptomless plants. Assessment of the resistance status within T0 inoculated plants was evaluated 5 weeks after inoculation. Infectivity assays of T0 progenies revealed that all inoculated NSR, HSC2, HSC3 and HSC4 lines as well as wild type plants are susceptible for the TYLCV challenge inoculation. However, SRG, SR and RW lines presented varying degrees of resistance from 8-28 percent.

Independent T1 transgenic plants were screened based on Kanamycin resistance. To assay virus resistance phenotype of T1 progenies, transgenic plants were agro-inoculated in 5-8 leaf stage. Inoculated plants were observed for disease symptoms development and assayed for presence of TYLCV DNA through molecular hybridization methods. Early symptoms in non-transgenic and sensitive plants appeared 2-3 wpi and developed further during the next weeks. Resistance response was evaluated at 4-5 wpi bases of symptom observation and DNA hybridization assays. A heterogeneous response was obtained within inoculated T1 progenies. These results indicated that all T1 progenies raised from RW14, RW22 and SR27 reveal typical TYLCV symptoms whereas SRG T1 plants expressing ScRep1-GFP recombinant protein showed a spectrum of symptoms ranging from a severely diseased to mild ones and the complete absence of any symptoms. The resistance phenotype was characterised by absence or remarkable reducing of disease symptoms and a concomitant substantial reduction or complete suppression of viral DNA replication. T1 plants developed from SRG28 and

SRG18 lines revealed highest resistance. Further analyses indicated that individual SRG transgenic plants emit varying intensity of fluorescence under excitation by UV light. Usually, elevated amounts of ScRep1-GFP transcripts are directly correlated with higher fluorescence intensity emitted from transgenic lines. Together comparative Q-PCR and fluorescence intensity analyses with data obtained from virus resistant assays indicated that mostly plants with higher transcripts level consistently exhibited a higher degree of virus resistance.

Because of high similarity of Rep protein among the begomoviruses, challenging of SRG resistant tobacco lines with other viruses such as ACMV is a promising idea. Due to high susceptibility of *N. benthamiana* against TYLCV and high inoculum pressure using for agroinoculation of transgenic plant, virus could easily overcome protective ability of recombinant scFv. Therefore, infectivity assays with transgenic tomato plants expressing scFv fragments which are naturally infected with *B. tabaci* could give more reliable results. Since the viral CP is critical for insect transmission, expression of those scFvs binding to virions in transgenic tomato plants may confer a resistance phenotype against TYLCV infections as well.

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I Introduction

Plant viruses are obligatory intracellular parasites entirely dependent on host machinery for all aspects of their life cycle. They were discovered in the last century when the science of virology was born. Out of 1200 known plant viruses less than 250 cause significant losses in crop yields and challenge plant breeders around the world (Nelson and Citovsky, 2005).

Viral infections can result in anything from mild or even symptomless infections to severe diseases resulting in complete loss of marketable crops. Geminiviruses cause severe diseases in important crop plants and have been spread worldwide in the past four decades. Several factors; recombination between co-infecting viruses, diversification of insect vector population and transportation of plant material, have caused the geminiviruses epidemics in last decades (Sung and Coutts, 1995; Moffat, 1999). As in the past, plant viruses remain a major problem for the cultivation of many vegetable and ornamental crops throughout the world.

I.1 Development of resistance against plant viral diseases

Protection against viral disease requires an understanding of the virus, its replication strategy, modes of infection, transmission vectors and identification of useful genes to make highly resistant cultivars. In contrast to the control of fungal diseases no chemical pesticides are available for use as direct antiviral agents, and alternative control strategies are required. For a long time, cultivation techniques like crop rotation, early detection and destruction of infected source plants, cross-protection, breeding for resistance, and chemical control of transmission vectors have been attempted. However, these strategies do not provide an effective defence against plant viruses and are usually time-consuming. Increasing knowledge of hosts natural defence systems and the molecular genetics of plant viruses have resulted in the development of a number of novel ways to control virus diseases in plants.

I.2 Natural resistance to plant viruses

Natural resistance against plant viruses are separated into two main categories: passive and active defence. In a passive defence system, existing natural barriers like the rigid cell wall prevent entrance of viruses into the plant cells, while active defence mechanisms are based on specific recognition of pathogens by the plant. Hypersensitive response (HR) is the most common mechanism associated with active defence. In HR, cells surrounding primary infection sites die rapidly and prevent development of infection. This reaction is usually

accompanied by formation of necrotic local lesions in infected parts of leaves. The induction of this response is preceded by a specific recognition of the virus based on dominant gene products of the plant produced by resistance genes (R genes) corresponding to avirulence genes of virus. A single dominant R resistance gene may recognize different categories of plant pathogens. Interestingly, independent of pathogen type (including viruses, bacteria, fungi and nematodes) all identified R genes encode proteins which can be categorized into on five classes (LZ-NB-LRR, NB-LRR, CC-NB-LRR, TIR-NB-LRR and LRR-TM) in a range of different crop species (Dangl and Jones, 2001). All R genes pertaining to plant virus resistance belong to the LZ-NB-LRR class, the largest class of R genes, and encode NB-LRR protein (nucleotide-binding site plus leucine-rich repeat) (Goldbach et al., 2003). Natural plant disease resistance genes can be used to generate resistant plants against pathogens, for example, transgenic tomato plants carrying the tobacco N gene are resistant to TMV (Whitham et al., 1996).

I.3 Engineered resistance to plant viruses

I.3.1 Pathogen-derived resistance (PDR)

Plant viruses have small genomes containing only a limited number of genes corresponding to crucial functions during the viral replication cycle. As such, these pathogens could be suitable targets for engineering resistance based on the principle of pathogen-derived resistance (PDR). In PDR, pathogen genomic sequences are deliberately engineered into the host plant's genome on the non-specific basis that the sequence may be expressed at an inappropriate time, in inappropriate amounts or in an inappropriate form during the infection cycle resulting in some form of resistance in the plant. The presence of the pathogen sequence may directly interfere with the replication of the pathogen or may induce some host defence mechanism.

The concept of creating virus resistant crops by incorporating part of a viral genome in the host plant genome was first presented by Hamilton (Hamilton, 1980). The principle involves the use of virus-derived genes or genome fragments to interfere virus replication in the plant. Approaches to PDR may require either the production of proteins or only the accumulation of viral nucleic acid sequences. The former is non-specific and may confer resistance to a broader range of virus strains, whereas the latter is quit specific and would provide very high levels of resistance to a specific virus strain. All virus genes and non genomic fragment of viruses could be used to confer resistance in PDR including those for coat proteins, replicases,

movement proteins, defective interfering RNAs and DNAs, and non-translated RNAs (Beachy, 1997).

The first use of PDR was coat protein mediated resistance (CPMR) against *Tobacco mosaic virus* (TMV) (Powell et al., 1986). Viral coat proteins have been widely applied and exploited successfully to confer resistance in several plants against a number of RNA viruses, including TMV, PVX, AlMV, CMV and TRV. Transgenic plants expressing their CP lead to high levels of operational resistance (Wilson, 1993; Baulcombe, 1996), which can only be overcome at extremely high inoculation pressure or infection with unencapsidated viral RNA. In CPMR, produced recombinant and native CP must be able to interact with each other but not necessarily to form virus particles (Clark et al., 1995). The role of transgene CP is not clear but it may interfere with the disassembly process of TMV and prevent release of infectious RNA (Register and Beachy, 1988). Furthermore, there is a direct correlation between the amounts of expressed CP and the level of resistance (Powell et al., 1990). Employment of certain mutants of the TMV CP may confer much greater levels of resistance than wild-type CP (Beachy, 1997). CPMR can provide different level of protection against related strains or viruses, as was demonstrated by the use of the CP of TMV as an effective agent to induce resistance against closely related strains, but providing decreasing levels of resistance to other tobamoviruses with greater variation from the TMV CP (Nejidat and Beachy, 1990). Transgenic potato expressing the CP gene of *Potato virus Y* (PVY) strain N605 provided resistance against original strain N605 and related strain 0803 (Malnoe et al., 1994), but the CP gene of *Papaya ring spot virus* (PRSV) strain HA provided resistance in papaya only to strain HA (Tennant et al., 1994). In contrast, transgenic tobacco plants producing the CP of *Soybean mosaic virus* (SMV), which is not able to infect tobacco, conferred resistance against two unrelated potyviruses, PVY and *Tobacco etch virus* (TEV) (Stark and Beachy, 1989). It is not clear that why some CP provides broad or strong degrees of CPMR while others provide only narrow or weak resistance. Sometimes combining CP genes from different strains confer resistance to those strains, for instance, to obtain broad resistance against three different strains of *Tomato spotted wilt virus* (TSWV), all nucleoprotein genes of these strains were combined in a single construct (Prins et al., 1995).

Movement proteins (MPs) are encoded by plant viruses and enable viruses to spread through the plant systemically by cell to cell. Plasmodesmata are involved in local spread of viruses in plants so they provide symplastic continuity between cells and tissues. Transgenic plants producing defective mutant of MP (dMP) from TMV confer resistance to several tobamoviruses (Cooper et al., 1995).

Expression of complete or partial sequence of viral replicase can confer some level of immunity to infection in transgenic plants. This type of resistance is generally limited to the virus strain that the gene was obtained. The mechanisms involved in Rep-mediated resistance are not completely known, but it is proposed that transgene protein produced by plant interfere with native replicase (Palukaitis and Zaitlin, 1997). Transgenic plants expressing replicase protein of *Potato leaf roll virus* (PLRV) are resistant to this virus, but it seems that the induced resistance is independent of the expression of transgene protein and it may be RNA-mediated rather than protein-mediated (Vazquez Rovere et al., 2001). Expression of a truncated replicase obtained from CMV sub-group I virus conferred high level of specific resistance in tobacco (Zaitlin et al., 1994).

One of the most important types of PDR is post-transcriptional gene silencing (PTGS). PTGS is based on processes of post-transcriptional control of gene expression found in plants, fungi and some animal species. It causes suppression of foreign genetic elements such as viruses and transposons through a specific RNA breaking down mechanism known as RNA silencing. PTGS in plants could act as an adaptive, antiviral defence system (Goldbach et al., 2003). The PTGS was first discovered in transgenic petunia plants carrying copies of the chalcon synthetase gene together with one endogenous gene that were co-suppressed (van der Krol et al., 1990). It was then proposed that PTGS could explain previous unexpected outcomes of virus resistance based on PDR (Lindbo et al., 1993). In some PDR experiments, untranslatable versions of viral genes imagined as a negative controls presented levels of resistance similar to translatable versions. It was revealed that in the resistant plant lines the transgenic RNA was rapidly broken down in a sequence-specific manner. Accordingly, foreign RNA sequence with no homology to the host's genome, with great efficiency, can trigger RNA silencing. To date transgenic resistance against virtually all major plant DNA and RNA viruses has been reported. It is suggested that PTGS acts as a natural antiviral defence system by surveying and destructing of aberrant foreign RNAs (Goldbach et al., 2003). It has been shown that some regions of viral genomes are more vulnerable to antisense RNA and resulted different level of resistance. Transgenic tobacco producing antisense RNA complementary to the 5' leader region of viral genome proved resistant against TMV infection (Nelson et al., 1993) while using the 3' end of TMV RNA as complementary antisense RNA resulted in lower level of resistance (Powell et al., 1989).

Although PDR has been successfully implemented against a number of plant viruses, it is not always an effective way and can be dependent on the mechanism of pathogenesis or host–virus interactions and may have undesirable consequences (Schuler et al., 1999). It is

generally accepted that RNA viruses can do recombination and, together with the high frequency of mutations in their replication, result in great viral genetic variability. New viruses with altered virulence, host range, or vector specificity may be occur by recombination with virus transgenes expressed in plants (Nagy and Simon, 1997). Furthermore, recombination events could restore some defects in viral protein functions of avirulent strains and lead to creation of virulent strains (Rubio et al., 1999). Nonhomologous recombination between invading viruses and transgenes from an unrelated virus could result in new hybrid viruses with expanded host ranges and new disease phenotypes (Masuta et al., 1998).

I.3.2 Antiviral agents and resistance genes

Another strategy is the use of specific and natural inhibitors of virus replication that could confer resistance when expressed in transgenic plants.

Potential antiviral proteins include ribonucleases 2′, 5′ oligoadenylate synthase, and ribosome-inactivating proteins (RIBs). Expression of a specific double-stranded RNA ribonuclease in transgenic plants inhibited TMV, CMV and PVY infections (Watanabe et al., 1995). A specific ribonuclease enzyme named as ribozeymes could be used against invading viruses to cleave and destroy their RNA. They contain a catalytic domain flanked by hybridising arms that are complementary to target RNA. Transgenic tobacco plants expressing a specific ribozyme showed resistance against TMV infection (de Feyter et al., 1996). Transgenic expression of 2′, 5′ oligoadenylate synthetase was proved as an effective approach to protect plants against CMV and PVY infection (Ogawa et al., 1996). Tobacco and potato plants expressing a ribosomal inhibiting protein were resistant to PVX, PVY and PLRV (Ogawa et al., 1996).

I.3.3 Antibody-mediated resistance

The expression of viral genes or using of viral nucleic acid as PTGS in transgenic plants could be a very effective tool to attenuate plant viral infection. The PDR strategies may lead recombination and transcapsidation between transgenes and incoming viruses. Nevertheless, risk issues concerned with their application in plants may limit the exploitation of this strategy.

An alternative approach to create plants resistant against pathogens that lack these drawbacks would be the expression of antibodies or antibody fragments that could bind to functional components of pathogens and inactivate pathogens and pathogen proteins in plants. The

effectiveness of this strategy is related to the antibody affinity and specificity to the target protein. This strategy has been developed with progress in understanding of plant diseases mechanism and by identification of many proteins critical to pathogen infection, development, replication and spread. The first successful use of antibodies to make plant virus resistant was the using of specific single-chain variable fragments (scFv) against *Artichoke mottled crinkle virus* (AMCV). This scFv was constructed from a monoclonal antibody against the AMCV virion. Before plant transformation, the single chain was expressed in bacteria to show that it retained the general characteristics of its parental antibody. The constitutive cytosolic expression of this scFv in transgenic tobacco caused a reduction in viral infection and a delay in symptom development (Tavladoraki et al., 1993).

Clearly, the choice of plant compartment for recombinant antibody expression is critical for engineering of viral resistance. Because most processes involving in viral replication and spread take place within the cytoplasm, cytosolic expression of the recombinant antibody fragments is desirable (Baulcombe, 1994). The main problem accompanying this issue is that recombinant antibodies or even their fragments (scFv) might not fold properly in the cytosol and have low stability (Fecker et al., 1996). Expression of antibody fragments in cell components other than the cytoplasm could provide better conditions for their folding and an associated increase in protein stability. Full-length antibody expression targeted to the apoplast conferred protection against TMV (Voss et al., 1995). When full size recombinant antibody obtained from TMV was expressed in cytosolic and apoplastic regions, expression level of functional full size antibody in apoplast remained high while cytosolic expression was barely detectable (Schillberg et al., 1999). Accumulation of scFv obtained against TMV CP expressed in apoplasm is 50000-fold higher than its cytosolic expression. However, even very low levels of cytosolic scFv expression (0.00002% of total soluble protein) led to remarkable enhancement in systemic viral resistance over that conferred by apoplastic targeting of scFv or full-size antibodies (Zimmermann et al., 1998). It has been shown that TMV neutralizing scFv targeted to endoplasmic reticulum and integrated into the tobacco cells plasma membrane (facing the apoplast) retained scFv antigen binding affinity and specificity. Transgenic plants expressing membrane targeted scFv were resistant to TMV infection, demonstrating that anti-viral antibody targeting to plasma membrane is functional *in vivo* and offer an effective method to create resistance against pathogens (Schillberg et al., 2000).

Expression of coat protein-specific scFv of *Beet necrotic yellow vein virus* (BNYVV) in the ER or an ER-associated compartment partially protected *N. benthamiana* against the pathogenic effects of BNYVV (Fecker et al., 1997).

Transgenic plants expressing functional scFv fragments were typically protected only against the specific viruses which the antibody was raised, but some scFv that could experimentally detect several viruses, normally within same virus family, might be used to protect plants from distinct viruses (Xiao et al., 2000).

Most of recombinant antibodies have been raised against the coat proteins. High variability of CP in plant viruses might prevent broad-range resistance and moreover, the amount of coat protein accumulated in plant cells is high and may not be completely neutralized by limited amount of scFvs expressed in plants cytosol. An alternative; Virus resistance based on antibodies to RNA-dependent RNA polymerase (RdRps), could be a beneficial approach. The replicases contain several conserved motifs and are multifunctional proteins occurring in low concentration in infected cells. Boonrod et al. used scFvs against a conserved domain of a *Tomato bushy stunt virus* (TBSV) RdRp. Transgenic *N. benthamiana* plants expressing different scFv either in the cytosol or in the endoplasmic reticulum showed varying degrees of resistance against four plant viruses from different genera, three of which belonged to the *Tobamoviridae* family (Boonrod et al., 2004).

I.4 *Geminiviridae* family:

The family *Geminiviridae* is one of the only two known plant virus families that have DNA genomes and replicate through DNA intermediates. The Geminiviruses, a fast growing viruses group, are pathogens of a large and diverse group of angiosperm viruses. The group is characterized by twin icosahedral capsids approximately 20X30 nm in size encapsidating a single molecule of covalently closed circular DNA (ssDNA) genomes of 2500 to 3000 bp that replicate in the nuclei of the infected cells via a double stranded DNA (dsDNA) intermediate (Lazarowitz, 1992).

The members of the family *Geminiviridae* are currently divided into four genera (summarized in Table I-1) on the basis of their insect vector, host range and genome organization (Fauquet et al., 2003).

	Mastrevirus	***Curtovirus***	***Begomovirus***	***Topocuvirus***
Genome organization	Monopartite	Monopartite	Mono- or Bipartite	Monopartite
Host range	Monocotyledonous	Dicotyledonous	Dicotyledonous	Dicotyledonous
Insect vector	Leafhoppers	Leafhoppers	Whiteflies	Treehoppers
Virus type member	MSV	BCTV	BGMV	TPCTV
Other members	WDV, SSV	HCTV	ACMV, SqLCV, TYLCV, ToLCV	_____

Table I-1: Classification of the Family *Geminiviridae*
MSV= *Maize streak virus*, BCTV= *Beet curly top virus*, TPCTV= *Tomato pseudo curly top virus*, BGMV= *Bean golden mosaic virus*, WDV= *Wheat dwarf virus*, SSV= *Sugarcane streak virus*, HCTV= *Horseradish curly top virus*, ACMV= *African cassava mosaic virus*, SqLCV= *Squash leaf curl virus*, TYLCV= *Tomato yellow leaf curl virus*, ToLCV= *Tomato leaf curl virus*

Members of the *Mastrevirus* genus are limited to monocotyledon hosts and have not been reported in America. The *Curtovirus* genus has arisen from a recombination event between a *Mastrevirus* and *Begomovirus*, the latter being the most diverse and widely distributed *Geminiviridae* lineage. Phylogenetic analysis of the begomoviruses has demonstrated distinct geographic lineages and two major clusters have emerged. The New World cluster for American viruses and the Old World cluster for viruses from Europe, Africa and Asia, but there is the third growing cluster including American SqLCV and *Texas pepper virus* (Arguello-Astorga et al., 1994).
Begomoviruses infections occurring in different continents have considerable serological relationship to each other. It has been demonstrated that begomoviruses isolated from one geographical region have more similar epitope profiles than those from different regions irrespective of the source host and type of diseases (Harrison and Robinson, 1999).

I.4.1 *Tomato yellow leaf curl virus* (TYLCV)

The TYLCV is the most devastating and fast spreading virus disease agent of tomato crops in tropical and warm temperate regions of the world including the Mediterranean, Middle east and tropical regions of Africa and Central America and causes up to total yield loss (Nakhla and Maxwell, 1998). The first reports of damage caused by this virus were in the late of 1930s and it was recorded as a whitefly-transmitted virus in tomato crops in the Middle east (Cohen and Harpaz, 1964) and later shown to be a *Geminivirus* member (Czosnek et al., 1988). It remained of minor importance and limited geographical distribution, until a severe outbreak of B biotype of *Bemisia tabaci* in tropical and subtropical regions occurred during the late 1980s. This biotype had a wider host rang than others, which apparently resulted in the spread of the viruses, which had originally infected only endemic plants and weeds, into adjacent and previously unaffected species (Rybicki and Pietersen, 1999). TYLCV is considered a phloem-limited virus and is not sap transmissible. It is confined to cells of phloem in the shoot apical, developing leaf, stem and floral tissues of tomato plants. The relatively inefficient interaction between TYLCV proteins (such as MP) to epidermal and mesophyl plasmodesmata (PD) may explain the phloem-limited nature of this virus in tomato. It seems that CP and C4 interact more efficiently with PD of phloem cells to mediate spread of virus (Rojas et al., 2001) and they can not suppress host defence responses in non-vascular tissue (Voinnet et al., 1999). Some immunolocalization studies suggest it may not be phloem-limited in all tissue type (Michelson et al., 1997). The virus can not be transmitted through seeds.

The symptoms of disease become visible in tomato 2-3 weeks after infection and consist of upward curling of leaflet margins, yellowing of young leaves, and abortion in flowers. Those leaflets that appear soon after inoculation are cupped down and inwards. Infected plants are severely stunted and resulting decrease of plant growth reduces total yield. Seedlings infected during the first month after planting are arrested in their growth and do not set fruits (Cohen and Harpaz, 1964). TYLCV-Is also can cause natural infection in 13 plant species throughout six botanical families. In addition to tomato, among cultivated crops, it was detected in common bean (*Phaseolus vulgaris*) (Navas-Castillo et al., 1999), pepper (*Capsicum annum*) (Reina et al., 1999) and lisianthus (*Eustoma grandiflora*) (Cohen et al., 1995). TYLCV is not wide-spread in weed hosts, although TYLCV-Sar, cause of severe epidemics in tomato plants since the late 1980s and early 1990s, has been found in some annual weed species including *D. stramonium*, *Solanum nigrum*, *S. luteum* and *Euphorbia sp*. (Bosco et al., 1993).

The name TYLCV is a generic name based off sequence comparison, and is given to a growing number of varyingly related viruses belonging to *Begomovirus* group of *Geminiviridae* and infecting tomato plants, causing similar diseases in widely separated geographical area in many tropical and subtropical regions (Czosnek and Laterrot, 1997; Nakhla and Maxwell, 1998; Moriones and Navas-Castillo, 2000). Epitope profiles of isolates from different geographical regions are different (Macintosh et al., 1992). Tomato geminiviruses are classified into three main clusters representing 1) the Mediterranean, Middle East, African region, 2) India, the Far East and Australia and 3) the Americas. In contrast to other members of the begomoviruses, all TYLCV isolates contain a single DNA-A-like genomic component, except for the isolate from Thailand which has a genome comprising two molecules, DNA-A and DNA-B (Rochester et al., 1994).

The TYLCV is transmitted by *B. tabaci* in a circulative manner. The adults and crawlers (first instar) are the most susceptible stages to transmit virus. The minimum effective acquisition and inoculation access periods are approximately 10-20 min. At least 8 hours are required that insect can transmit virus after acquisition (latent period). Female insects are sufficiently more efficient vector (Caciagli et al., 1995). Virus could be associated with the insect for its whole life and transmitted through the eggs for at least two generations and between individuals through copulation. Thus, TYLCV seems to be a pathogen of *B. tabaci* that suffers deleterious effects on life expectancy and fertility (Ghanim and Czosnek, 2000).

The TYLCV genome is transcribed to result in 2 virion and 4 complementary-sense transcripts, designed V1, V2 and C1 to C4. The V1 encodes the 30 kDa coat protein (CP) covering the viral genome and V2 encodes a 13.3 kDa movement protein (MP) that together with the 11 kDa protein product of C4 involve in cell-to-cell movement of viral DNA (Rojas et al., 2001). The C1 gene on the complementary strand encodes for a 41 kDa replication initiator protein (Rep). The C2 gene produces a 15.7 kDa transcription activator protein (TrAP) contributed in viral pathogenicity and localized into the nucleus (van Wezel et al., 2001) and it could be a suppressor of post-transcriptional gene silencing (PTGS) (van Wezel et al., 2002). The C3 gene product (REn) act as a replication enhancer (Hanley-Bowdoin et al., 2000). Generally, geminivirus genes encoded on the complementary strand are involving in virion replication, regulation of viral transcription and shifting of cellular processes to favour the viral replication cycle, but, the genes encoded on virion sense participate in the intra and intercellular movement of viral genomes and in encapsidation of genome.

Because TYLCV particles occur in low concentration in infected plants, traditional methods are severely limited for detection in crude extract. Methods like immunosorbent electron

microscopy (ISEM) (Stanley et al., 1997), double antibody-sandwich form of ELISA (Sequeira and Harrison, 1982) based on polyclonal antibodies, and triple antibody-sandwich ELISA (TAS-ELISA) (Thomas et al., 1986) using monoclonal antibodyies are viable tools for detection of viral particles. In some cases it is essential to include reducing agents such as sodium sulphite or 2-mercaptoethanol in tissue extraction medium to solubilize proteins and preventing re-formation of disulfide bonds (Macintosh et al., 1992).

I.4.2 Geminiviruses genome structure

Monopartite geminiviruses including mastreviruses, curtoviruses and some old world begomoviruses, like TYLCV, contain four to six overlapping genes encoding all viral proteins necessary for replication, transcription, virus movements and encapsidation. These genes are arranged in two divergent clusters separated by a large intergenic region (LIR) that contains the promoters for both transcription units.

The genes on the right side are called virion sense (v-sense) because they are transcribed from a DNA strand with the same polarity as the encapsidated viral DNA that also called plus strand. Similarly, genes on the left side (opposite orientation) are called complementary sense (c-sense) because they are transcribed from a DNA strand that is complementary to the encapsidated DNA and present only in the dsDNA intermediate. In general genes encoded on the c-sense portion of the genome, like *rep*, *TrAp* and *REn,* are involved in virus replication and transcription, whereas genes encoded in the virion sense have a function in encapsidation (CP) and virus movement (MP) (Lazarowitz, 1992; Arguello-Astorga et al., 1994; Hanley-Bowdoin et al., 1999; Hanley-Bowdoin et al., 2000).

Bipartite begomoviruses have a genome composed of two ssDNAs, designated as components A and B, 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 the common region (CR), which is the only highly conserved region between both components. All 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 *rep* (AC1) (replication initiation protein), *REn* (AC3) (replication enhancer protein), and *TrAP* (AC2) (transcriptional activator protein) are found in the complementary sense. A fourth gene (AC4) has been described in some geminiviruses, although 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 development. The BC1 gene product [movement protein (MP)] seems to be involved in cell-to-cell transport of the virus, probably by increasing the plasmodesmata 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).

It seems that geminiviruses move in between plant cells as virions or nucleoprotein complexes (Gafni and Epel, 2002) but the form of viral DNA (ss or ds) involved in this movement is unknown. In BDMV, a bipartite begomovirus mesophyll-invassive, both ss- and ds-DNA are involving in movement (Rojas et al., 1998), while in SqLCV, a phloem-limited bipartite begomovirus, NSP and MP bind strongly to ss-DNA (Pascal et al., 1994). In contrary to other geminiviruses, ss-DNA of ToLCV accumulates in the cytoplasm of infected phloem cells (Rasheed et al., 2006). It is likely that different tissue tropisms in bipartite geminiviruses is result of different movement mechanisms (Morra and Petty, 2000).

In addition to genomic DNA, many begomoviruses contain defective or nanovirus-like circular ssDNA encapsidated by CP (Saunders et al., 2000). Unlike other plant viruses begomoviruses predominantly rely on protein-protein and protein-DNA interactions for the stability of their particles (Harrison et al., 2002)

I.4.3 Geminiviruses replication

Most plant viruses replicate in the cytoplasm of infected cells, while geminiviruses replicate in the nucleus, a feature that makes them an excellent model for studying of plant genome replication. The replication of the geminivirus genome occurs entirely through DNA intermediates and within the nucleus of the infected cell. It is largely dependent on cellular factors, and it can be divided into two steps: 1) the conversion of ssDNA (virion DNA) into a dsDNA form and 2) the production of virion-sense ssDNA from dsDNA intermediate.

Little is known about the first process in begomoviruses and curtoviruses. However, in the case of the mastreovirus, a small oligoribonucleotide complementary to a sequence located in the SIR is found associated with the virions. This oligoribonucleotide may prime the synthesis of the complementary strand (Donson et al., 1987).

The process of generation of new viral ssDNA molecules from dsDNA intermediate is better known. Geminiviruses use a rolling circle (RC) replication mechanism, similar to the one used by ssDNA bacteriophages such as ϕX174 (Stenger et al., 1991), in which the viral

protein Rep, considered a replication initiator protein, play a leading role and is multifunctional protein, but does not display DNA polymerase activity. The intergenic region in all geminiviruses contains cis-acting signals required for transcription and initiation of rolling-circle DNA replication. To initiate RC replication, Rep cleaves the viral plus-strand DNA between position 7 and 8 of the nonameric motif (5'-TAATATT AC-3') universally conserved in all geminiviruses. This invariable sequence is found in the loop of the conserved 30 bp hairpin (stem loop) element located in the virus intergenic region. The structure of the stem-like loop sequence is also important. Point mutation analysis revealed that base pairing, which contributes to the maintenance of the stem-loop, is crucial for viral DNA replication (Orozco and Hanley-Bowdoin, 1996). A set of directly repeated sequences are found in the genome of most dicot-infecting geminiviruses (Arguello-Astorga et al., 1994). In ToLCV, these sequences are involved in virus specific Rep-binding (Behjatnia et al., 1998). This motif in the TGMV IR is required for recognition of the virion sense origin and negative regulation of the overlapping promoter for leftward transcription that is located on the left side of common region. This includes the hairpin element and five additional *cis*-acting elements contributing to replication origin function (Fontes et al., 1992). The key elements in the origin are the specific Rep-binding sites with a directly repeated motifs, whose nucleotide sequence varies greatly among geminiviruses, but are positioned similarly in all the known begomoviruses and curtoviruses (Arguello-Astorga et al., 1994). The TGMV Rep binding site is located between the c-sense genes TATA box (including Rep) and their transcription start site (Fontes et al., 1992). Other elements in the TGMV intergenic origin which are necessary for origin function but have little or no effect on AL1 promoter activity include a hairpin structure, AG-motif locating between AL1 binding site and hairpin, and CA-motif is located outside of minimal origin leading to increase replication efficiency to 20-fold (Orozco et al., 1998). The role of AG and CA motifs in the TGMV origin is not clear, but they might bind to host factors to facilitate initiation of plus strand replication. Rep binding site of geminiviruses are related but they have distinct DNA sequence and bind to their cognate Rep protein, specifically, showing that these sites act as origin of recognition elements (Fontes et al., 1994). The TATA-box and G-box transcription factor binding sites in the AL1 promoter act as replication enhancer elements (Eagle and Hanley-Bowdoin, 1997).

After nicking of the origin, Rep becomes covalently linked to the 5'-end (-PO4) of the nicked strand, via a phosphor-tyrosine linkage. The 3'-end (-OH) of nicked DNA primes the plus-strand synthesis by host DNA polymerases, which uses the minus strand as a template, and displaces the parental plus strand linked to the Rep protein. After reconstitution of the origin

of replication, produced full-length linear ssDNA (plus strand) is displaced from the dsDNA intermediate. Rep performs both a second cleavage in the new nonanucleotide sequence and a ligation of two ends of the linear ssDNA. This ligation of Rep-linked 5'-PO4 and 3'-OH releases Rep and generates a circular ssDNA molecule (Heyraud-Nitschke et al., 1995). Molecular details of elongation and termination steps in viral DNA replication are largely unknown. Host cellular replication factors may interact with viral protein and DNA motifs to achieve complete synthesis of viral DNA.

Completion of geminivirus DNA replication requires the cellular DNA replication machinery which is frequently absent or inactive in non-proliferating cells. Therefore, replication of geminiviruses must be limited to dividing cells containing all factors needed for DNA replication. Alternatively, they have evolved a strategy to replicate in some cells and activate specific sets of cellular genes required for DNA replication. In this way geminiviruses act like human oncogenic viruses like SV40 or adenoviruses. Geminiviruses activate differentiated cells by interacting with a host protein, the retinoblastoma related protein (pRBR) and induce transcription of genes encoding host replicative enzymes (Arguello-Astorga et al., 2004). It has been shown that TGMV Rep can induce accumulation of proliferating cell nuclear antigen (PCNA), the processivity factor of DNA polymerase δ, in terminally differentiated cells (Nagar et al., 2002).

I.4.4 Geminiviruses infection cycle

Most geminiviruses are able to infect a variety of differentiated cells (Nagar et al., 1995; Michelson et al., 1997; Sudarshana et al., 1998) while others, including SLCV and abutilon mosaic virus, are confined to vascular tissue and may be restricted to pro-vascular and cambial cells that can support DNA replication (Sanderfoot and Lazarowitz, 1996). The first stage in the infection cycle involves the injection of viral ssDNA into plant cell by an insect vector, *B. tabaci,* that deliver virus into the phloem sieve tubes, from where virus start to spread.

Since the geminiviruses replicate in the nucleus of plant cells, they must have a mechanism that enables them to deliver their genome into the nucleus. As only viral CP is available, movement of the genome into the nucleus must be entirely CP and/or host-machinery dependent. It is not clear that genomic material of virus moves to the nucleus as an encapsidated virion or as a nucleoprotein complex (Gafni and Epel, 2002). Once in the nucleus, the viral ssDNA is converted into dsDNA, the form that serves as a template for both transcription and replication. Synthesis of complementary DNA is exclusively accomplished

by host proteins, since dsDNA is a transcriptionally active template. Host histones then associate with viral dsDNA and are packaged into so-called minichromosomes structures (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, like AC1, AC2, and AC3, are expressed earlier than the virion-sense genes (CP and BV1). After the expression of the early viral genes transcribed from the complementary sense (DNA), the multiplication of the virus genome by a rolling-circle mechanism generates new viral ssDNA molecules from the dsDNA intermediate. An ssDNA molecule produced in this process has one of two fates, depending upon the stage of infection. In an 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; late gene products CP and NSP (BV1) are able to bind viral ssDNA and either encapsidate or simply transport it out of the nucleus, permitting the virus genome to cross from nucleus to cytoplasm. In the bipartite geminiviruses, BV1 gene product serves as a shuttle protein involved in the transport of viral genome into and out of the cell nucleus (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 a BV1 homologue, and other viral proteins like CP must act in the shuttling of DNA between nucleus and cytoplasm (Kunik et al., 1998; Rojas et al., 2001).

For long-distance transport, geminiviruses move from cell to cell and enter the phloem tissues. It is not clear if virus transported as ss- or ds-DNA, or whether it moves encapsidated or as nucleoprotein. Considering the long intracellular distance, there is probably a transport mechanism by which both plant and virus protein interact together to allow the viruses genome to reach the plasmodesmata. BV1 and BC1 genes of bipartite geminiviruses encode two protein involved in transporting the viral genome from the nucleus to the cell wall and through it to adjacent cells (Sanderfoot and Lazarowitz, 1995). In TYLCV infected cells, it seems that CP export viral ds-DNA from nucleus to cytoplasm for cell-to-cell and long distance movement of virus within the plant whereas it encapsidates ss-DNA within the nucleus to form the virions required for insect transmission (Rojas et al., 2001)

In the case of the monopartite geminivirus TYLCV, the movement protein and protein encoded by C4 may act as BC1 homologues to deliver viral genome to the cell periphery and PD. It has been shown that some viruses require the CP for systemic spread in some hosts,

whereas in other hosts the CP is dispensable. In addition MP, C2 and C4 gene products may be directly or indirectly involved in movement of monopartite geminiviruses (Rojas et al., 2001). *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 and Rivera-Bustamante, 1999).

The last stage of the cycle corresponds to the uptake of the virions by the insect vector. In this case, it has been shown that the CP and virus particles are indispensable for insect transmission (Noris et al., 1998; Morin et al., 2000).

I.4.5 Replication initiator protein (Rep)

All geminiviruses encode a replication initiator protein (Rep) also named C1:C2 in mastreviruses, C1 or L1 in curtoviruses and AC1 or AL1 in begomoviruses. This is a 40 kDa protein that is necessary for replication of viral DNA (Hanley-Bowdoin et al., 1990). The geminivirus replication initiator protein has no similarity to known polymerases but it contains conserved sequences of the Rep protein of bacterial plasmids involved in initiation and termination of RC replication (Koonin and Ilyina, 1992). Because of these functional similarities it was designated a Rep protein. Rep is a multifunctional protein with the capacity for sequence specific DNA-binding (Fontes et al., 1992), site-specific endonucleolytic and joining activity (Heyraud-Nitschke et al., 1995), regulation of viral gene expression (Hanley-Bowdoin et al., 2000), self-oligomerization (Orozco et al., 1997), Ren protein (A13) binding (Settlage et al., 1996), interaction with host retinoblastoma related protein and induction of the expression of the host DNA synthesis protein (Nagar et al., 1995). The C-terminal part of Rep also contain a NTP-binding domain (Gorbalenya and Koonin, 1989) and ATPase activity (Desbiez et al., 1995).

Comparison of geminivirus Rep to initiator proteins in prokaryotic rolling-circle replication, parvoviruses, circoviruses and plant nanoviruses revealed high similarity within their N-terminal amino acids containing three highly conserved motifs (RCR-I to III). The function of RCR-I (FLTYPxC) is not known, but RCR-II motif (HLHxxxQ) through an invariant His residue involves in the coordination of a bivalent cation. A Tyrosine residue located in the RCR-III (VxDYxxK) motif is crucial for Rep nicking activity. The Hydroxyl group (-OH) of this tyrosine attack to phosphodiester bond between the last T and A in the invariant loop structure producing a dsDNA circle with a nick that provide 3'-OH needed for making new virion sense (DNA) by host DNA polymerase in the viral replication process, whereas Rep

seems to remain covalently attached to the 5' ends via an energy-conserving ester bond. After one round of replication, the newly synthesized origin is cleaved again by Rep, the new 3' hydroxyl group is linked to 5' phosphate group of the strand linked to Rep, and one ssDNA molecule is released. In this sense, the Rep behaves as topoisomerase I-like site specific cleavage/ligation enzymes (Laufs et al., 1995).

The C-terminal part of TYLCV Rep contains a fourth conserved motif GxxxxGKT/S, specifying the phosphate binding fold (P-loop) that is found in many NTP binding proteins and contributing to its ATPase activity. Point mutations in these conserved motifs cause a substantial decrease or loss of its ATP and GTP hydrolysis activity resulting in reduced viral DNA accumulation (Desbiez et al., 1995). The Exact role of ATPase activity has not yet been identified but was hypothesized that Rep acts as helicase to displace the viral-strand DNA or to unwind the origin, like the Rep68 protein of adeno-associated viruses (Hofer et al., 1992).

The oligomerization domain located in the middle region of Rep is also essential for origin recognition and DNA binding. This region displays strong sequence and structural homology between geminiviruses (Orozco et al., 2000). Rep oligomer containing eight monomers is involved in nucleoprotein complex of REP-DNA. It appears Rep oligomerization is not required for its cleavage and joining activities (Orozco et al., 1998; Sanz-Burgos and Gutierrez, 1998).

The DNA binding domain of Rep has been mapped to its amino terminal moiety (Choi and Stenger, 1995; Jupin et al., 1995). Despite a high sequence homology and functional conservation between geminiviruses of Rep, it shows high specificity for replication of their cognate genome. This specificity is particularly determined by the high-affinity binding site of DNA locating in IR and replication specificity domain of Rep localized in the N-terminal region (Choi and Stenger, 1995; Jupin et al., 1995; Chatterji et al., 1999). In TGMV, this domain maps to the N-terminal 181 amino acids and overlaps the DNA cleavage (amino acids 1-120) and oligomerization (amino acids 134-181) domains. Almost identical amino acids contribute to DNA binding and cleavage activities. However, DNA binding is distinguished from cleavage and ligation domains by its dependence on Rep-Rep interactions (Orozco and Hanley-Bowdoin, 1998). TGMV AL1-AL1 interaction might be prerequisite for DNA binding, because the DNA binding domain includes the oligomerization domain (Orozco et al., 1997). In TYLCV, the catalytic domain of REP is composed of a central five-stranded anti parallel β-sheets which is flanked by a small two-stranded β-sheets, a β-hairpin and two α-helices and is related to a large group of RNA or DNA binding proteins (Arguello-Astorga and Ruiz-Medrano, 2001; Campos-Olivas et al., 2002).

Rep can induce expression of some host genes required for DNA synthesis and accumulation of proliferating cell nuclear antigen (PCNA), the processivity factor of DNA polymerase δ, in non-dividing cells (Nagar et al., 1995). The biochemical role of PCNA in RCR of geminiviruses has not been examined in detail. Mastrevirus RepA contains a LxCxE motif that can interact with human retinoblastoma proteins (cell cycle regulators) (Collin et al., 1996). Begomoviruses Rep lack this binding motif but can interact with maize protein related to human retinoblastoma (Ach et al., 1997). They interact with plant retinoblastoma-related protein (pRBR) through a novel amino acid sequence mapped between amino acids 101 and 180 including two α-helices (Arguello-Astorga et al., 2004). PCNA is also expressed in differentiated cells of transgenic plants expressing Rep, demonstrating that Rep is sufficient for host induction (Kong et al., 2000). In animals pRb family members can negatively regulate cell cycle progression and facilitate their differentiation (Sidle et al., 1996).

In begomoviruses, it has been shown that Rep can interact with other viral proteins like REn. This interaction has not been seen *In vivo,* although in Replication of TGMV DNA molecules containing point mutations in the Rep binding site, Rep can act only in the presence of REn protein provided in trans. This suggests that Rep-REn interaction is required in DNA replication (Settlage et al., 1996).

Rep down-regulates the expression of c-sense genes. Since the presence of the Rep binding site is necessary for its down-regulating activity, it is possible that Rep interferes in transcription machinery (Eagle et al., 1994). In some geminiviruses, Rep could enhance transcription of late genes (Hofer et al., 1992).

I.4.6 Coat protein (CP)

Coat protein, with a mass of 30 kDa, is the second largest viral protein and contains 260 amino acids rich in arginine, valine, serine and lysine resulting in a net positive charge at neutral pH. There are probably 110 CP molecules per geminate particle (Azzam et al., 1994). The CP of geminiviruses is a multifunctional protein, as its primary function is encapsidation of ssDNA and formation of viral capsule to protect viral genome during transmission. Unlike many viruses, the CP of bipartite geminiviruses is not required for cell-to-cell and long-distance movement leading to systemic infection (Pooma et al., 1996) whereas in monopartite geminiviruses, it is crucial for systemic infection as well as insect transmission and capsid formation (Lazarowitz, 1992; Noris et al., 1998). TYLCV CP, like BV1 encoded protein of bipartite geminiviruses, acts in the shuttling of viral genome between nucleus and cytoplasm (Rojas et al., 2001) and this may protect viral genome from intracellular nucleases

(Palanichelvam et al., 1998). Disruption of the TYLCV CP gene prevented accumulation of viral DNA and symptom development in *N. benthamiana* and tomato (Wartig et al., 1997). Geminiviruses CP determines vector specificity (Hofer et al., 1997) and protect viral genome during transmission by insect vector (Azzam et al., 1994) or mechanical transmission (Frischmuth and Stanley, 1998). Other properties of CP include self-binding and binding to a GroEL homologue, a protein that is secreted into whitefly haemolymph by endosymbiotic bacteria that seems to be crucial for insect transmission and may stabilize virus particles in insect haemocoele during transmission by insect (Harrison et al., 2002).

CP expression is regulated by viral TrAP which is encoded by AC2 (Sunter and Bisaro, 1991). CP can allow virus to pass through the whitefly gut wall as well as from white fly haemocoele to salivary gland (Harrison et al., 2002).

CP in both mono- and bi-partite of geminiviruses can import viral genome into the nucleus (Kunik et al., 1998; Unseld et al., 2001). Microinjection and transient expression experiments have shown that TYLCV CP can be localized to nucleus of plant and insect cells (Kunik et al., 1998; Rojas et al., 2001). Nuclear import in karyophilic proteins is generally mediated by a nuclear localization signal (NLS) sequence which typically belong to one of three groups, 1) monopartite motif like SV40 large T antigen PKKKRKV (Kalderon et al., 1984), 2) bipartite motif including two basic motifs separated by some spacer amino acids (but not less than four) such as nucleoplasmin NLS with KR-X10-KKKL residues (Robbins et al., 1991), most NLS found in plant proteins belong to bipartite type (Raikhel, 1992), 3) tripartite motif that is suggested for l-periaxin (Sherman and Brophy, 2000). The NLS of TYLCV CP belongs to the bipartite motif group and is mapped in N-terminal amino acids 3-20 containing KR-X11-KvRRR residues (Gafni, 2003) while ACMV CP contains tripartite NLS whereby the third basic region alternatively facilitate nuclear import with domain one or two (Unseld et al., 2001). The most likely means of nuclear import of TYLCV CP is mediated by karyopherinα (Kunik et al., 1999). Experiments show that another supplementary NLS lies between residues 36 and 61 that facilitates nuclear import (Kunik et al., 1998).

In vitro experiments show that TYLCV CP, like BV1 product of bigeminiviruses, could act as a nuclear shuttle to export DNA from the nucleus to the cytoplasm (Rojas et al., 2001). Nuclear export signal (NES) is normally mediated by a common motif rich of leucine residues such as LPPLERLTL in the HIV-1 Rev molecule (Fischer et al., 1995). Unlike some bipartite geminiviruses in which a leucine-rich sequence was found on the BV1 protein (Ward and Lazarowitz, 1999), no corresponding sequence to serve as an NES have been identified in the CP of monopartite geminiviruses like TYLCV. ACMV CP, as a bipartite geminivirus,

mediates both nuclear import and export of viral genome, its NLS mapped in the both end and middle part of CP, while one NES motif was detected in the central part of CP (Unseld et al., 2001).

The primary function of CP is protection of genomic DNA during transmission. It is possible that the ssDNA binding ability of CP facilitate the coating of virion sense viral DNA as well as its nuclear shuttling functions into and out of nucleus (Gafni, 2003). *In vitro* analysis has shown that TYLCV CP could bind to ssDNA, but not dsDNA, in a highly co-operative and sequence non-specific manner (Palanichelvam et al., 1998). After translation of CP in the cytoplasm, it enters into the nucleus to encapsiadate viral ssDNA. The DNA binding domain of CP is located in N-terminal half of the protein (Liu et al., 1997).

Coat protein is the only known protein constructing the viral particles. Several experiments have shown that full-length CP could interact with itself. N- and C-terminal sequences as well as amino acids concerned in insect transmission are involved in multimerization (Noris et al., 1998; Hallan and Gafni, 2001; Unseld et al., 2004). It seems that for virus assembly, N-terminal amino acids of CP interact with C-terminal amino acids (Hallan and Gafni, 2001). The N-terminal part of CP is located on the surface of the virion, is particularly immunogenic and varies considerably among begomoviruses while the C-terminal of the sequence is strongly conserved and predicted to be mostly buried (Harrison et al., 2002).

CP has a crucial role in insect transmission and in determining insect specificity. Exchange of ACMV CP gene with that of BCTV altered the insect specificity of ACMV from whitefly to leafhopper (Briddon et al., 1990), and it seems that whiteflies do not transmit geminiviruses containing CP mutants unable to form capsid (Azzam et al., 1994). Replacing CP genes of non-transmissible AbMV with that of the relatively closely related whitefly transmissible S*ida golden mosaic virus* (SGMV) resulted in transmission of AbMV (Hofer et al., 1997). As in luteoviruses, the CP may interacts with receptors in the salivary glands of *B. tabaci* (Gildow, 1987). Sequences important for insect transmission are in the central regions of CP (Noris et al., 1998).

Begomovirus CP and virus particles accumulate in the nucleus and it seems virus assembly occurs in the nucleus (Harrison et al., 2002).

I.5 Antibody structure

Antibodies are the host proteins produced in response to foreign molecules in the mammalian body. They may exist as membrane-bound (on B-cell surface) or as soluble antigen receptors, and assist in the process of detection and subsequent elimination of foreign antigens.

Antibodies belong to the immunoglobulin supergene family and constitute the humoral immune response, accounting for approximately 20% of the plasma proteins of blood. The immunoglobulin supergene family are transmemberane glycolproteins specified by a common structural motif with functional domains mediating antigen recognition (Calame, 1986).

The basic structure of all immunoglobulin, independent of their specificity, are similar and consist of two identical light (L) and two identical glycosylated heavy (H) chains. L chains are composed of 220 amino acid residues (25 kDa) while H chains are composed 440-450 amino acid residues (50 kDa). A disulfide bond covalently joins a heavy and a light chain together. The two H chains forming antibody molecule also joined together by a disulphide bond located in a flexible region of the heavy chain known as "hinge". Other disulphide bonds occur in the folding of whole polypeptide H and L chains of antibody leading to form globular regions termed "domain". Both H and L chains contain constant (C) and variable (V) regions which are found at their carboxyl and amino terminals, respectively.

The carboxyl half of light chain and three-quarters of the heavy chain show relatively limited variability and make up the constant regions. Each light chain has one constant region while heavy chain has three constant regions named CH1-CH3. The first 110 amino acids of the amino terminal in both heavy and light chains are considered variable regions (V_H and V_L) and are responsible for the antigen-binding specificity of antibodies. The V region of both H and L chains combine each other to form two identical antigen binding sites. Hypervariable regions or complementary determining regions (CDRs) are found in the variable regions of both H and L chains that recognize and bind specifically to antigens (Kabat et al., 1977). The region of variable domains outside the CDR are called the framework, and do not directly interact with antigen. They are highly conserved and required for correct folding of V domains and maintaining the integrity of the binding site (Morea et al., 1997; Morea et al., 2000).

Partial digestion of immunoglobulin generates antibody fragments that are still biologically active and can be used to elucidate antibody structure or as specific reagents. Digestion of immunoglobulin by papain creates two Fab and one Fc fragment. Pepsin digestion creates a fragment F(ab')2 containing two antigen binding sites comprised of two Fabs and the hinge. Other antibodies fragments include Fv fragment, an unstable fragment comprising V_L and V_H that could bind to antigen, Fd fragments which contain the N-terminal half of H chain, and single chain Fv fragment (scFv) that is a stable form of Fv produced by recombinant antibody technology, in which a peptide linker connects the two V regions. scFv is the most commonly used in research and therapy (Bird et al., 1988)

Immunoglobulin molecules are divided into 5 distinct classes (isotypes) according to their H chain type, known as IgG (γ), IgA (α), IgM (μ), IgD (δ) and IgE (ε). These classes are different in size, charge, amino acid composition and carbohydrate components. The human IgG group possess four subclasses, namely IgG1, IgG2, IgG3 and IgG4. There are also known to be two subclasses of human IgA, but none have described for IgM, IgD, and IgE. The light chains, base on their C region, are divided within two subgroups, κ and λ.

IgG is the most important class of immunoglobulin in the secondary immune responses and dominant (70-75%) immunoglobulin in human serum. It is a monomer, containing two γ heavy chains and two κ or λ light chains and is the only immunoglobulin transported across placenta. IgM is predominant antibody in primary immune responses, accounts for approximately 10% of the immunoglobulin pool and is the first immunoglobulin expressed by B-lymphocytes. Monomeric IgM is the principal antigen receptor on B cells. IgA represents 15-20% of the human serum immunoglobulin pool. It occurs in monomer and dimmer forms, which more than 80% of IgA are as a monomer. IgA monomer is produced by plasma cells in the bone marrow while the dimmer is the most common immunoglobulin in adult serum. IgD is similar to IgG and accounts for less than 1% of the total plasma immunoglobulin but is a major component of the surface membrane of many B-lymphocytes. IgE has a four chain polypeptide structure and is scarce in serum and found on the surface membrane of basophiles and mast cells.

The primary function of an antibody is binding to antigen, which can lead to important consequences depending upon the nature of antigen. In a few cases these binding have a direct effect and may lead to neutralizing of bacterial toxin or prevention of viral attachment to host cells, abilities independent of immunoglobulin isotype which reflect antigen binding capacity of variable regions. In contrast, other antibody functions, which are dependent upon the immunoglobulin isotype, lead to activation of the classical pathway of the complement system.

Particular residues of the hypervariable region of antibodies specifically interact with antigens. Multiple non-covalent bonds involve in the interaction between antigen and amino acids of the binding site. The strength of a non-covalent bond is dependent to the distance of interacting groups. Situations occur when antibody and antigen contain suitable atomic groups on opposing parts of the epitope and paratope and the shape of combining sit must fit the epitope, so that several non-covalent bonds can form simultaneously. In this way, antigen and combining site of antibody are complementary and they have sufficient binding energy to resist thermodynamic disruption of the bond. The strength of binding between a single

antigenic determinant and an individual combining site of antibody is known as antibody affinity while the strength with which a multivalent antibody binds a multivalent antigen is termed antibody avidity. Antibodies clearly recognize the overall shape of an epitope rather than particular residues. Antibodies are able to distinguish between small differences in the primary amino acid sequence of antigens as well as differences in charge, optical configuration and steric conformation. Many antibodies will bind only to native antigens or other fragment folded sufficiently to form multiple interactions between antigen and antibody. Antibodies which bind to these discontinues epitopes often do not bind to denatured antigen (Sakurabayashi, 1995).

Antibodies are remarkably diverse and have the capacity to recognize and respond to millions of antigenic shapes in the environment. In fact, produced types of antibodies are more than our total genes. This huge diversity in antibodies is formed by gene rearrangement process, i.e. recombination between different gene sequences. Separate sets of V genes encode the variable domain of L and H chains that are produced separately. Mammalian DNA germline contain three different segments corresponding to light chain. The first segment, V, encode first part of L chain. There are up to 200 different V sequence linked in tandem to DNA germline. The second segment, J, consist 5 different DNA sequences and the third segment is the constant region (C) of the light chain. During B cell differentiation in bone marrow, one of 200 V segments joins to one of 5 J segments and constitute variable region of light chain (Melchers, 1995). The heavy chain genes contain more segments than light chain genes including V, D and J segments containing 200, 12 and 4 different sequences, respectively. The variable H chain forms by recombination and adjoining of one segment from each V, D and J sequences (Early et al., 1980). Thus, a functional immunoglobulin is constituted from two gene created during the development of B-lymphocyte that is antigen-independent stage. Through these rearrangements, about 10^3 different light chain genes and 10^4 heavy chain genes can be formed individually leading to create 10^7 different types of antibodies.

I.6 Phage display technology

Phage display is a powerful technique applied for selection of peptides with specific binding properties from a vast number of variants. Foreign peptides are presented on the surface of a bacteriophage and are isolated by an affinity selection known as panning (Smith, 1985). The advantage over the synthetic libraries is the physical coupling of phenotype and genotype. This enables the identification of a single binding molecule, displayed as protein or peptide fused to the surface of a bacteriophage by sequencing the encoding genome after

amplification. Up to 10^{14} M13-like bacteriophage can be contained in one ml, but, library size is primarily limited by the efficiency of transformation of *E. coli* enabling realistic library size up to 10^{11} different variants.

Filamentous bacteriophage (M13, fd, f1, IKe) of *E. coli* possess a circular, covalently closed ssDNA, surrounded by a cylinder of coat proteins. The genome consists of 9 genome encoding 11 proteins (pI-pXI). The minor coat protein pIII of filamentous bacteriophage is essential for infectivity. The C-terminal domain of pIII is known to be required for its incorporation into phage particle and mediating its release from the inner membrane (Rakonjac et al., 1999). Plaques formed by these phages on an *E. coli* lawn layer appear turbid, because the infected bacteria are not lysed, their growth is simply impaired. This distinguishes the filamentous phages from the most other bacterial viruses which are icosahedral in shape, accumulated in the cell cytoplasm and release from the host cell by lysing it.

For phage display, peptides are usually fused to the N-terminus of either minor coat protein pIII or major coat protein pVIII. Additionally, cDNA libraries can be displayed by a fusion to the C-terminus of pVI (Jespers et al., 1995). Phage particles can either contain a phage genome, or transduce a phagemid which consists of a plasmid carrying the phage origin of replication and one gene encoding a coat protein fusion. A resistance marker gene allows for the selection of library-containing *E. coli* cells for propagation. Phagemids have to be propagated with the aid of a super-infecting helper-phage providing all the necessary genes needed for particle formation but itself defective in replication. A phage, depending on the length of encapsidated genome, usually possesses 3-5 copies of the pIII and 3000 copies of the pVIII coat protein. With a phagemid, the number of fusion protein copies per phage particle can be adjusted by the promoter preceding the gene. There are several advantages for the use of phagemids, especially if the protein to be displayed is large and reduces the infectivity of the phage particles. Such an impediment from the fusion protein could lead to an accumulation of non-displaying (deletion) phage, elevating the non-specific background in the selection process. Depending on the coat protein used as fusion partner and the choice of the system, different proteins can be effectively displayed. The minor coat protein pIII tolerate N-terminal fusions with proteins as large as scFv (McCafferty et al., 1990). Larger peptides and proteins like Fab were efficiently introduced by the use of hybrid phage producing wild type and fused protein pVIII (Kang et al., 1991).

The scFv libraries have been developed base on scFv fused to pIII minor coat protein. These scFv have been derived by amplification of V_L and V_H region of different animal species such

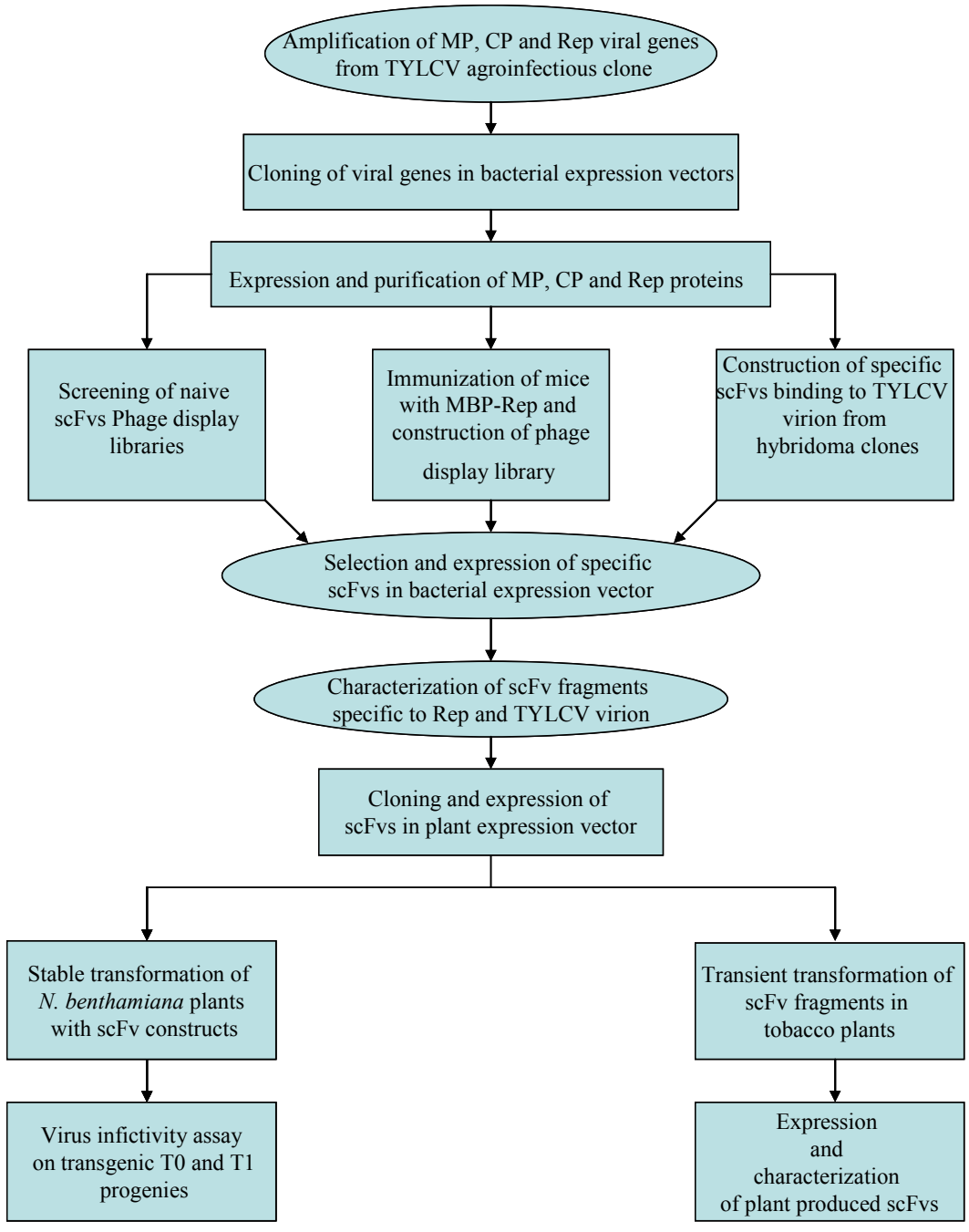
as mice (Hoogenboom et al., 1991) human donors (Sheets et al., 1998) rabbits (Ridder et al., 1995) and camels (Hamers-Casterman et al., 1993) followed by cloning in plasmid vectors. Sources of immunoglobulin genes include the spleens and peripheral blood lymphocytes of immunized animals (Clackson et 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 and animal donors (Clackson et al., 1991; Marks et al., 1991). The phage display libraries from immunized 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 antibody selected by affinity maturation also has 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 of the individual from which the samples were taken. 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 (Griffiths et al., 1994; De Kruif et al., 1995).

Selection of specific scFv from a phage library is done by their presentation to target protein in a process known as bio panning. In bio panning, the target molecule is often immobilized on a solid support. Phage population is incubated with immobilized target protein to capture specific phages. Non-specific immobilisation can mask the antigen of interest or alter the structure of the immobilised target and lead to false positives in the course of selection. Previous blocking of the solid support with BSA or skimmed milk reduces the background binding of phages which do not recognise the target. After incubation, several washing steps are performed to select for the correct binding variants. Elution of these variants is often performed unspecifically by the addition of an acidic buffer. For enrichment of the target binding variants, the eluted phage are allowed to infect *E. coli* cells which are grown under antibiotic selection either separately on a petri dish, or subjected directly to an erlenmeyer flask. In the case of phagemid system, *E. coli* has to be super-infected by the helper phage to initiate the phage particle production. The produced phage are then harvested by PEG/salt precipitation and re-suspended in the incubation buffer to start the next cycle of panning.

Usually, three to five cycles of panning are necessary to enrich specific clones which can then be isolated and sequenced.

I.7 Research objectives

In this study the generation of resistant transgenic plants against TYLCV through recombinant antibody-mediated resistance approach was anticipated. The main objectives of this research were generation of specific scFv fragments binding to viral proteins and sebsequent cytosolic and/or nuclear expression of these scFvs within the plant cells to ideally block viral functions involved in coating/uncoating, transmission, replication, and nuclear import and export. A schematic overview of this Ph.D thesis is presented in below figure.



II Materials and Methods

II.1 Materials

II.1.1 Chemicals and consumables

All chemicals were supplied from the following companies: BioRad (München), Boehringer (Mannheim), Duchefa (Haarlem, Netherlands), GibcoBRL (Eggenstein), Gerbu (Gaiberg), ICN (Eschwege), Merck (Darmstadt), Lehle, Net Fluid Contents (Lehle, USA), Pharmacia (Uppsala), Pierce (Illonois, USA), Amersham Pharmacia Biotech (Freiburg), Roth (Karlsruhe), Roche (Mannheim), Serva (Heidelberg), Sigma (Deisenhofen) and Fluka (Neu-Ulm).

The consumables were purchased from the following companies: Becton Dickenson (Fraga, Spanien), BioSepra (München), Biozym (Hessisch Oldendorf), Dianova (Hamburg), DIFCO (Detroit, USA), Eppendorf (Hamburg), Greiner (Solingen), Hewlett Packard (München), Kodak (Stuttgart), Millipore (Eschborn), Novagen (Darmstadt), Nunc (Bieberich), Pharmacia (New Jersey, USA), Premier Brands (Moreton, UK), Schott (Mainz), Stratagene (Amsterdam, Niederlande) and Whatman (Maidstone, England).

II.1.2 Buffers, media and solutions

Buffers, standard media and stock solutions were prepared according to standard procedures (Ausubel et al., 1995; Coligan et al., 1995; Sambrook et al., 1996) using deionized water. All media and some solutions were prepared and sterilised by autoclaving (25min/121°C/2bar). Heat-sensitive components, such as antibiotics, were prepared as stock solutions, filter-sterilised (0.2 µm) and added to the medium after cooling to 50°C.

II.1.3 Matrices and membranes

Glutathione sepharose 4B from Amersham Pharmacia Biotech was used for purification of GST fusion proteins (II.2.2.1).

Amylose resin (New England BioLabs) is a composite amylase/agarose beads and was used for isolation of protein fused to maltose-binding protein (MBP) (II.2.2.2).

Ni-NTA agarose matrix (BioRAD) was used for purification of scFv fragments from large scale cultures by immobilized metal ion affinity chromatography (IMAC) (II.2.2.3.2). 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.2.3.3.1). Hybound-N^+ membrane (Amershame pharmacia biotech) was used for southern blot analysis (II.8.8).

II.1.4 Enzymes and kits

Restriction enzymes were used from either the company New England Biolabs (NEB, Schwalbach) or the Fermentas (St. Leon-Rot). *Taq* DNA polymerase produced by the Institute of Molecular biotechnology, RWTH-Aachen was used for common amplification and clony-check PCR amplification. The *Taq* DNA polymerase from Fermentas (St. Leon-Rot) was used for amplification of mouse heavy and light chain fragments. All enzymes were used according to the instructions of the company.

The following kits were used:

QIAprep Spin Miniprep Kit (Qiagen)

QIAquick Gel Extraction Kit (Qiagen)

QIAquick PCR Purification Kit (Qiagen)

RNeasy® Mini-Isolation Kit (Qiagen)

RNeasy® plant mini kit (Qiagen)

DNeasy plant mini kit (Qiagen)

SuperScript™ II Reverse Transcriptase (RT) (Invitogen)

SuperScript™ III Reverse Transcriptase (RT) (Invitogen)

GeneTailor™ Site-Directed Mutagenesis Kit (Invitrogen)

Genaxxon Bioscience T/A Cloning Kit (Genaxxon)

TOPO TA Cloning® Kit (Invitrogen)

Omniscript Reverse Transcription Kit (Qiagen)

II.1.5 Primary antibodies, enzyme conjugated secondary antibodies and substrates

Rabbit anti-GST polyclonal antibody prepared by Dr. Michael Monecke (Institut of Molecular Biotechnology, RWTH-Aachen, Germany) was used for analysis of GST and GST fusion protein expression. Rabbit anti-MBP polyclonal antibody (New England Biolabs) was used to detect MBP fusion proteins. Mouse anti-*c-myc* tag monoclonal antibody (9E10) (prepared by Gottfried Himmler, Institute of Apllied Microbiology, Vienna, Austria) and the mouse-anti-penta-His tag (Qiagen and Sigma) were used for detection of scFv-fragments by dot blot (II.2.3.3.1.2), Western blot (II.2.3.3.1.1) and ELISA (II.2.3.3.2) experiments. Polyclonal antibody (AS-0588) reacting against begomoviruses and monoclonal antibodies (AS-0542 and

AS-0546) specifying TYLCV strains (DSMZ-Braunschwieg, Germany) were used at recommended dilution for detection of TYLCV infected leaves by TAS-ELISA (II.2.3.3.2).
Goat anti mouse (GAM) and goat anti rabbit (GAR) antibodies, conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRP) (Dianova, Hamburg), which could react against constant region (Fc) of mouse and rabbit antibodies, were used as a secondary antibodiy in immunoblot (II.2.3.3.1) and ELISA (II.2.3.3.2) analysis.
NBT/BCIP (Bio-Rad) and pNPP (Bio-Rad) were used as substrate for detection of immobilized proteins in Westernblot (II.2.3.3.1.1) and ELISA (II.2.3.3.2), respectively. ABTS (Roche) substrate containing L-tartaric acid/sodium carbonate was used as a substrate for horse radish peroxidase enzyme in ELISA (II.2.3.3.2).

II.1.6 Synthetic oligonucleotides

All synthetic oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany). Oligonucleotides used for sequence analysis and amplification of DNA are listed below.

1. Primers used for amplification of *C1* and *V1* and *V2* genes and cloning into TOPO vector (Invitrogen):

C1 primers:
Forward primer (*SalI*): 5′- GAC GTC GGT CGA CGC AGC CCC CAA TCG G -3′ (28-mer)
Reverse primer (*NotI*): 5′- CGC TGA ACG GCG GCC GCT TAC GCC TTA TTG -3′ (30-mer)
V1 primers:
Forward primer (*SalI*): 5′- GAC GTC GGT CGA C TC GAA GCG ACC AGG CG -3′ (29-mer)
Reverse primer (*NotI*): 5′- CGT CAC CGC GGC CGC TTA ATT TGA TAT TGA ATC -3′ (33-mer)
V2 primers:
Forward primer (*SalI*): 5′- GAC GTC GGT CGA CTG GGA CCC ACT TCT AAA TG -3′ (32-mer)
Reverse primer (*NotI*): 5′- CGT CAC CGC GGC CGC TCA GGG CTT CGA TAC -3′ (30mer)

2. Primer used for amplification and cloning of *C1* gene as fusion with DsRed in pTRAkt vector:

C1 primers:
Forward primer (*AflIII*): 5′- ATT AAC ATG TAT GGC AGC CCC CAA TC -3′ (26-mer)
Reverse primer (*AflIII*): 5′- TAAT ACATGT TCGCCTTATTGGTTTCTTC -3′ (29-mer)

3. Primer used for amplification and cloning of scFv-RWAV gene as cytosolic expression in pTRAkt vector:
Forward primer (*NcoI*): 5′ ATG CCC ATG GCC CAG GTG CAG CTT C -3′ (25-mer)
Rewerse primer (*Xba*I): ATG CTC TAG ATT AGT GAT GGT GAT GGT GAT GAT TC -3′ (35-mer)

4. Primer used for amplification and cloning of scFv-RWAV gene with nuclear targeting signal in pTRAkt vector:

Forward primer with NLS signal (*BspHI*): 5′ - ATG CTC ATG AAT GGC TCC CAA GAA GAA GAG AAA GGT ACC CAT GGC CCA GGT GCA GCT TC -3′ (59-mer)
Rewerse primer (*Xba*I): ATG CTC TAG ATT AGT GAT GGT GAT GGT GAT GAT TC -3′ (35-mer)

5. Primers used to restore frame in V_H region of pHENHI-HScCP1:

5.1 Restoring of frame in V_H frame work 3:
Forward primer: 5′- CAC CCT GTT CCT GCA AAT GAA CTA CCC TCA C -3′ (31-mer)
Reverse primer: 5′- CAT TTG CAG GAA CAG GGT GTT CTT GGG ATT G -3′ (31-mer)

5.2 Restoring of frame in V_H frame work 4:
Forward primer: 5′- GCC GCT GGA TTG TTA TTA CTC GC -3′ (24-mer)
Reverse primer: 5′- AGA CGG TGA CCG AGG TCC TTC AC -3′ (23-mer)

6. Primers used for making cDNA and subsequent amplifying by real time PCR to quantify scFv transcript in transgenic plants:
Forward primer (pA35S): 5′- CCG CAA AAA TCA CCA GTC -3′ (18-mer)
Reverse primer (pA35S): 5′- CAA CAC ATG AGC GAA ACC -3′ (18-mer)
7. Primers used for cDNA synthesis and PCR amplification of mouse V_H - and V_L - domains:

Table II-1: Murine phage display primer sequences for first strand cDNA generation from total RNA in 5′-3′ orientation.

Name	Sequence
COH30 (V_H IgG1)	GGC CAG TGG ATA GAC AGA
COH 32 (V_HIgG2a/2b)	TAA CCC T(TA)G ACC AGG CAT CC
MuPD31 (κV_L)	GCT GAT GCT GCA CCA ACT GTA TCC GTC GAC GCG GCC GCG ACT AGT
MuPD32 (λV_L)	TTT CCA CCT TCC TCT GAR GAG CTT GTC GAC GCG GCC GCG ACT AGT

Table II-2: Sequences of murine V_H-domain specific forward primers MPDVHF1-MPDVHF16 in 5′-3′ orientation (degeneracy codes: K= G or T; M = A or C; S = C or G; R = A or G; W = A or T).

Name	Overhang region	Restriction enzymes (*SfiI*/*NcoI*)	Annealing region
MPDVHF1	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAK GTR CAG CTT CAG GAG TCR GGA
MPDVHF2	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	CAG GTG MAG CTG AWG GAR TCT GG
MPDVHF3	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAG GTC CAG CTR CAR CAR TCT GGA CC
MPDVHF4	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	CAG GTW CAG CTS CAG CAG TCT G
MPDVHF5	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	SAG GTC CAR CTG CAG SAR YCT GGR
MPDVHF6	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAG GTT CAG CTG CAG CAG TCT GGG
MPDVHF7	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAR GTG AAG CTG GTG GAR TCT GGR
MPDVHF8	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAG GTG AAG STY MTC GAG TCT GGA

MPDVHF9	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAR GTG AAG CTK GAK GAG WCT GR
MPDVHF10	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAV GTG MWG CTK GTG GAG TCT GGK
MPDVHF11	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAG GTG CAR CTK GTT GAG TCT GGT G
MPDVHF12	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	SAG GTY CAG CTK CAG CAG TCT GGA
MPDVHF13	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	CAG ATC CAG TTG GTG CAG TCT GGA
MPDVHF14	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	CAG GTS CAC STG RWG SAG TCT GGG
MPDVHF15	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	CAG GTT ACT CTR AAA GWG TST GGC C
MPDVHF16	C ATG CCA TGA CTC GC	G GCC CAG CCG GCC ATG GCC	GAT GTG AAC TTG GAA GTG TCT GG

Table II-3: Sequences of murine V_L-domain specific forward primers MPDVLF1-MPDVLF15 in 5′-3′ orientation (degeneracy codes: K= G or T; M = A or C; S = C or G; R = A or G; W = A or T).

Name	**Overhang region**	**Restriction enzymes (*Asc*I)**	**Annealing region**
MPDVLF1	CAT GCC ATG ACT CGC	GGC GCG CCT	GAC ATT GTG MTG WCH CAG TCT CCA
MPDVLF2	CAT GCC ATG ACT CGC	GGC GCG CCT	GAC ATT CAG ATG ATT CAG TCT CC
MPDVLF3	CAT GCC ATG ACT CGC	GGC GCG CCT	GAC ATT GTT CTC WHC CAG TCT CC
MPDVLF4	CAT GCC ATG ACT CGC	GGC GCG CCT	GAC ATT GTG MTG WCH CAG TCT CAA
MPDVLF5	CAT GCC ATG ACT CGC	GGC GCG CCT	GAT RTT KTG ATG ACC CAR RCK GCA

MPDVLF6	CAT GCC ATG ACT CGC	GGC GCG CCT	GAT RTT KTG ATG ACC CAR RCK CCA
MPDVLF7	CAT GCC ATG ACT CGC	GGC GCG CCT	GAC ATT GTG ATG ACC CAR BHT G
MPDVLF8	CAT GCC ATG ACT CGC	GGC GCG CCT	GAT ATT KTG ATG ACC CAR AYT CC
MPDVLF9	CAT GCC ATG ACT CGC	GGC GCG CCT	RAM ATT GTG MTG ACC CAA TYT CCW
MPDVLF10	CAT GCC ATG ACT CGC	GGC GCG CCT	SAA AWT GTK CTS ACC CAG TCT CCA
MPDVLF11	CAT GCC ATG ACT CGC	GGC GCG CCT	GAY ATY CAG ATG ACM CAG WCT AC
MPDVLF12	CAT GCC ATG ACT CGC	GGC GCG CCT	GAY ATY CAG ATG ACH CAG WCT CC
MPDVLF13	CAT GCC ATG ACT CGC	GGC GCG CCT	GAC ATT GTG ATG ACT CAG GCT AC
MPDVLF14	CAT GCC ATG ACT CGC	GGC GCG CCT	CAR SYT GTK STS ACT CAG KAA T
MPDVLF15	CAT GCC ATG ACT CGC	GGC GCG CCT	CAR SYT GTK STS ACT CAG KCA T

Table II-4: Sequences of murine V_H-domain specific reverse primers MPDVHB1-MPDVHB5 in 5′-3′ orientation (degeneracy codes: K= G or T; M = A or C; S = C or G; R = A or G; W = A or T).

Name	**Overhang region**	**Restriction enzymes (*BstE*II)**	**Annealing region**
MPDVHBII-1	TGM RGA	GAC GGT G	AC CGT RGT C
MPDVHBII-2	TGM RGA	GAC GGT G	AC CGT RGT G
MPDVHBII-3	TGM RGA	GAC GGT G	AC CAG RGT C
MPDVHBII-4	TGM RGA	GAC GGT G	AC CGA GGT T
MPDVHBII-5	TGM RGA	GAC GGT G	AC CGA RAT T

Table II-5: Sequences of murine κ and λ V_L -domain specific reverse primers MPDVLB1-MPDVLB5 in 5′-3′ orientation (degeneracy codes: K= G or T; M = A or C; S = C or G; R = A or G; W = A or T).

Name	**Overhang region**	**Restriction enzymes (*Sal*I/*Not*I)**	**Annealing region**
MPDVLB1	CT AGT GGT ACT CCA C	GC GGC CGC GTC GAC	AGC MCG TTT CAG YTC CAR YTT
MPDVLB2	CT AGT GGT ACT CCA C	GC GGC CGC GTC GAC	AGC MCG TTT KAT YTC CAR YTT
MPDVLB3	CT AGT GGT ACT CCA C	GC GGC CGC GTC GAC	AGC MCG TTT BAK YTC TAT CTT TGT
MPDVLB4	CT AGT GGT ACT CCA C	GC GGC CGC GTC GAC	AGC MCG AGC MCG TTT TAT TTC CAA MKT
MPDVLB5 (λ)	CT AGT GGT ACT CCA C	GC GGC CGC GTC GAC	CTG RCC TAG GAC AGT SAS YTT GGT

Table II-6: Primers used for DNA sequencing [LI-COR IR2-DNA sequencer, labelled with IRD 700 or IRD 800 (USB/Amersham)]. Sequences are in 5′-3′ direction.

Name	**Sequence**
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 forward	ATC CTT CGC AAG ACC CTT CCT CT
pSS reverse	AGA GAG AGA TAG ATT TGT AGA GA
LMB3	CAG GAA ACA GCT ATG AC

8. Primers used for cDNA synthesis and Q-PCR analysis of transgenic plants by amplification of pA35S region:

Forward primer: 5′- CCG CAA AAA TCA CCA GTC -3′ (18-mer)

Reverse primer: 5′- CAA CAC ATG AGC GAA ACC -3′ (18-mer)

9. Primers used for detection of pBIN19 sequence in the agroinoculated plants:

Forward primer: 5´- CCG AGA TAG GGT TGA GTG -3´ (18-mer)

Reverse primer: 5´- CTG GCC GTC GTT TTA CAA C -3´ (19-mer)

II.1.7 Bacterial strains

The following bacterial strains were used for *in vivo* amplification of plasmid DNA, phage display, protein expression and plant transformation. *E. coli* strains DH5α and XL1-blue (II.1.7) were used as a host cells for all intermediate cloning constructs. *E. coli* strain BL21 (λDE3) was used for expression of GST and MBP fusion proteins (II.2.2.1 and II.2.2.2). *E. coli* strain XL1-blue was used for generation of phage-displayed scFv library. *E. coli* strain TG1 was used for amplifying eluted phages after solid-phase panning of phage libraries (II.2.6). *E. coli* strain HB2151 was used for expression and purification of soluble scFv-fragments (II.2.2.3.2) (Table 7).

The bacterial strain *Agrobacterium tumefaciens* GV3101 [pMP90RK, Gm^R, Km^R, Rif^R (Konz and Schell, 1986) was used for agrobacterium-mediated gene transformation (II.2.8.3).

Table II-7: Names and genotypes of *E. coli* strains used throughout the work.

Strain	**Source**	**Genotype**
DH5α	(Ausubel et al., 1995)	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., 1995)	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 laclIq Z*Δ*M*15 Tn10 (Tetr)]
TG1	Stratagene	*supE thi*-1 Δ(*lac-proAB*) Δ(*mcrB-hsdSM*) 5(*rK-mK*-) [F´*traD*36 *proAB lacIq Z*Δ*M*15]
BL21(λDE3)	Novagen	F- *ompT hsdSB* (rB- mB-) *gal dcm* (DE3)
$DH5\alpha^{TM}$-$T1^R$	Invitrogen	*F´ φ80lacZ*Δ*M15* Δ*(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44thi-1 gyrA96 relA1 tonA*

II.1.8 Plants and animals

Nicotiana tabacum L. cv. Petite Havana SR1 was used for transient protein expression after agroinfiltration (II.2.8.3.1). *N. benthamiana* grown from seeds, was used for stable transformation of scFv fragment constructs (II.2.8.3.2.1). *Lycopersicum esculentum* (cv. Super Marmande) was used for stable transformation with pTRAkt-ScRep-GFP (II.2.8.3.2.2). 6-8 weeks old female BALB/c mice were used for immunization with MBP-Rep fusion protein (II.2.4).

II.1.9 Helper phages

M13KO7 helper phage is an M13 derivative phage carrying the mutation Met40Ile in *gene*II. The p15A origin of replication and the kanamycin resistance gene from Tn903 were inserted within the M13 origin of replication (Vieira and Messing, 1987).

II.1.10 Plasmids and phagemides

The following plasmids were used in this thesis. Furthermore, schematic presentations of all vector maps used in this thesis are provided in the Appendix.

The pCR2.1-TOPO is a cloning vector designed for cloning of PCR products directly from a PCR reaction. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. The plasmid vector is linearized with single 3' T overhangs for TA cloning and topoisomerase I covalently bound to each 3´ phosphate of vector. PCR products must contain a single 3' adenine overhang which allows the PCR inserts to ligate efficiently with the vector.

The pMBL-T/A is a cloning vector (2.9 kb) for direct cloning of PCR products. The vector prepared by cutting with *Eco*RV and adding 3´ terminal thymidine to both ends. These singe 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of PCR.

The pGEX-5X-3 (Amersham Pharmacia Biotech) was used for subcloning of C1, V1 and V2 viral genes for expression as a GST fusion protein. The cloned gene introduced downstream of GST gene.

The pMAL-c2X (New England Biolabs) is an expression vector that 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.

The pHENHI is a phagemid vector [4,6 kBp, Amp^R, ori [M13], *lac*I, *lac*Z, *pel*B, c-myc, His-6, Gen III, pMB1 ori (rop^-)] (Hoogenboom et al., 1991). This vector contains a 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 pIT2 (HIS-myc tag) is a phagemid vector used for construction of Tomlinson I and J libraries. It contains *Sfi*I/*Nco*I-*Xho*I, *Sal*I-*Not*I cloning sites.

The pTRAkt (8,4 kbp, Amp^R, Cb^R, Km^R), kindly provided by Thomas Rademacher (Institute of Molecular biotechnology, RWTH-Aachen,Germany), is an optimized binary plant expression vector containing the p35SS promoter, 5´ untranslated region (5´-UTR) from *Tobacco etch virus* and pA35S polyadinelation signal (3´-UTR) from CaMV. A matrix attachment region was introduced to improve transcription.

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).

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).

Thermocyclers: Primus and Primus PCR 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.

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

Software: Windows NT 4.0 operating system (Microsoft); Microsoft Office 2000 (Microsoft); Adobe Photoshop 6.0 (Adobe); Chromas; 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}.

Mini hybridization oven (Biometra, Göttingen,Germany)

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 including PCI (phenol/chloroform/isoamyl alcohol), 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 Isolation of plasmid-DNA from *E.coli*

Recombinant plasmid DNA was purified using QIAprep® Plasmid Isolation Mini (II.1.4) according to the manufacturers' manual based on the alkaline lyses method (Sambrook et al., 1996). Quality and quantity of DNA was confirmed by reading the absorbance at 260 nm and 280 nm in a spectrophotometer according to (Müller et al., 1993) and (Sambrook et al., 1996) or analytical agarose gel electrophoresis (II.2.1.3). Isolated DNA samples were stored at –20°C.

II.2.1.2 PCR amplification

Polymerase chain reaction (PCR), a procedure for rapid *in-vitro* enzymatic amplification of a specific segment of DNA, was used for the amplification and modification of genes of interest as well as for the insertion or short nucleotide sequences. The reactions were performed in 0.2ml PCR tubes (Biozym, Oldenburg), using a PCR thermocycler (MWG Biotech). The

cycler contained a heated lid to avoid the use of mineral oil. PCR reactions were carried out in a total volume of 50 µl as described in the following table:

Components	Volume	Final concentration
10X PCR buffer	5 µl	1X
50 mM $MgCl_2$	2.5 µl	2.5 mM
2.5 mM dNTPs	1 µl	0.25 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)	0.5 µl	2.5 units
dd H_2O	To 50 µl	

Amplification was carried out under the following conditions:

10 min 95°C
1 min 95°C
1 min 55°C } 35 X
1.5 min 72°C
10 min 72°C

The optimal annealing temperature (T_P) of the primer was experimentally optimized (temperature gradient) or calculated by the empirical formula (WU *et al.* 1991). $T_P = 22 + 1.046 [2x(G +C) + (A + T)]$ PCR products were resolved on a 1-1.2% (w/v) agarose gel (II.2.1.3) with appropriate DNA markers to confirm the successful amplification and integrity of the amplified product.

For generation of highly sensitive hybridization probes suitable for detection of low copy target sequences in southern-blot hybridization (II.8.8), DIG-11-dUTP (alkali stable) was incorporated into V1-PCR product according to the manufacturees instructions (Roche).

Recombinant *E. coli* and Agrobacteria clones harboring plasmid DNA containing the gene of interest were identified by PCR as described by (Jesnowski et al., 1995). Single colonies were picked with sterile toothpicks and dipped each in a PCR tube containing 10µl of sterile water. 15µl of the PCR mix were added to each 10µl bacterial suspension giving a final volume of 25µl. Specific primers annealing to the 5' and 3' ends of the cloned gene or primers specific

for the vector backbone were used for PCR reaction. Thermocycler conditions were used as described above. 10µl of the PCR product were analyzed on a 1.2% (w/v) agarose gel (II.2.1.3).

II.2.1.3 Analytical agarose gel electrophoresis

Plasmid DNA, PCR fragments and restriction enzyme digested DNA (II.1.4) were electrophoretically separated on 0.8-1.2% (w/v) agarose gels prepared in TBE buffer containing 0.1µg/ml ethidium bromide as described by (Sambrook et al., 1996).

Known amount of DNA molecular marker such as 100 bp DNA ladder or *Pst*I-digested lambda DNA were used for evaluation of sample size, integrity and determination of DNA concentration. The DNA was visualized on an UV transilluminator at 302nm and documented by a black and white E.A.S.Y 429K camera (Herolab).

II.2.1.4 Preparative gel electrophoresis

Preparative gel electrophoresis was used for isolation of DNA fragments after restriction enzyme digestion (II.1.4) or PCR amplified DNA (II.2.1.2) prior to cloning in the appropriate vectors. After electrophoresis, the desired DNA fragments were excised from the gel and purified using the "QIAquick Gel Extraction Kit" (Qiagen) (II.1.4) according to the manufacturers' protocol.

II.2.1.5 Quantification of nucleic acids

The amount of RNA or DNA in a sample was estimated by analytical agarose gel electrophoresis or by measuring the OD_{260nm}. The OD_{260nm} of 1 corresponds to ~50µg/ml of dsDNA or ~40µg/ml of ssDNA and RNA. Purity of the nucleic acid was ascertained by the OD_{260nm} / OD_{280nm} ratio of the measured optical density, which is 1.8 for pure DNA and 2.0 for pure RNA.

II.2.1.6 Restriction of DNA

Restriction endonucleases, appropriate buffers and BSA solution were obtained from New England Biolabs (Schwalbach). Single or double restriction of DNA fragments were performed at suitable buffer and temperature according to the manufacturers' protocol.

II.2.1.7 Dephosporylation

To prevent auto-ligation of plasmid vector, dephosphorylation of restricted vector-DNA was accomplished with CIP (Calf Intestine Phosphatase, NEB) at 37°C for one hour according to the manufacturers' protocol.

II.2.1.8 Klenow Fill-in

For the fill-in of single-stranded DNA 5'-fragments, the Klenow Fragment of DNA polymerase I of *E.coli* was used (Joyce and Grindley, 1983). The Klenow-Fill-in reaction was done for making blunt ends according to the manufacturers' protocol.

II.2.1.9 Ligation of DNA

Restriction enzyme digested DNA (II.2.1.6) was ligated using 80 U T4 DNA-Ligase or 1 µl Quick T4 DNA-Ligase (NEB) in buffer systems recommended in the manufacturers' protocol in a final volume of 20 µl. Sticky-end ligations were carried out at 22 °C for 30 min whereas blunt ligations were incubated at 4 °C (overnight). Ligation product was used for transformation of *E.coli* (II.1.7).

II.2.1.10 DNA sequencing and sequencing analysis

Fluorescently labelled 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.

The universe primer (II.1.6) was used for sequencing of viral gene cloned in the TOPO vector. The pGEX forward and backward primers were used for confirming of the truncated viral genes (NC1 and NV1) cloned in the pGEX-5X-3 vector. Forward and backward pHEN primers were used for sequencing of scFv-fragments in the pHENHI phagemid vector. The LMB3 primer was used to determine sequence of scFv genes harboured in pIT vector. The pSS forward and reverse primers were used for sequence analysis of genes in pTRAkt vector.

Chromas software package was used for displaying the chromatogram files from LI-COR automated DNA sequencer. The sequences were edited and exported for further analysis with the Wisconsin Package^{TM} of Genetic Computer Group (GCG).

II.2.1.11 *In vitro* site-directed mutagenesis

GeneTailor™ Site-Directed Mutagenesis Kit (Invitrogen) was used for making DNA mutation in framework 3 of V_H-scFv-HScCP1 according to the manufacturer`s protocol. The methylation reaction was prepared as follow:

Reagent	**Amount**
Plasmid DNA	100 ng
Methylation Buffer	1.6 µg
10X SAM	1.6 µg
DNA methylase (4U/µl)	1 µl
Steril, distilled water	to 16 µl

The methylation was accomplished for 1 hr at 37°C. The PCR reaction was obtained as following table:

Component	**Volume**	**Final concentration**
10X High fidelity PCR buffer	5µl	1X
10mM dNTP	1.5µl	0.3 mM each
50 mM $MgSO_4$	1µl	1 mM
Primers (10µM each)	1.5µl	0.3µm each
Methylated DNA (12.5-31.25 ng)	2-5µl	As required
Platinum® *Taq* high fidelity (5U/µl)	0.2-0.5 µl	1-2.5 units
Autoclaved, distilled water	to 50 µl	

Amplification was carried out under the conditions described below:

Segment	Cycle	Temperature	Time
1	1	94 °C	2 minutes
2	20	94°C	30 seconds
		55°C	30 seconds
		68°C	5.5 minutes
3	1	68°C	10 minutes

After the DNA amplification, 2 µl of PCR product was used for transformation into 50 µl of DH5α^{TM}-T1^{R} competent cell (II.1.7).

II.2.1.12 Growth and maintenance of bacterial strains

II.2.1.12.1. Culturing and maintenance of *E. coli*

Individual colonies of all *E. coli* strains were obtained by plating the corresponded strain on LB agar plates. Strains carrying an F′ factor were spread on M9 plates. Incubation was performed at 37°C. LB medium containing the suitable antibiotics and 2% glucose was inoculated with a single recombinant colony of *E. coli* and grown overnight at 37°C with vigorous shaking (225 rpm). The plates were stored at 4°C for short periods (less than 2 weeks). For long term storage of bacterial strains, 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.2.1.12.2 Culturing and maintenance of *Agrobacterium*

Single colonies of *A. tumefaciens* were examined for the presence of plasmids by clony check PCR (II.2.1.2). Positive colonies were inoculated in 10 ml of YEB-Rif-Km-Carb medium and cultivated at 28°C for 2 days with vigorous shaking at 250 rpm. The culture was transferred to Falcon tubes and Agrobacteria cells were precipitated 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 stock media (GSM). The suspension was aliquoted (100µl) and stored at –80°C.

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Glycerol stock media (GSM):

Glycerol	50% (v/v)
$MgSO_4$	100 mM
Tris-HCl pH 7.4	25 mM

II.2.1.13 Bacterial transformation

II.2.1.13.1 Preparation of competent *E. coli* cells for heatshock transformation

E.coli competent cells were prepared for $CaCl_2$-mediated heatshock transformation as described by (Hanahan, 1985). Briefly, a single bacterial colony was inoculated in 5 ml of LB broth and cultured at 37°C overnight (overnight). 0.5 ml of the overnight 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 OD_{600nm} 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.2.1.13.2 Transformation of *E. coli* by heat-shock

After thawing of the competent cells (II.2.1.13.1), plasmid DNA (up to 100 ng) 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.

II.2.1.13.3 Preparation of competent *E. coli* cells for electroporation

Electrocompetent *E. coli* were prepared from the following strains, DH5α, BL21(λDE3) and XL1-blue (II.1.7) as described (Dower et al., 1988). A single bacterial colony from an LB plate was cultured in 5 ml LB-broth and cultured at 37°C overnight. Three ml of fresh overnight 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.2.1.13.4 Transformation of *E. coli* by electroporation

Electrocompetent cells (II.2.1.13.3) were thawed on ice and 50 µl of the cells were 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 overnight.

II.2.1.13.5 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.2.1.13.6 Transformation of *Agrobacterium* by electroporation

0.2-1.0 µg of plasmid DNA (II.2.1.1) in sterile dH_2O was added to a thawed aliquot of electrocompetent *Agrobacterium* cells (II.2.1.13.5) 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.2.2 Expression and purification of recombinant proteins

II.2.2.1 Expression and purification of GST fusion proteins

pGEX plasmids are designed for inducible, high level intracellular expression of gene or gene fragments as fusion with *Schistosoma japonicum* GST. GST-fusion proteins were expressed and purified via affinity chromatography using Glutation Sepharose 4B 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 overnight at 37°C with vigorous shaking. The following day 1L of fresh 2YT medium containing 0.1% (w/v) glucose and 100 µg/ml ampicillin media were inoculated with 10 ml overnight culture and grown at 37°C and 225 rpm till $OD_{600\ nm}$ reaches to 0.6-0.8. Expression of recombinant proteins was then induced by addition of IPTG to a final concentration of 0.25 mM. Cells were cultured 4 hr at 25°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 (50 ml/1000 ml bacterial culture) supplemented by protease inhibitor cocktail (1 tablet/50 ml PBS) and DTT to final concentration of 5 mM. The cells were 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/11000g/4°C) and the supernatant subjected to glutathione affinity chromatography according to the manufacturers instructions (Amersham Pharmacia Biotech). Elution buffer was used to release bound fusion proteins from the column. The purified fractions were immediately stored at -20ºC before use.

Elution buffer: 0.154 g Glutation dissolved in 50 ml Tris-HCl 50 mM, pH 8

II.2.2.2 Expression and purification of MBP fusion proteins

The pMAL^{TM} vectors provide a method for expressing and purifying a protein. The cloned gene is inserted downstream from the male gene of *E. coli* which encodes maltose binding protein (MBP). The 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 overnight at 37°C with vigorous shaking. The following day 1L of fresh 2YT/0.1% (w/v) glucose and 100 µg/ml ampicillin media were inoculated with 10 ml overnight culture and grown at 37°C and 225 rpm to an OD_{600nm} of 0.5-0.8. Expression of recombinant proteins were then induced by addition of IPTG to a final concentration of 0.25 mM and were cultured 4 hr more at 25°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 overnight at -20°C. The samples were thawed in cold water and sonicated four times in short pulses of 30 sec (150W with 30 sec interval). The crude extract (supernatant) was obtained by centrifugation at 9000 x g for 30 minutes at 4°C. Supernatant was subjected to amylase resin according to the manufacturer` s instructions (New England Biolabs). To elute the bound proteins, 3ml 100mM-maltose containing column buffer was added to the column and the eluted protein was collected in separate fractions and stored at -20ºC.

Column buffer: 20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT

II.2.2.3 Expression and purification of soluble scFv antibodies

II.2.2.3.1 Mini-induction of scFv in pHENHI phagemid vector

A single recombinant colony (II.2.1.13.2) of *E. coli* strain XL1-blue or HB2151 harboring the pHENHI phagemid containing the scFv gene was inoculated in 2 ml of 2YT medium containing 100 µg/ml ampicillin and 0.1% (w/v) glocuse and cultivated at 37 °C for 3-4 hr with shaking until the $OD_{600\ nm}$ reached 0.4-0.6. The induction was carried out at 30°C for 16

hr after addition of 0.5 mM IPTG. The cells were removed by centrifugation (4000g/4°C/10 min) and the supernatant were used for immunoblotting (II.2.3.3.1) and ELISA (II.2.3.3.2).

II.2.2.3.2 Large scale expression and purification of scFvs by IMAC

Recombinant pHENHI plasmid harbouring scFvs genes were initialy transformed into *E. coli* strain HB2151. A single recombinant colony was inoculated in 10 ml of 2YT medium containing 1% (w/v) glucose and 100 µg/ml ampicillin (2YTGA) and cultivated overnight at 37°C with shaking (200 rpm). Three ml of the overnight culture were transferred into 500 ml of 2YT media containing 0.1% (w/v) glucose and 100 µg/ml ampicillin and grown at 37°C until the OD_{600nm} reached 0.7- 0.8. Expression of scFv fragments were induced by addition of IPTG to a final concentration of 1 mM for 3 hours at 30°C. The culture was centrifuged (4000 g/4°C/20 min) and the pelleted bacteria were resuspended in 10 ml of resuspension buffer (30 mM Tris, 20 % (w/v) sucrose, pH 8.0). EDTA was added to a final concentration of 1 mM. The suspension was incubated at 4°C for 10 min with gentle agitation followed by centrifugation at (8000 g/4°C/20 min). The supernatant S1 was taken and kept on ice. The pellet was resuspended in 9 ml of ice cold 5 mM $MgSO_4$ and incubated at 4°C for 8-9 min on a shaker. EDTA was added to a final concentration of 1 mM and the suspension was agitated for 3 more minutes followed by centrifugation at (8000 g/4°C/20 min). The second supernatant (S2) was mixed with the first supernatant (S1) 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 wash buffer. Imidazol and sodium chloride were added into the dialyzed periplasmic fraction to a final concentration of 10 mM and 500 mM, respectively. The sample was passed twice through the pre-equilibrated Ni-NTA matrix. The column was washed with 20 volumes of wash buffer and the proteins were eluted in three fractions with 700 µl of elution buffer. Collected fractions were immediately dialysed against PBS (pH 7.4) to remove imidazol and salt. To control the yield and purity of dialyzed scFv fragments 15 µl were run in a SDS-PAGE (II.2.3.2). The concentration was determined using the Bradfrod protein assay (II.2.3.1).

Wash buffer: 1x PBS, 30 mM imidazol; 500 mM NaCl

Elution buffer: 1x PBS, 250 mM imidazol pH 4.5

II.2.3 Protein analysis

II.2.3.1 Quantification of total soluble protein

The concentration of purified protein was determined by Bradford assay (Bradford, 1976) or visual comparison with a purified protein of known concentration in Coomassie-stained gel (II.2.3.2) and/or Western blot (WB) (II.2.3.3.1.1) assays.

For Bradford assay the protein solution of interest was serially diluted. BSA was also serially diluted and used as standard. 10 µl of each dilution was transferred into the wells of a low binding microtiter plate (Greiner, Solingen, Germany). 10 µl of the buffer was used as a blank. 200 µl of Bradford reagent were added to each well, mixed with the proteins and incubated at RT for 10 min followed by the measurement of OD_{595nm}. For each dilution, measurements were performed in duplicate and the average was taken for the calculation of the protein concentration.

II.2.3.2 SDS-PAA gel electrophoresis and Coomassie brillant blue staining

Discontinuous SDS-polyacrylamide gels (stacking gel 4%, pH 6.8; separating gel 12%, pH 8.8) (Ausubel et al., 1995) were used for separation of protein samples. Before loading onto the gel, protein samples were denaturated in SDS-PAA-sample buffer and heated 3min at 95°C. 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 30 min in Coomassie staining solution at RT under constant rocking. Coomassie staining was removed by adding destaining solution until the protein bands were clearly visible.

10X SDS-PAA-sample buffer:

Tris-HCl (pH6.8)	62.5 mM
Glycerol	10% (V/V)
SDS	2%(W/V)
β-Mercaptoethanol	5%(V/V)
Bromephenol Blue	0.05(W/V)

SDS-PAGE running buffer (pH 8.3):

Tris-HCl (pH 8.3)	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.2.3.3 Immuno assays

II.2.3.3.1 Immunoblot analysis

II.2.3.3.1.1 Western-blot analysis (WB)

Electrophoretically separated proteins (II.2.3.2) were transferred from an SDS-PAGE gel to PVDF or Hybond^{TM}-C nitrocellulose membrane (0.45 µm). The membrane was blocked with PBS buffer containing 3% (w/v) skimmed milk powder (MPBS) and blotted proteins probed with a primary antibody (anti *c-myc*, anti-His, anti GST, or anti MBP) that reacted specifically with antigenic epitopes displayed by the target protein attached to the membrane. The bound antibody was detected by addition of appropriate 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)

AP buffer (pH 9.6):

Tris-HCl, pH9.6	100mM
NaCl	100mM
MgCl2	5mM

II. 2.3.3.1.2 Dot-blot analysis

About 5 µl of supernatant (II.2.8.5) containing soluble scFv fragments extracted from plants were immobilized on Immobilon-P PVDF membrane. Immobilized scFv fragments were detected using 1:5000 diluted anti-*c-myc* monoclonal antibody 9E10 followed by 1:10000 diluted AP-labelled goat anti-mouse polyclonal antiserum. Binding of AP-conjugated secondary antibodies was revealed by adding BCIP/NBT substrate.

II.2.3.3.2 ELISA (Enzyme-linked immunosorbent assay)

Indirect ELISA was used for analysis of bacterially and plant expressed soluble scFv fragments. About 10-50 µg/ml antigens in PBS were directly coated on high-binding microtitre plates by incubating at 4°C overnight followed by a blocking step using 2% (w/v) skimmed milk in PBS. 100 µl of scFv solutions were then applied to the plates and incubated at 37°C for 2 hr. Bound scFvs were detected using 1:5000 diluted anti-*c-myc* monoclonal antibody 9E10 followed by 1:5000 diluted horseradish peroxidase (HRP)-conjugated goat-anti-mouse polyclonal antibodies. ABTS substrate dissolved in ABTS buffer (Boheringer Mannheim) was used for color development at 37°C for 30 min followed by the measeurment of OD_{405nm}.

For titre determination of polyclonal antibodies from sera of mice, direct ELISA was performed using GST-Rep, GST-NRep, MBP and GST proteins as antigens/controls. 10µg/ml of antigens was coated onto high binding ELISA plates. GST and MBP (10 µg/ml) were 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 (1:500-1:1024000) 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 $OD_{405\ nm}$.

For detection of TYLCV infected leaves, triple antibody sandwich ELISA (TAS-ELISA) was used. This ELISA is based on monoclonal antibodies which react with all TYLCV species. It employs a polyclonal antibody (IgG) for coating and monoclonal antibody (MAbs) for decorating of the virus coat protein. Since the monoclonal antibody is not labeled, a secondary

mouse specific antibody (RAM^{AP}) is used to react with the bound MAb. Polyclonal antibody (DSMZ AS-0588) with monoclonal antibody (DSMZ AS-0546/2) kindly prepared by S. Winter from DSMZ, Braunschweig, Germany were used according to manufacturer's protocol.

II.2.4 Immunization of mice

The treatment and maintenance of laboratory animals was approved by the 'Regierungspräsidium des Landes NRW' (RP-Nr.: 50.203.2 AC 02/06). Three female mice (Balb/c) each were immunized with 100 µg of MBP-Rep fusion protein mixed with 40µl GEBRU's adjuvant. Seven further 50µg injections into the tail vein were given at weekly intervals with 20µl GEBRU's adjuvant per mouse. One boost more was performed 1 day prior to sacrifice with concentration of 50µg. After final boosting, blood was taken from the tail vein using a 26 gauge needle (20 µl) and a capillary for picking up blood. The blood was diluted up to 2-fold with PBS for estimation of antibody titre by ELISA (II.2.3.3.2).

II.2.4.1 Isolation of mouse spleen and spleen cell preparation

The immunized mouse (II.2.4) was sacrificed with Isofluran, sterilized with 70% (v/v) ethanol and then dried with some paper towels. The spleens from immunised mice were removed and dissected. Spleenocytes were prepared by disrupting the spleen using a mechanical homogenizator in 7 ml Trizol.

II.2.5 Construction of phage-displayed ScFv libraries

II.2.5.1 Isolation of total RNA from spleen cells

Isolation of total RNA was carried out using Trizol extraction (Invitrogen). Frozen homogenized spleenocytes incubated in RT to complete dissociation of nucleoprotein complexes. Total RNA was purified using chloroform and subsequent precipitation by Isopropyl alcohol and ethanol according to the manufacturers' protocol.

II.2.5.2 The first strand cDNA synthesis

To remove genomic DNA, about 3 µg total RNA was treated with DNase I enzyme (Fermentas). First-strand cDNA was synthesised from 2 µg of total RNA using Super Script™ II Reverse Transcriptase (RT) (Invitogen) and oligo dT primer according to the manufacturers instructions.

II.2.5.3 Construction of scFv libraries

Variable heavy and light regions were amplified by PCR using two specific sets of primers binding to the murine framework region 1 and 4 (II.1.6). For each forward primer separate PCR reactions were performed whereas backward primers were used as a cocktail. PCR reactions and amplification program were accomplished as mentioned in tables below:

Components	Volume	Final concentration
10X PCR buffer	5 µl	1X
$MgCl_2$ (50 mM)	1.5 µl	2.5 mM
dNTPs (2.5 mM)	4 µl	0.2 mM each
Forward primer (10 pmol/µl)	1 µl	10 pmol
Reverse primer (10 pmol/µl)	1 µl	10 pmol
cDNA template	4 µl	
Taq DNA polymerase (5U/µl)	0.5 µl	2,5 units
dd H_2O	To 50 µl	

PCR reaction was carried out under the following conditions:

5 min 95°C
1 min 95°C
1 min 52°C } 35X
1.5 min 72°C
5 min 72°C

PCR amplified heavy (V_H) and light (V_L) chain fragments were gel purified using "QIAquick gel extraction kit" (Qiagen) (II.1.4) and digested with *Sfi*I/*Bst*EII (V_H) or *Asc*I/*Not*I (V_L) enzymes, respectively. To create the sublibraries, the pHENHI phagemid DNA was digested with *Sfi*I and *Bst*EII or *Asc*I and *Not*I and gel purified. 200 ng of purified vector were ligated with a five fold molar excess of purified fragments. Ligation products were electroporated into electrocompetent *E. coli* XL1Blue cells (II.1.7). The scFv fragment libraries were constructed by recovering V_H fragments from the V_H sublibrary and cloning into the V_L sublibrary.

II.2.5.4 *Bst*NI fingerprinting

The variability of the generated scFv libraries was tested by restriction analysis with *Bst*NI. The PCR-amplified scFv fragments from 17 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). Digested fragments were separated in a 2% (w/v) agarose gel (II.2.1.3) and the variability of the library was estimated.

II.2.6 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 strains. For isolation of phages exposing antigen specific antibodies, panning procedures were performed. GST/MBP-Rep fusion proteins (~50-100µg/ml) were immobilized overnight 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 h under rotation on an under and over turntable and then 1.5 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 7.5. Nine ml of log phase *E. coli* TG1/XL1-blue cells were infected with eluted phages and plated on 2xYT agar plates containing 1% (w/v) glucose and 100 µg/ml ampicillin (2x YTGA-agar). The plates were incubated overnight 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.2.7 Cloning of specific scFvs interacting to TYLCV virion from hybridoma cell lines

II.2.7.1 Purification of mouse mAbs from the hybridoma supernatants

Hybridoma clones producing monoclonal antibodies against TYLCV virion, briefly named HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 (kindly provided by Dr. S. Winter, DSMZ-Braunschweig, Germany) were grown in 200 ml tissue culture flasks. The culture supernatants were harvested when the medium became acidic. 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. Protein-A, a 43 kDa cell wall protein produced by the bacteria *Staphylococcus aureus* contains four binding sites for the Fc regions of IgG located in the interface between the CH2 and CH3 domains and is commonly used for antibody purification. Protein-A was isolated and purified first by (Sjoquist et al., 1972).

About 1.6 ml of Protein A matrix was packed in a column and equilibrated with 5 column volumes 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 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.2.3.3.2), WB (II.2.3.3.1.1) and SDS-PAGE analysis (II.2.3.2).

II.2.7.2 Construction of scFv fragmnets

Starting from the hybridoma cells, corresponding scFvs were made from each hybridoma lines by extraction of total RNA from hybridoma cells following the cDNA synthesis, V_H and V_L amplification by specific primers (II.1.6) and cloning into pHENHI by the same way used for construction of scFv phage library (II.2.5.3).

II.2.7.3 Soluble expressions of scFv fragments and indirect ELISA

Screening of scFv-fragment libraries was performed after the third round of panning by small scale induction of scFv expression from pHENHI or pIT2 phagemid vectors in ELISA plates. 96 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, 5 µl of bacterial culture was transferred from the master plate to a second plate containing 200 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 detection of positive clones by indirect ELISA as it was described (II.2.3.3.2).

II.2.7.4 Characterisation of selected scFv by western blotting

Selected scFvs from the last step (II.2.7.3) were analyzed by western blot. Recombinant clones of *E. coli* strain HB2151 were inoculated in 50ml of 2xYT, 100µg/ml ampicillin, 1% (w/v) glucose in sterile tubes. The Next day, 50µl of this culture were transferred to 50 ml fresh 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 overnight by addition of IPTG to a final concentration of 1 mM. The cells were removed by centrifugation and the supernatant was used for western blot as described (II.2.3.3.1.1).

II.2.8 Generation and characterisation of transgenic plants

II.2.8.1 Growth and maintenance of tobacco plants

Tobacco plants were grown in a greenhouse in ED73 standard soil (Patzer, Sinntal-Jossa) with 0-30% (v/v) sand under supplementary illumination of 10 000 Lux (plus the sun light), 70-90% humidity and 16h photoperiod at 24°C (or higher depending on the outside temperature). To prevent pollination from other plants, flowers were covered with plastic bags with micro pores. Mature, dried seeds were stored in paper bags at RT.

II.2.8.2 Preparation of recombinant Agrobacteria

100 ml of YEB-Km-Rif-Carb medium was inoculated with 100 µl of glycerol stock of the selected recombinant Agrobacteria harbouring a plant expression vector. The culture was grown at 28°C overnight with shaking at 250 rpm. Next day the cells were precipitated by centrifugation at 5000 g for 10 min at 15-25°C and transferred into 250 ml of induction

medium and cultivated at 26°C overnight with shaking at 250 rpm. Agrobacteria cells were centrifuged (4000g/15-25°C/15 min) and resuspended in 50 ml of MMA solution and kept at RT for 2 h. The OD_{600nm} was measured after 1:10 dilution and the cell suspension was adjusted to an OD_{600nm} of 1. 100 ml of the diluted cell suspension was used for vacuum infiltration.

Induction medium:

YEB medium

MES 10mM

pH was adjusted to 5.6 and 2 mM MgSO4, 50 µg/ml kanamycin, 50 µg/ml rifampicin, 100 µg/ml carbenicillin, 20 µM acetosyringone were added after autoclaving and cooling.

MMA buffer:

MS-salts (Murashige & Skoog, basic salt mixture) 0.43% (w/v)

MES 10mM

pH was adjusted to 5.6 and Sucrose 2% (w/v) and Acetosyringone was added directly before use 200 µM.

II.2.8.3 Recombinant agrobacterium-mediated transformation

II.2.8.3.1. Transient transformation of intact leaves

Transformation of young *N. tabacum* cv. Petite Havana SR1 and *N. benthamiana* plants was transiently accomplished by vacuum infiltration and inoculation by syringe.

In vacuum infiltration young leaves were placed in 100 ml of agrobacteria suspension in a "Weck" glass and a continuous vacuum (60-80 mbar) was applied for 15-20 min. The applied vacuum was released rapidly and the leaves were briefly rinsed in tap water and kept on wet Whatman paper no. 1 with adaxial side upwards. The plastic tray was sealed with saran wrap and placed at 22°C with a 16 h photoperiod for 3 days. As control, leaves were infiltrated with agrobacteria suspension, which did not contain pTRAkt plasmid.

In syringe inoculation, whole tobacco plants in pots were used. So, lower epiderm of intact leaf was scratched by pipet tip and agrobacterium suspension was injected into beneath space. Tobacco pots were kept at 22°C with a 16 h photoperiod for 3 days.

II.2.8.3.2 Stable transformation of *N. benthamiana*

Stable transformation of *N. benthamiana* was performed with the help of Dr. Flora Schuster (Institute of Molecular Biotechnology, RWTH-Aachen, Germany). Transgenic *N. benthamiana* was generated by leaf disc transformation using recombinant Agrobacteria transformed with pTRAkt plasmid carrying cytosolic or nuclear taregeting of different scFv genes with and without fusion to GFP (II.2.1.13.6). Transgenic T0 plants were regenerated from transformed callus as recomended (Fraley et al., 1983) and (Horsch et al., 1985). Briefly, wild type plants were grown on MS medium in "Weck" glasses and the youngest leaves (length up to 4 cm) were used for transformation. The agrobacteria suspension was prepared as described above (II.2.1.12.2) and the OD_{600nm} was adjusted to at least 1.0 after dilution in MMA buffer. The leaves were cut into 8-10 pieces and transferred into "Weck" glasses containing 50- 100 ml of agrobacteria suspension and incubated at RT for 10-15 min. The leaf pieces were then transferred onto sterile pre-wetted Whatman filters in petri dishes closed with saran wrap and incubated at 26-28°C in the dark for two days. Following washing with distilled water containing 100 µg/ml kanamycin and 200 µg/ml claforan, leaf pieces were transferred onto MS II-plates and incubated at 25°C in the dark for one week and with a 16 h photoperiod for 2-3 weeks. After shooting, the shoots were removed and transferred onto MS-III-plates and incubated at 25°C with a 16 h photoperiod for 10-14 days until roots developed and transferred into soil. The young leaves from regenerated transgenic plants were used for immunoblot analysis of expressed scFvs.

MS medium:

MS-salts	0.43% (w/v)
Myo-Inosite (SERVA)	0.1% (w/v)
Sucrose	2% (w/v)
Thiamin-HCl	0.4 mg/l
Distilled water add to 1000 ml	

The pH was adjusted to 5.8 with 1 N NaOH (for preparation of solid medium, 0.8% (w/v) agar were added), autoclaved and 500 µl of vitamin solution I were added upon cooling to 55°C.

MS-II medium:

MS medium supplemented with:

BAP (in DMSO, from Sigma)	1 mg/l

NAA (from Sigma)	0.1 mg/l
Kanamycin	100 mg/l
Claforan	200 mg/l
Betabactyl	200 mg/l

MS-III medium:

MS medium supplemented with:

Kanamycin	100 mg/l
Claforan	200-250 mg/l
Betabactyl	200-250 mg/l

Vitamin solution I:

Glycin	0.4% (w/v)
Nicotinic acid	0.1% (w/v)
Pyridoxin	0.1% (w/v)

Filter sterilized and stored at 4°C.

II.2.8.4 Fluorescent microscopy

The infiltrated tobacco leaves (II.2.8.3.1) were checked for GFP, DsRed fluorescence using Olympus BX41 flourescent microscope. 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.

II.2.8.5 Preparation of total soluble proteins from plant leaves

For the extraction of transiently expressed scFv in infiltrated tobacco leaves (II.2.8.3.1) or in stable transformed tobacco plant (II.2.8.3.2), third leaf from apical was displaced and grounded with a mortar and pestle in liquid nitrogen to a fine powder. Total soluble proteins were extracted using 2 ml of extraction buffer per gram leaf material. Cell debris was removed by two rounds of centrifugation (16000g/4°C/30 min) and the supernatant was used for subsequent analyses (II.2.3.3).

Extraction buffer:

PBS pH 7.5	1X
EDTA	5 mM
β-mercaptoethanol	5 mm

II.2.8.6 Quantification of scFv transcripts in transgenic plants

II.2.8.6.1 Extraction of total RNA from transgenic plants and cDNA construction

Total RNA of transgenic plants were extracted by RNeasy mini kit (Qiagen) according to the manufacturers' instructions. Quality and yield of extracted RNA was examined by running on agarose gele electrophoresis and reading the absorbance at 260 nm and 280 nm in a spectrophotometer. Isolated total RNA was stored at -20°C.

3 µg of total RNA was subjected to DNA digestion through DNaseI enzyme (Fermentas, St. Leon-Rot). cDNA synthesis was performed using 2 µg of total RNA, SuperScript™ II Reverse Transcriptase (RT) (Invitogen) and specific reverse pA35S primer (II.1.6) recognizing 3´ untranslated region according to the manufacturer's instruction.

II.2.8.6.2 Quantitative (real-time) PCR assays

Real-time PCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) reaction buffer containing 2X SuperMix, 50X ROX reference dye, 50 mM $MgCl_2$ and 20X BSA. Specific primer recognizing 3`-UTR (pA35S) region were designed (II.1.6) to target the same amplicon presented in all transgenic plants. Real time PCR was done in total 20 µl reaction mixture including 1X qPCR Mastermix plus 10 µM for each primer and 2 µl of cDNA with dilution 100 as a template. Amplifications were accomplished in 96 well plates with optical caps on ABI Prism® 7000 sequence detection system (SDS) (Applied biosystem, Foster City, USA). PCR reaction was carried out under the following conditions:

2 min 50°C
10 min 95°C
15 sec 95°C
1 min 60°C } 40X

The results were analysed with ABI Prism 7000 SDS and Exel softwares and relative quantities of the transcripts were calculated by using the standard curve method.

II.2.8.7 Extraction of total DNA from transgenic plants

Plant total DNA was isolated from transgenic and wild type plants based on alkaline lysis method. Tissue from new emerged leaves (50- 100 mg) was ground with mortar and pestle in liquid nitrogen and extracted in 500 µl extraction buffer. Then vortex briefly and add 500 µl of phenol-chlorophorm-isoamylalcohol (25:24:1). After being mixed and centrifuged, the

supernatant was phase-separated with 300 µl chloroform. The nucleic acids in the resulting supernatant were precipitated by adding 40 µl Sodium aceate 3M pH 5.2 and 1 ml ethanol 96%. After removing of all liquids in the tube, the pellet was resuspended in 100 µl TE buffer 1 µl RNase (10 mg/ml) was added and solution was mixed and incubated 15-30 min at 37°C. Following RNA digestion, DNA was precipitated by adding sequentially 10 µl Sodium acetate 3M pH 5.2 and 250 µl ethanol 96%. The pellet resulting from centrifugation was dried and resuspended in 60 µl TE buffer. The concentration of purified DNA was calculated by $OD_{260/280}$ measurements on a spectrophotometer. The integrity of purified DNA was visualized on agarose gel.

Extraction buffer:

Tris-HCl (pH 8.5)	100mM
NaCl	100 mM
EDTA	10 mM
SDS	0.2%

TE buffer:

Tris-HCl (pH 8)	10mM
EDTA	1mM

II.2.8.8 Sothern blot hybridization assay

This assay is based on chemiluminescent detection of viral and plasmid DNA using digaxigenin-labled probes on nylon membrane. The full-length of TYLCV-CP (777 bp) was used for preparation of probe. The probe contained both plus and minus strand obtained by incorporating digoxigenin-dUTP (Roche, Mannheim) in PCR amplification using CP specific primers (II.1.6) according to the manufacturer's instruction. The probe was used at a final concentratin of 1 ng/ml in hybridization buffer.

S1 nuclease treatment was performed to distinguish between ssDNA and dsDNA form of virus. About 5µg of total DNA was used for S1 treatment. S1 nuclease was added to reaction mixture at 1.2 units/µg of DNA and incubated for 30 min at 37°C. The reaction was stopped using 0.1 vol. of 100 mM EDTA (pH 8.0) and used for southern hybridization.

About 1 µg of total DNA extracted from control and transgenic plants was submitted on a 1 % agarose gel electrophoresis in 1XTBE buffer for 3 hr at constant voltage 80. To facilitate blotting of big DNA fragments, de-purination step was done to take the purines out with HCl

0.25M for 15 minutes. Before transferring DNA onto membrane, the double-stranded DNAs must be denatured because only single stranded nucleic acids could bind to the membrane and furthermore, DNA must be single stranded to be able to hybridize with the probe. Therefore, the gel was emerged sequentially in denaturing and naturalising buffer and exposed to Hybound-N^+ membrane (Amershame pharmacia biotech) over and connected to a big chamber containing 20X SSC buffer. By capillary action, the DNA molecules are moved from the gel to the membrane, where they bind to the membrane. The transferring was left overnight. The blotted DNA was cross linked with UV light at 254nm and 125mJ energy by means GC Gene linker^{TM} (BioRad, München). Residual EtBr was removed by 3 times washing with 2X SSC buffer at RT for 3-5 hour. Pre-hybridization step is required before hybridization to block non-specific sites. For this mean, the membrane was incubated with 10 ml Dig Easy Hyb (Roche, Mannheim) in hybridization tube for 30 min at 37°C inside Mini hybridization oven (Biometra, Göttingen,Germany). Single-stranded DNA is required to base-pair with complementary sequences, for this mean, the dig-labelled probe is denatured by heating 5 min at 95°C and immediately added to new Dig Easy Hyb. The hybridization solution is added to the membrane and incubated at 37°C overnight. This long incubation allows the single stranded probe DNA molecules to hybridize (base-pair) to their complementary DNA sequences on the membrane.

The next day, pour off the hybridization solution and membrane is subjected to a series of washes that remove any of the DNA probes that is not correctly base-paired to its complementary sequences. Firstly, membrane was washed once with 2X SSC buffer for 3 min and twice with 2XSSC+0.1% SDS for 5 min at RT. Another washing was done twice at 65°C with 0.5X SSC+0.1% SDS. Then the membrane was washed with 1X Maleic acid buffer and blocked with 1% Southern blocking buffer for 30 min at RT. Anti Dig-AP-conjugate (Roche, Mannheim) antibody was used for interacting against dig-labelled probe. The next washing with 1X Maleic acid+0.3% Tween20 removes non-bound conjugate. The membrane was soaked into Alkali-phosphatese detection buffer for 5 min at RT and subjected to substrate CDR-Star (Roche, Mannheim) by dropping 1 ml on membrane. Finally the membrane was exposed to CCD-camera.

Denaturing buffer:

NaOH	500mM
NaCl	1.5 M

Neutralizing buffer:

Tris-HCl (pH 7.5)	1M
NaCl	1.5M

20X SSC (pH 7):

NaCl	3M
Tri-Citrate Dihidrate	0.3M

10X Maleic acid buffer (pH 7.5):

Maleic acid	1M
NaCl	1.5M

10 % Southern-Blocking buffer: 10% Casein in 100 ml 1X Maleic acid buffer

Alkali-Phosphatase detection buffer:

Tris-HCl (pH 9.0)	0.1 M
NaCl	0.15 M

II.2.8.9 Agroinoculation of *N. benthamiana* plants with TYLCV-Ir

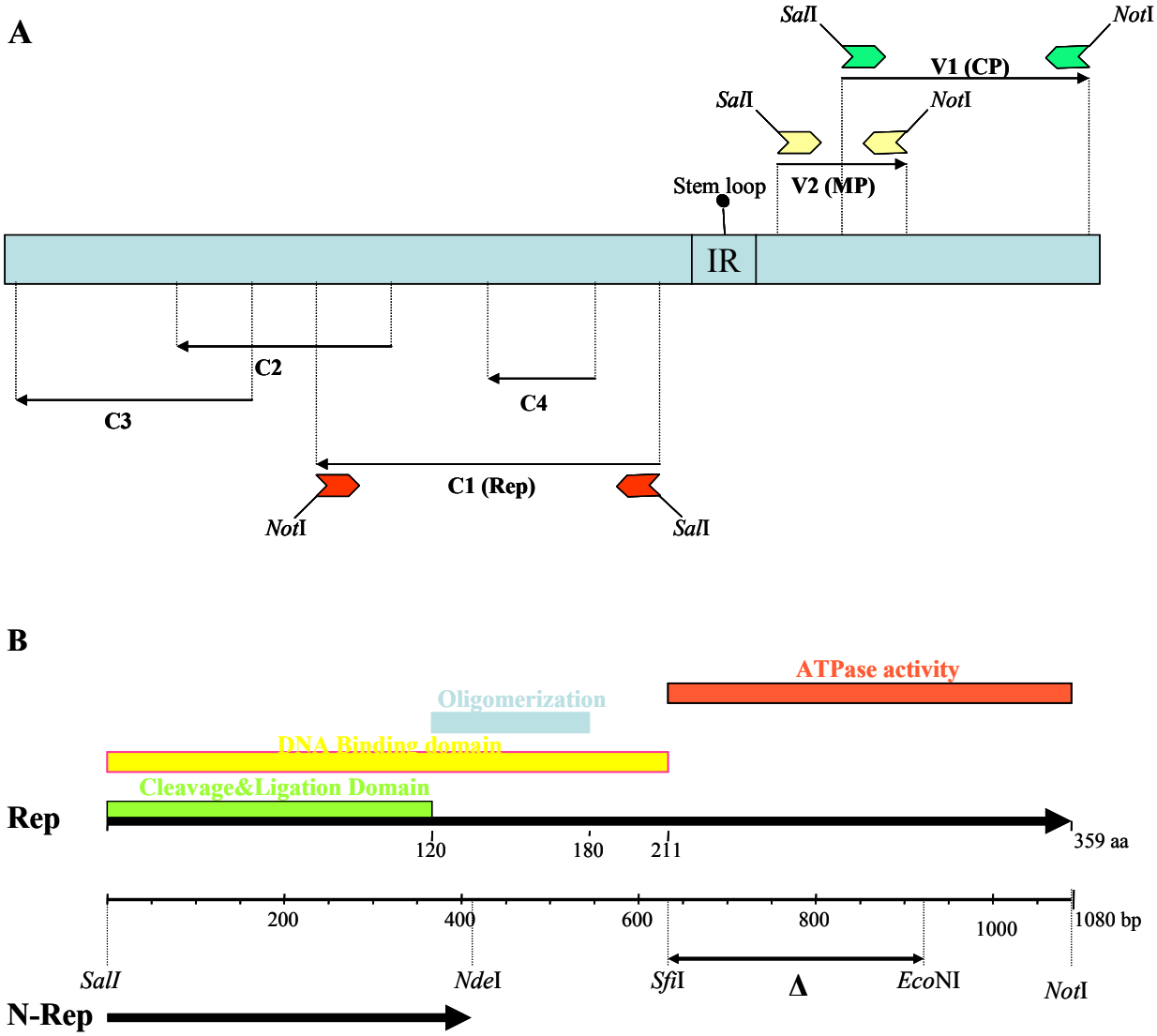
To assay virus resistance in *N. benthamiana*, transgenic and wild type plants were infected with pBIN19-2TYLCV-Ir agroinfectious clone. Young plants at the 4-5 leaf stage were inoculated. *A.tumefaciens* cultures were grown at 28°C for about 48 hr on YEB plate supplemented by kanamycin 50mg/lit and rifampicin 50 mg/lit. The plant apex was excised at the time of agroinoculation and used for subsequent molecular analysis. A yellow pipet tip was touched to bacterial culture and rubbed on the decapitated surface of plants. After inoculation, plants were observed for disease symptoms, weekly for 2 months, and assayed for presence of TYLCV genome using molecular hybridization or PCR.

III Results

III.1 Cloning and expression of the viral genes in bacterial expression vectors

III.1.1 Cloning of viral C1, V1 and V2 genes into TOPO vector

The viral genes V1 (777bp), V2 (351bp) and C1 (1081bp) encoding Rep, CP and MP, respectively, were amplified from a pBIN19-2TYLCV infectious clone (Figure 1) (kindly provided by Dr. A. Kheyr-Pour, CNRS, France) using specific primers (II.1.6). Restriction sites *Sal*I and *Not*I were introduced into the forward and reverse primers for sub-cloning into desired vectors (II.1.10). The PCR products were directly cloned into pCR2.1-TOPO vector and sequences verified from the pCR2.1-TOPO-C1, pCR2.1-TOPO-V1, pCR2.1-TOPO-V2 clones.



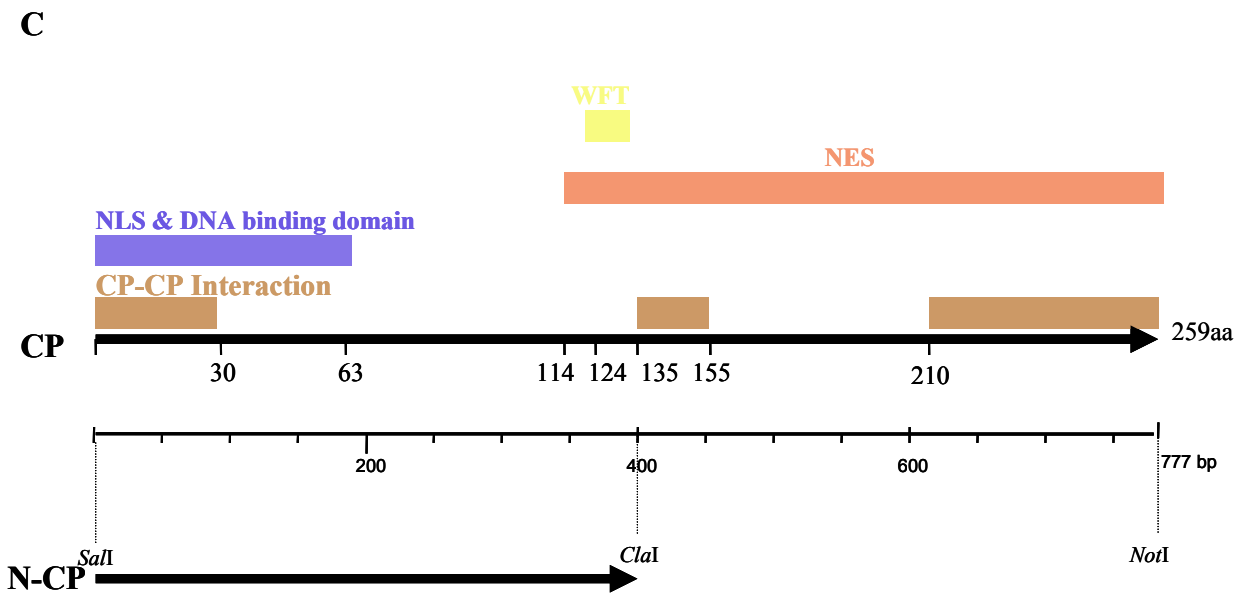


Figure 1: Schematic representation of TYLCV viral genes used for cloning and expression.
A: Linearized TYLCV depicting the genes of interest, transcription direction and the primers used for amplification of viral genes. B and C: Functional domains of Rep and CP and restriction sites used for making deletion NRep and NCP constructs, respectively. Δ: The portion of Rep removed for making of pTRAkt-DRep-DsRed construct (III.6.1.1); NLS: Nuclear localization signal; NES: Nuclear export signal; WFT: White fly transmission domain.

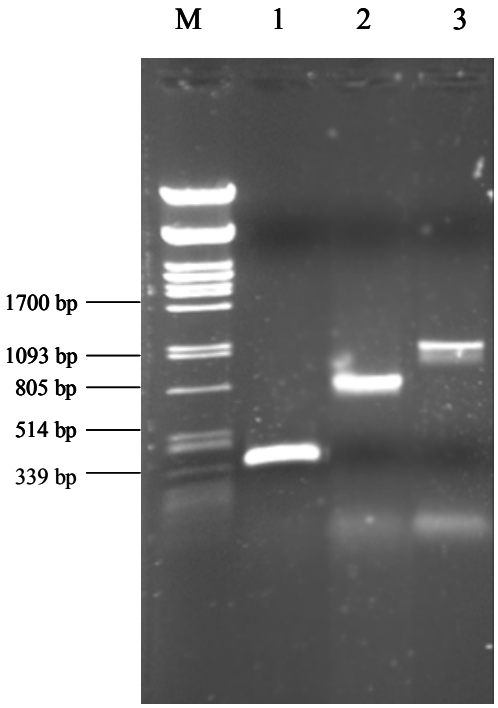


Figure 2: PCR amplification for cloning of C1, V1 and V2 genes.
Viral C1, V1 and V2 genes were amplified using specific primers (II.1.6) and PCR products were separated on a 1.2% (w/v) agarose gel. 1: C1 (1081bp), 2: V1 (777 bp) and 3: V2 (351bp) genes. M: λ *Pst*I DNA size ladder.

III.1.2 Cloning and expression of viral C1, V1 and V2 genes as GST fusion

To produce GST fusion proteins, the C1, V1 and V2 genes were cloned via *Sal*I/*Not*I digestion to the C-terminus of GST gene in pGEX5x-3 (Amersham/Pharmacia) expression vector. New constructs were named pGEX-C1, pGEX-V1 and pGEX-V2 encoding fusion GST-Rep, GST-CP and GST-MP proteins, respectively.

The one third amino terminal parts of C1 gene (133aa) carrying cleavage/linkage and DNA binding domains was cloned as fusion with GST. For this purpose, the C-terminal part of the C1 gene in pGEX-C1 was removed by *Nde*I/*Not*I digestion followed by modifying non-compatible overhang ends using Klenow fragment DNA polymerases and subsequent blunt-end ligation (Figure 1B). The resulting plasmid expressing the GST-NRep fusion protein was named pGEX-NC1. In addition, approximately half of the C-terminal component of V1 (144aa) was removed from pGEX-V1 by *Cla*I/*Not*I digestion and new pGEX-NV1 construct was produced. DNA sequencing results revealed that the modified NCI and NV1 gene sequences were in frame with the C-terminus of GST gene.

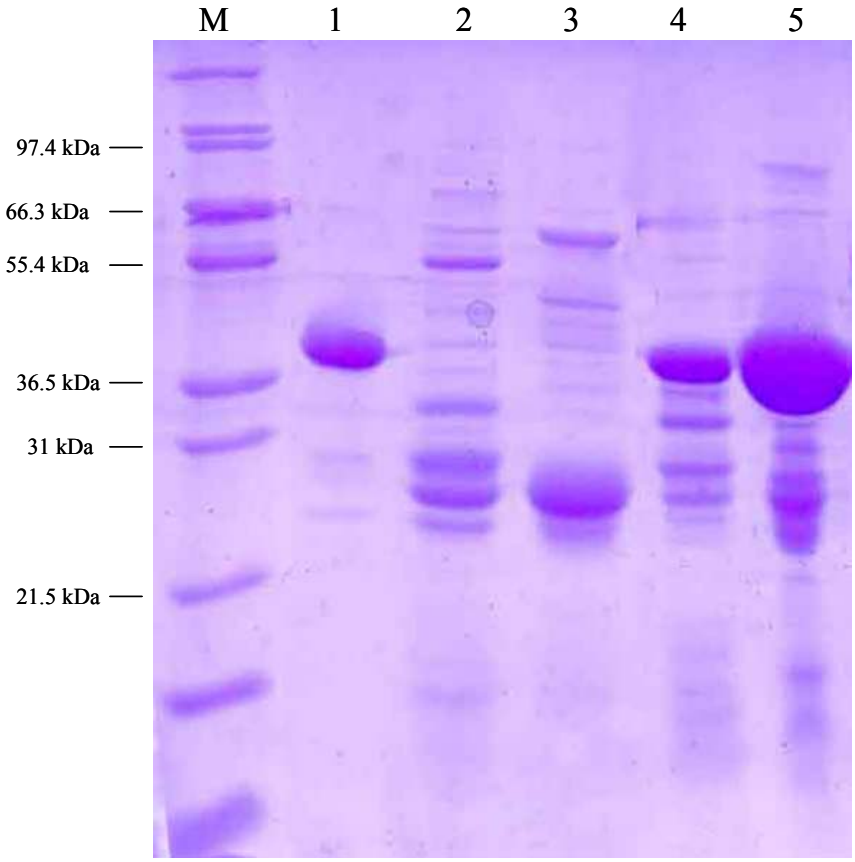


Figure 3: SDS-PAGE analysis of affinity purified GST fusion protein.

GST fusion proteins were affinity purified using glutathione sepharose matrix (II.1.3). Purified proteins were separated on 12% (w/v) SDS-PAA gels stained with Coomassie brilliant blue (II.2.3.2)

1: GST-MP (40.55kDa), 2: GST-CP (57.2kDa), 3: GST-Rep (67.75kDa), 4: GST-NCP (41.2kDa), 5: GST-NRep (42.6kDa), M: Mark 12 protein marker.

The resulting recombinant plasmids were transformed separately into *E. coli* strain BL21λ-DE3 (II.1.7). The expression and purification (II.2.2.1) of the GST-MP and GST-NRep fusion proteins were carried out according to the manufacturers' instructions. When expression of GST-Rep, GST-CP and GST-NCP were induced with 1mM IPTG at 30°C, most part of fusion proteins were produced as insoluble inclusion bodies and degraded (data not shown). The following modifications were carried out to decrease degradation and improve solubility of the yield. The IPTG concentration was reduced to 0.25 mM and the incubation temperature was lowered to 22°C in 4 hr. The yield of purified fusion proteins varied from 0.5-1mg per litre culture medium for GST-Rep, GSR-NCP and GST-CP to almost 5mg/L for GST-MP and GST-NRep. Affinity purified fusion proteins showed high purity for GST-MP and GST-NRep while majority of GST-CP and GST-Rep fusion proteins are degraded. Some degradation was detected in purified GST-NCP in Coomassie-stained SDS-polyacrylamide gels (SDS-PAGE) as well (Figure 3).

Purified proteins GST-Rep, GST-NRep, GST-MP, GST-CP and GST-NCP were used for panning of naive and immunized phage display libraries (II.2.6).

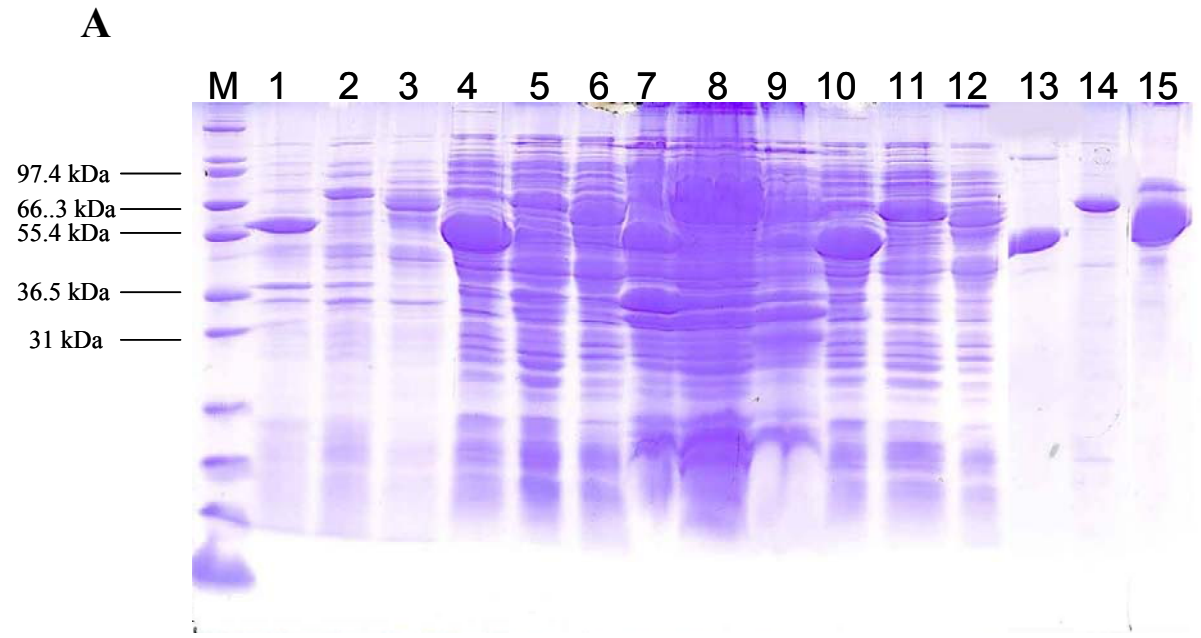
III.1.3 Cloning and expression of viral genes as fusion proteins with MBP

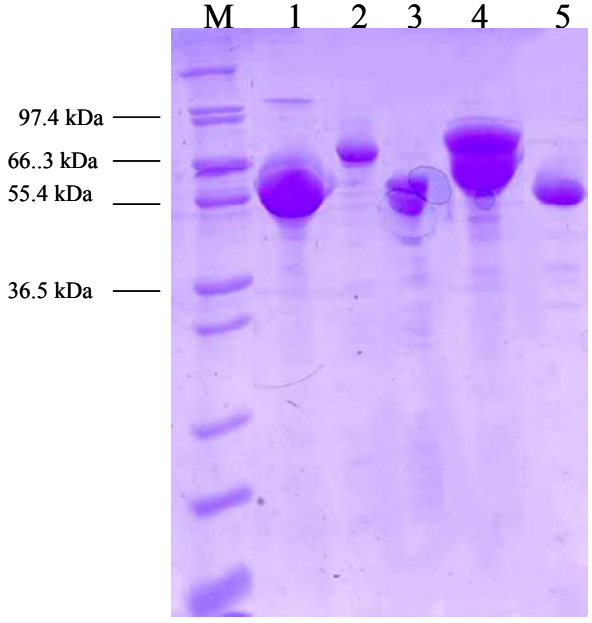
The gene of interest was introduced in frame downstream from the *mal*E gene, which encodes maltose-binding protein (MBP) of *E. coli*, resulting in the expression of a MBP fusion protein. The C1, NC1, V1, NV1 and V2 genes were cloned in frame with the MBP coding sequence into pMAC1 (kindly provided by Adel Zakri, Institute of Molecular Biotechnology, RWTH-Aachen, Germany) obtained from pMAL-c2x vector (New England Biolabs). The cloning was performed by *Sal*I/*Not*I digestion and subsequent replacing AC1 in pMAC1 with C1, NC1, V1, NV1 and V2 genes from pGEX-C1, pGEX-NC1, pGEX-V1, pGEX-NV1 and pGEX-V2, respectively. The new constructs were named pMAL-C1, pMAL-NC1, pMAL-V1, pMAL-NV1 and pMAL-V2. The *E. coli* strain BL21λ-DE3 (II.1.7) was used for expression of recombinant MBP fusion proteins.

The expression and purification of MBP-NRep and MBP-MP were carried out using the manufacturer protocols (II.2.2.2) while for expression and purification of MBP-Rep, MBP-CP and MBP-NCP the following changes were made: induction time and temperature were optimized to decrease the level of inclusion body formation and degradation of fusion proteins

(data not shown). The amount of purified fusion protein ranged from ~1mg per litre culture medium for MBP-Rep, MBP-CP and MBP-NCP to up to 4 mg/L culture in MBP-NRep and MBP-MP.

The purified fusion proteins were used for phage display panning (II.2.6) and screening the scFv libraries for specific binders. The MBP-Rep was also used for immunization of mice.





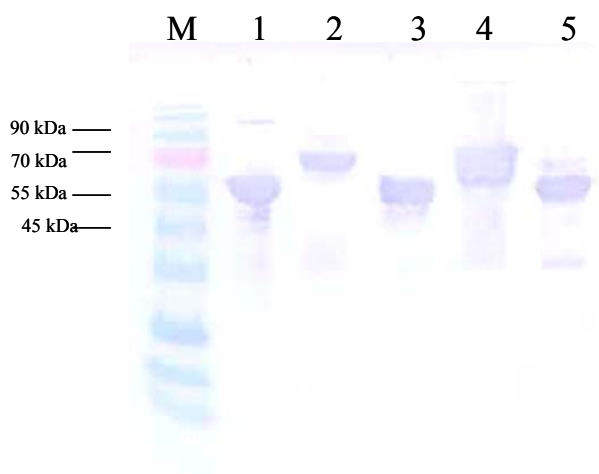


Figure 4: SDS-PAGE and immunoblot analysis of affinity purified MBP fusion proteins.

MBP fusion proteins were expressed and affinity purified using the amylase matrix and separated on 12% (w/v) SDS-PAA gel and stained with Coomassie blue (A and B) (II.2.3.2) or blotted onto Hybond^{TM}-C nitrocellulose membrane (C) (II.1.3). Immunoblot was performed using a polyclonal rabbit antiserum (New England Biolabs) raised against MBP as primary antibody and goat-anti rabbit conjugated to alkaline phosphatase as a secondary antibody followed by NBT/BCIP staining for 10 min at RT.

A) M: Mark 12 protein size marker; 1, 2 and 3: induced cells expressing MBP fusion MP, CP and Rep ; 4, 5 and 6: cell lysate ; 7, 8, and 9: pellet ; 10, 11 and 12: crude extract; 13,14 and 15: elution fractions

B) M: Mark 12 protein size marker; 1, 2, 3, 4 and 5: elution fractions of MBP-MP, MBP-CP, MBP-NCP, MBP-Rep and MBP-NRep, respectively

C) M: pre-stained protein size marker; 1, 2, 3, 4 and 5: elution fractions of MBP-MP (56.86kDa), MBP-CP (73.5kDa), MBP-NCP (57.53kDa), MBP-Rep (84.06kDa) and MBP-NRep (58.9kDa), respectively

III.2 Immunization of mice and determination of antibody titre

Affinity purified MBP-Rep fusion protein was used to immunize three mice. Immunization was repeated eight times at one week intervals, subcutaneously (II.2.4). After each boosting, polyclonal antisera from mouse were prepared by blood sampling from the tail vein and the titre determined by indirect ELISA (II.2.3.3.2). The antibody titer refers to the highest dilution at which antigen-specific binding was detectable above background binding to the negative control. To estimate cross reactivity of antibodies against MBP fusion partner, ELISA tests (II.2.3.3.2) were performed with GST-Rep protein and MBP was used as a control.

After the seventh boosting, the final polyclonal antibody titre was over 1:150,000. A high cross reactivity was observed with polyclonal antibody against MBP.

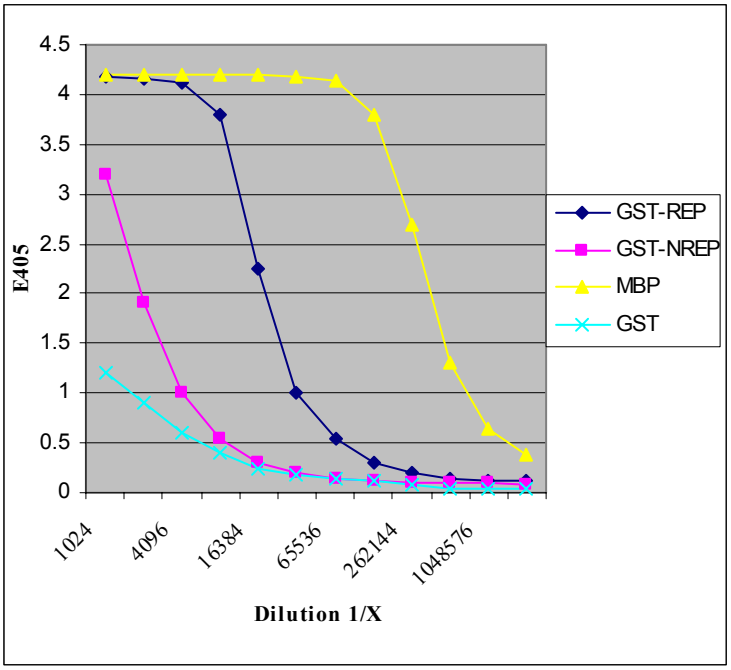


Figure 5: Determination of polyclonal antibody titers obtained from mouse immunized by MBP-Rep by direct ELISA.

The recombinant purified fusion proteins GST-Rep, GST-NRep, MBP and GST (10 µg/ml each) were coated to ELISA plates (II.2.3.3.2). Serial dilutions of sera were added to the coated plates and incubated for 2 hours. Bound antibodies were detected by addition of GAM^{HRP} polyclonal antibody (1:5000). ELISA readings were performed at OD_{405nm} after one hour incubation with ABTS substrate at 37°C. X indicates the antiserum dilutions.

III.3 Construction of phage displayed scFv libraries

III.3.1 Isolation of total RNA from mouse spleen cells

Total RNA was isolated from spleen cells of mice immunized with MBP-Rep (II.2.4). Agarose gel (1.2%) analysis showed good integrity of the isolated RNA (Figure 6). The 28S and 18S ribosomal RNA were visible as distinct bands indicating the quality and integrity of the total RNA. The RNA concentration and the purity of isolated total RNA was determined by spectrophotometry. The yield of total RNA isolated from an individual mouse, from 10^8 spleen cells, was around ~1 mg.

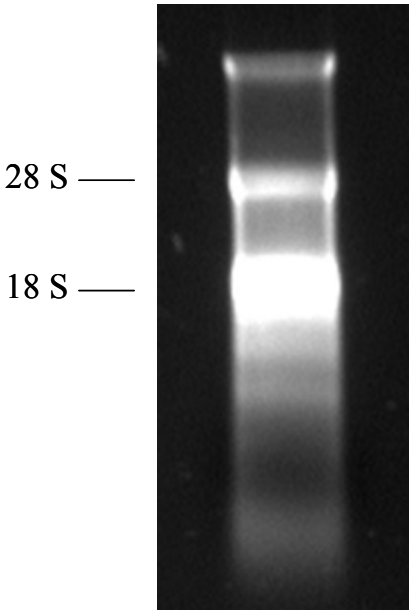


Figure 6: Analysis of total RNA isolated from mouse spleen cells.
Total RNA (1 µg) was isolated from spleen cells of immunized mouse (II.2.4) and separated on a 1.2% (w/v) agarose gel. The major ribosomal RNA species (28S and 18S rRNA) could be visualized by etidium bromide showing dsRNA molecules.

III.3.2 Synthesis of cDNA strand and amplification of variable heavy and light chain fragments

Total RNA isolated from spleen cells was used for RT-PCR amplification of variable heavy (V_H) and light (V_L) chain fragments and subsequent construction of scFv library. 3 µg of total RNA was terated with DNase I enzyme and reverse transcribed to synthesize cDNA, using oligo-dT primers.

Mouse antibody heavy and light chain variable regions were amplified by PCR using cDNA as template. Eight individual PCR reactions were performed to amplify heavy and light variable fragments. All PCR products had the expected size of 400 to 450 bp (Figure 7). No visible band was detected when λ light chain primers were used (data are not shown). Re-amplification of λ variable domains failed to amplify a PCR product.

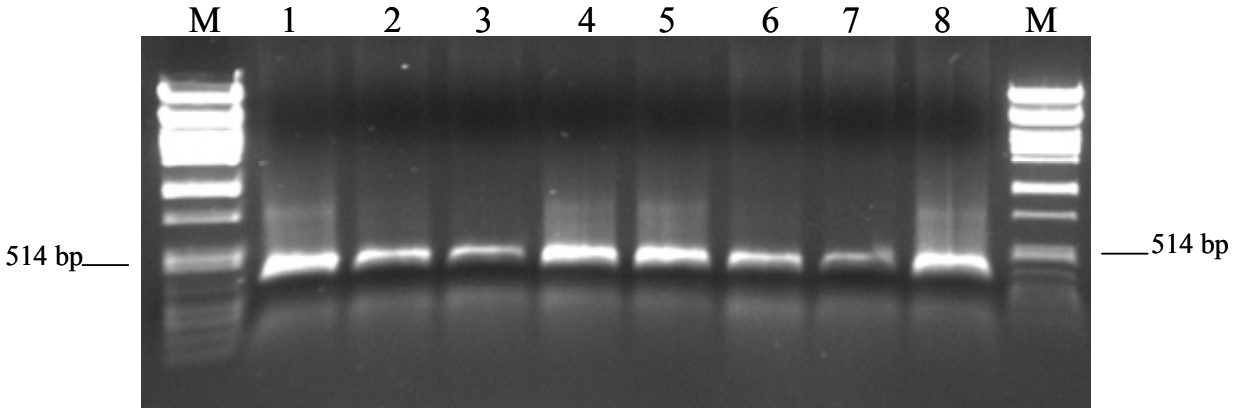


Figure 7: Amplification of variable heavy and light chain fragments from MBP-Rep immunized mice using primers specific for murine.
Nucleic acids were separated on a 1.2% (w/v) agarose gel**.** M= λ *Pst*I DNA ladder. Lines 1-4 and 5-8 show variable heavy and light fragments amplified using specific murine forward and reverse primers, respectively (II.1.6).

III.3.3 Construction of phagemid-scFv libraries

The strategy shown in Figure 8 was used to establish the scFv library expressing recombinant antibodies against Rep (II.2.5.3). The PCR amplified V_H and V_L fragments were gel purified and digested with *Sfi*I/*BstE*II and *Asc*I/*Not*I, respectively. To make heavy and light chain sub-libraries, digested V_H and V_L fragments were separately cloned into pHENHI vector. The constructed sublibraries contain inserts with a size around 380 bp.

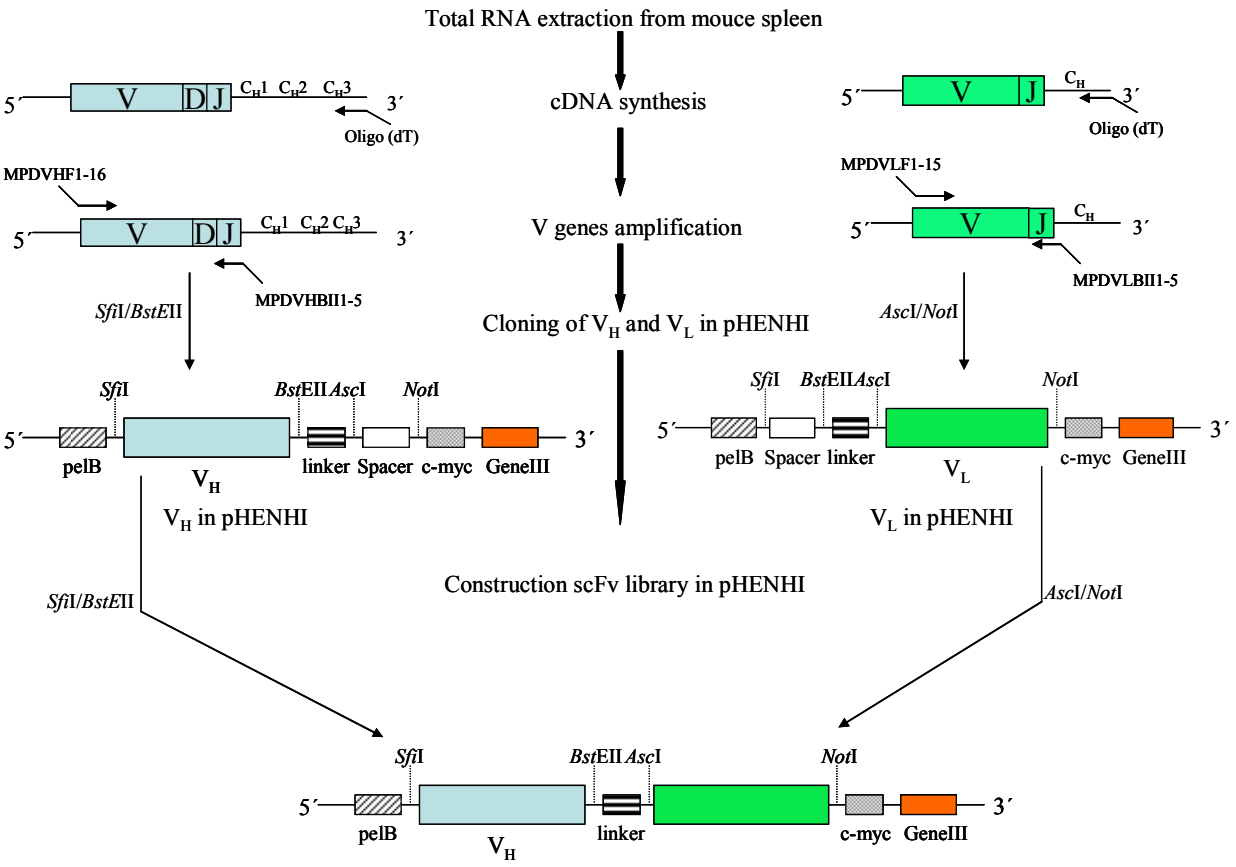


Figure 8: Schematic presentation of cloning procedure for the construction of murine scFv libraries.
The V_H and V_L amplification mixes were digested with *Sfi*I/*Bst*EII or *Asc*I/*Not*I, respectively and ligated into the linearized pHENHI vector. Then the V_L was excised with *Asc*I/*Not*I and sub-cloned into the pHENHI vector containing the corresponding V_H fragment. *pel*B: *pel*B leader peptide; *c-myc*: myc epitope tag for detection.

The construction of scFv libraries were performed by recovering of VL fragments from the VL sub-libraries and cloning them into the linearized pHENHI vector containing the corresponding VH fragments and transformed into electrocompetent *E. coli* XL1-Blue cells (II.1.7). After electroporation, cells were plated on LB medium containing 1% (w/v) glucose and 100 mg/ml ampicillin and incubated overnight at 37°C. All grown colonies 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 ARep library, 17 individual colonies were randomly selected and tested by colony check PCR (II.2.1.2) using pHEN specific primers (II.1.6) (Figure 9). The amplified PCR products revealed that all selected clones contain scFv fragment with expected size 900-950 bp length. To verify the library diversity, PCR-amplified scFv fragments were subjected to *Bst*NI digestion (II.2.5.4). RFLP (restriction fragment length polymorphism) analysis indicated that ~ 75% of the clones had different

restriction patterns (Figure 10). In some cases, sequencing results obtained form selected clones showed framshifts located within coding region of scFv which in turn led to failure expression. These frame shifts occurred in primer binding sites of V_H and V_L regions in result of incorrect binding of specific primers (data not shown).

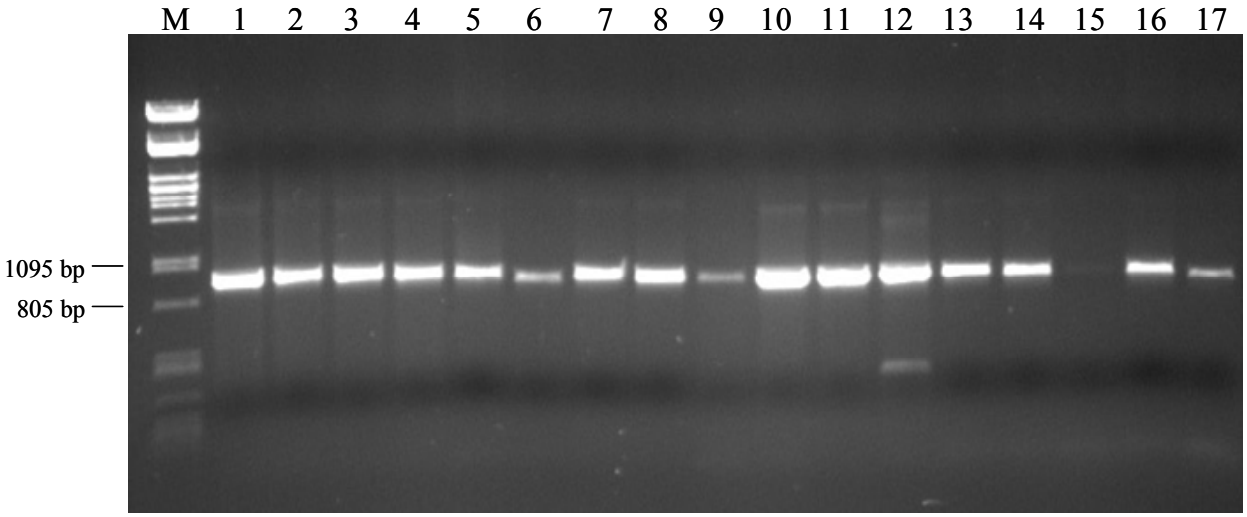


Figure 9: Analysis of PCR product of randomly selected colonies from murine scFv libraries with specificity for Rep.
1-17: PCR amplification of scFv-fragment of 17 independent colonies separated on a 1.2% (w/v) agarose gel. M: λ *Pst*I DNA ladder

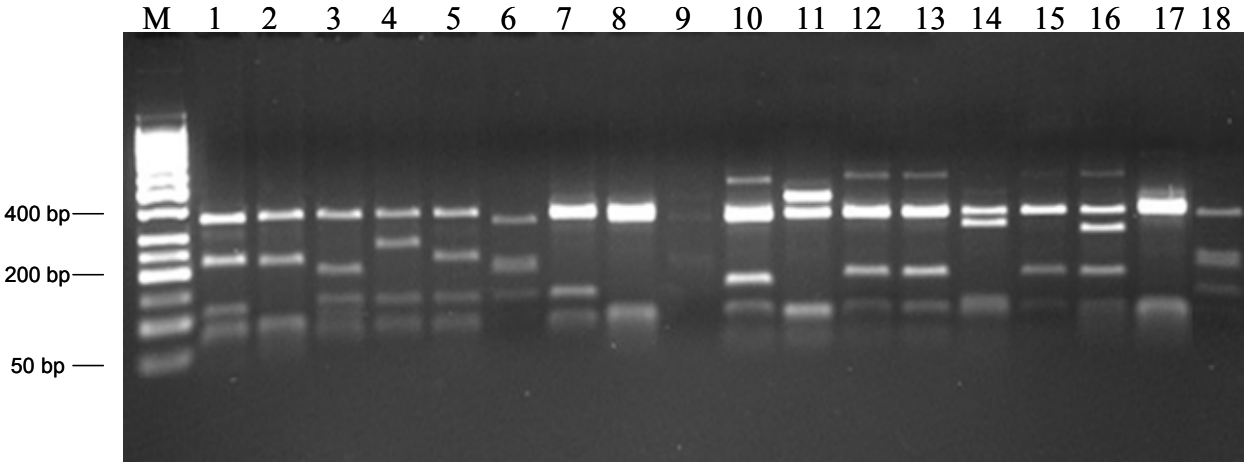


Figure 10: Analysis of murine scFv fragments after *Bst*NI digestion.
Samples were analyzed on a 2% (w/v) agarose gel. 1-18: *Bst*NI fingerprinting of 18 randomly selected colonies from ARep scFv library. M: 50 bp ladder.

III.4 Selection and characterisation of specific scFvs

III.4.1 Solid phase panning of ARep library against recombinant Rep fusion protein

The phage-displayed scFv library ARep was panned three times against 50 µg/ml GST-Rep (1st and 3rd round) and MBP-Rep (2nd round) immobilized on the surface of the high binding immuno-tubes (II.2.6). In each round of panning around 10^{13} cfu of recombinant phages were used. During three rounds of panning, population of recovered phages increased from 2.5 x 10^4 to 1.2 x 10^7.

III.4.2 Solid phase panning of naive Tomlinson I and J libraries against recombinant fusion Rep, CP and MP proteins

The Tomlinson I and Tomlinson J scFv libraries (MRC Laboratory of Molecular Biology and the MRC Centre for protein engineering, Cambridge, UK) comprise over 10^8 different scFv fragments. They had been cloned in phagemid (pIT2) and transformed into TG1 *E. coli* cells. The Tomlinson I and J libraries were screened for Rep, CP and MP binders using MBP-NRep/GST-NRep, MBP-CP/GST-NCP and MBP-MP/GST-MP as antigens, respectively. 3 rounds of panning were performed in each case and, in order to remove unspecific GST/MBP binders, MBP fusion proteins (II.2.2.2) were used in the first and the 3rd panning rounds whereas GST fusion proteins (II.2.2.1) were used in the 2nd panning round.

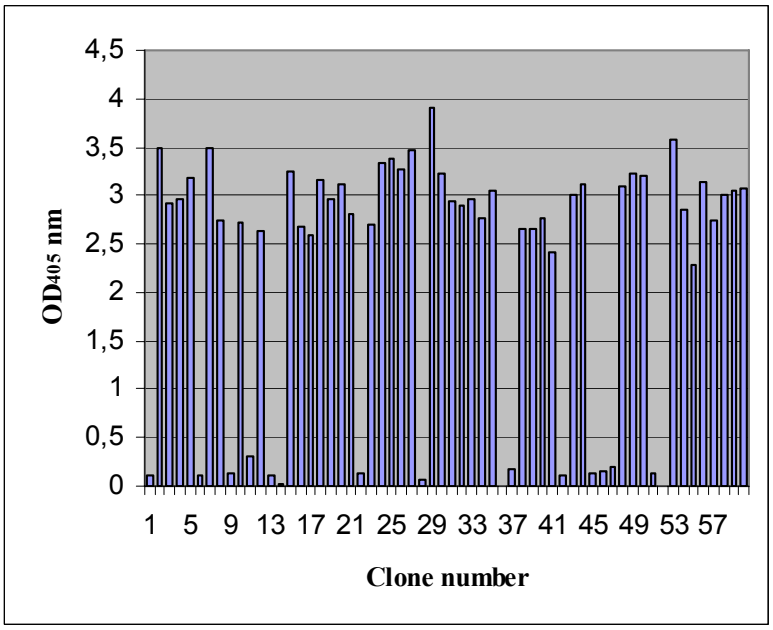
Table III-1: Enrichment of scFv fragments specific Rep through multiple rounds panning of phage display libraries.

scFv phage library	Antigen	Panning round	Input phage	Output phage
ARep	GSR-Rep	1	10^{13}	$2.5x10^4$
	MBP-Rep	2	10^{13}	$3.1x10^6$
	GST-Rep	3	10^{13}	$1.2x10^7$
Tomlinson I	MBP-NRep	1	10^{13}	$3.2x10^3$
	GST-NRep	2	10^{13}	$4.1x10^4$
	GST-NRep	3	10^{13}	$7.8x10^5$

III.4.3 Screening and characterisation of selected scFv-fragments

After the third panning round of Tomlinson I, Tomlinson J and ARep phage display libraries with different fusion proteins, 96 colonies were randomly selected from each plate and grown in microtitre plates. Expression of soluble scFv-fragments was induced by 1 mM IPTG. The specificity of scFv-fragments was tested in direct ELISA assays against bacterially expressed GST fusion proteins (GST-NRep, GST-Rep, GST-MP and GST-CP).

A



B

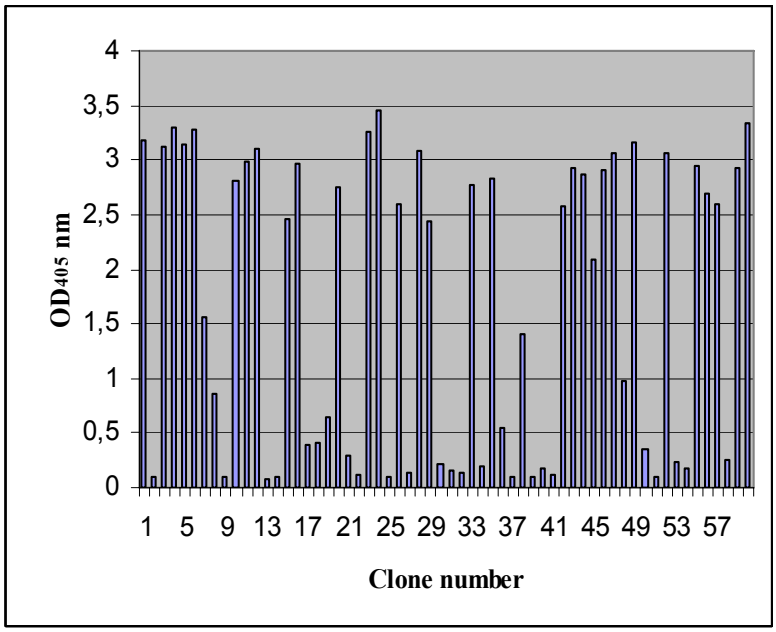


Figure 11: Screening of selected clones from the scFv libraries Tomlinson I (A) and ARep (B) in soluble ELISA.

Binding activity of 60 randomly selected soluble murine scFv binding to Rep protein after the third round of panning was revealed by direct ELISA (II.2.3.3.2). Around 50 µg/ml of MBP-Rep was coated on microtitre plates. Bacterial supernatant was added in 2% MPBS to a final volume of 100µl. The specific scFv were detected by adding of 1:5000 diluted 9E10 monoclonal antibody, and goat anti-mouse polyclonal antibody conjugated to horse radish peroxidase as secondary antibody (1:5000). ELISA readings (OD_{405nm}) were performed after 30 min incubation with ABTS substrate at 37°C. A and B: soluble scFvs selected from Tomlinson I and ARep libraries were used, respectively.

To overcome the differences based on expression in micro-titre plates, 12 clones with distinct absorbance profiles were subjected to mini scale periplasmic expression. Their reactivity to the recombinant MBP-Rep, MBP-NRep, GST-NRep and MBP was evaluated by direct ELISA.

Approximately 60% of the positive clones selected from scFv library ARep displayed activity against Rep protein (Figure 11). These specific scFv exclusively reacted to GST-Rep and MBP-Rep fusion proteins (Figure 16) but not to GST-NRep and MBP-NRep. This shows that corresponding scFvs have failed to bind to the amino-terminal part of Rep and no cross reactivity to MBP and GST was observed.

To verify the integrity and differentiate between scFv-fragments, DNA of 10 clones was PCR-amplified and purified. All clones consisted similar DNA sequence producing scFv around 29.9 kDa.

In the case of the clones selected by panning the Tomlinson I library, around 70% of the analyzed clones showed a specific binding to both Rep and NRep fusion proteins. No MBP or GST binders were found which indicates the efficiency of using two different antigen fusions for excluding all the binders against the fusion partners. To verify the presence of full size scFv-fragments, the DNA inserts of 10 clones that showed a high reactivity to GST-Rep were PCR-amplified followed by *Bst*NI fingerprinting. All clones analyzed showed an insert of about 1 kb and *Bst*NI digestion indicated the presence of only one banding pattern (Figure 12). In addition, sequencing results verified that these clones contained identical scFv sequences.

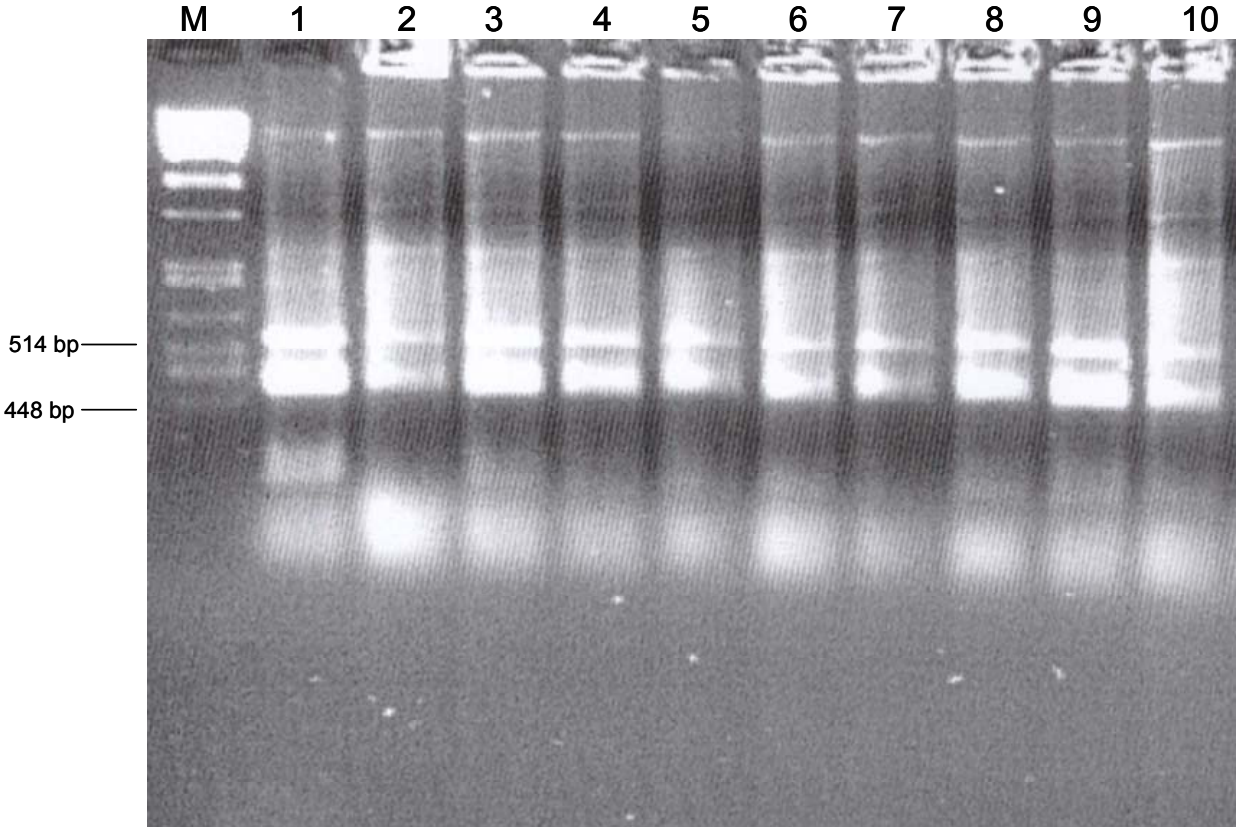


Figure 12: Finger printing analysis of Rep binder clones selected from Tomlinson I phage display library.

The scFvs gene was amplified by specific pHEN primers (II.1.6) and subjected to *Bst*NI digestion. Samples were analyzed on a 2% (w/v) agarose gel. 1-10: RFLP patterns of 10 selected clones reacting to Rep. M: DNA ladder.

Panning of Tomlinson I library through immobilized recombinant MP led to select some positive clones detected in direct ELISA. But further analysis revealed that they are specifying against MBP fusion partner. No specific scFv was selected from panning of Tomlinson libraries I and J using immobilized recombinant fusion CP.

III.4.4 Sequence analysis of scFv fragments specific to Rep

The DNA of different clones selected from the Tomlinson I library showing high activity against Rep were sequenced by pHEN specific primers. The sequencing results showed that all clones contained an identical sequence and named scFv-ScRep1.

In the same way, ten specific clones reacting against Rep selected from the ARep phage display library were sequenced. Sequencing results revealed they, too, contained an identical sequence that named scFv-ScRep2.

Both scFv-ScRep1 and scFv-ScRep2 had open reading frames encoding proteins of 28.1 kDa, and 29.9 kDa, respectively. The sequence alignment of the two scFv-fragments with the consensus sequence of IMGT databases (http://imgt.cines.fr/IMGT_vquest/) indicated that variable heavy and light chain fragments were members of different groups. Notably divergences were observed from closely related germline V-gene segments both in the nucleotide and amino acid level. Table III-2 summarizes the sequencing results of the two selected scFvs.

Amino acid comparison of the selected scFv showed no major similarity in the framework and CDR regions of both heavy and light chain fragments. The deduced amino acid alignment of two scFv is presented in Figure 13.

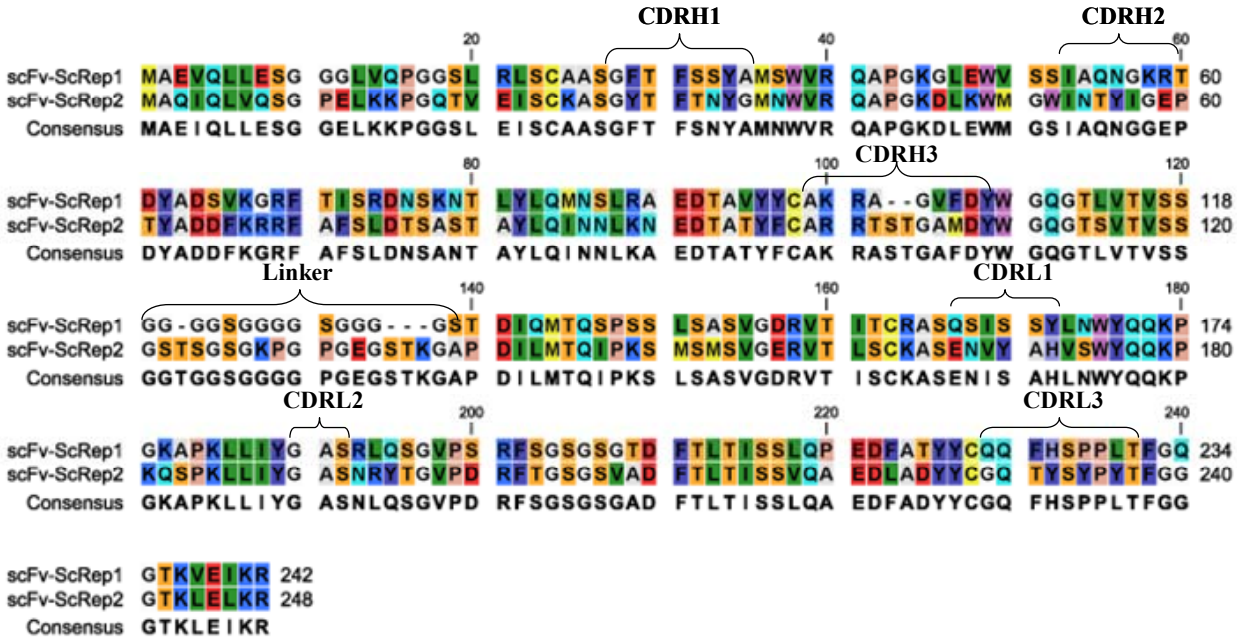


Figure 13: Alignment of the deduced amino acid sequences of selected murine scFv fragments interacting with Rep (ScRep1 and ScRep2).
Complementary determining regions (CDRs) were determined according to IMGT databases. - : no amino acid at this position. The complementary determining regions (CDR) and polylinker have been indicated.

Table III-2: Summary of nucleotide length and deduced molecular weight of derived scFv with specificity for Rep

Designation of scFv	Nucleotide length (bp)	Deduced MW (kDa)
scFv-ScRep1	726	25.5 kDa
scFv-ScRep2	744	27.09 kDa

Table III-3: V-gene family of two scFvs specific for Rep. The alignment of the selected scFvs to the germline gene sequences most closely related to theirs performed using the IMGT database (http://imgt.cines.fr./imgt_vquest/).

scFv	Mouse/Human IgG Family		Closest germline gene sequences		Identity (Nucleotides)	
	V_H	V_L	V_H	V_L	V_H	V_L
scFv- ScRep1	IGHV3	IGKV1	M99660	X59315	94.44% (272/288 nt)	96.77% (270/279 nt)
scFv-ScRep2	IGHV9	IGKV6	AJ851868	Y15981	96.87% (279/288 nt)	92.83% (259/279 nt)

III.4.5 Large scale expression and IMAC purification of soluble scFvs from phage libraries

The scFv-ScRep1 gene was sub-cloned into pHENHI vector via *Nco*I/*Not*I digestion. Expression of scFv-ScRep1 and scFv-ScRep2 was performed in *E. coli* strain HB2151. The His6 tagged scFvs were purified by IMAC as described in material and methods (II.2.2.3.2).

The scFvs were diluted from Ni-agarose matrices with buffer containing 200 mM Imidazole. To remove the Imidazole from the purified scFvs, they were dialysed with cold PBS solution. During the dialysis a lot of white precipitate was observed in the tube, which was removed by centrifugation. The SDS-PAGE analysis indicated that the precipitate was scFv (data are not shown) and caused to decrease total scFv yield. To reduce precipitate formation, dialysis was performed using PBS buffer containing 15 mM Imidazole. This treatment led to reducing of white precipitate formation and, as shown in Figure 15, increased total yield of scFv.

SDS-PAGE analysis of the affinity purified scFv-ScRep1 and scFv-Rep2 revealed the presence of a band of approximately 30 kDa for scFv fragments (Figure 14).

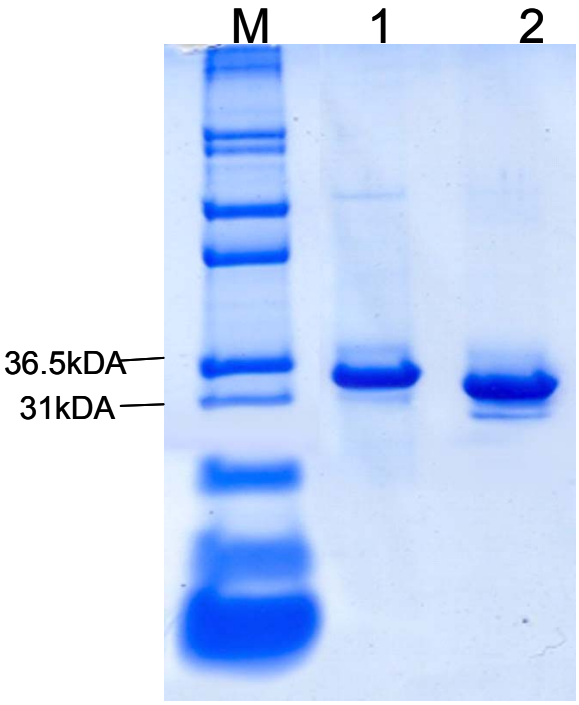


Figure 14: SDS-PAGE analysis of affinity purified scFvs.

The scFv-ScRep1 and scFv-ScRep2 were expressed in the *E. coli* strain HB2151 (II.1.7). The expressed scFvs were purified by IMAC (II.2.2.3.2). 1: scFv-ScRep2 and 2: scFv-ScRep1. M: Protein marker.

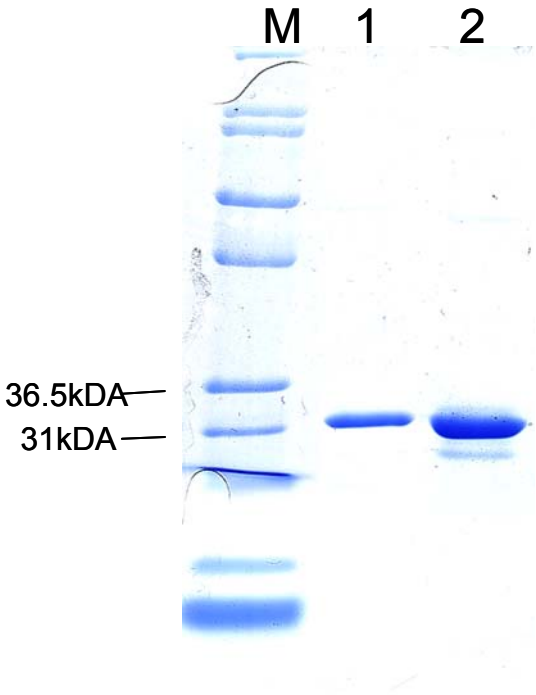


Figure 15: The effect of buffer containing Imidazole on total scFv yield through dialysis. The purified scFv-ScRep1 protein was dialyzed three times either by PBS buffer (1) or PBS buffer containing 15 mM Imidazole (2). After dialysis white precipitate was removed by centrifugation and 20 µl of supernatant subjected to SDS-PAGE. Dialyzed proteins were separated on 12% (w/v) SDS-PAGE (II.2.3.2) and stained with Coomassie brilliant blue. M: Protein marker.

III.4.6 Comparative analysis of the binding activities of scFv-ScRep1 and scFv-ScRep2 to fusion Rep protein

Direct ELISA (II.2.3.3.2) using bacterially expressed scFvs revealed high specificity of both scFv-ScRep1 and scFv-ScRep2 against full Rep fusion proteins, while only scFv-ScRep1 reacts against fusion NRep proteins. WB analysis (II.2.3.3.1.1) shows that scFv-ScRep2 binds to both entire and degraded MBP-Rep bands but not to MBP-NRep, indicating it interacts with the middle segment of Rep.

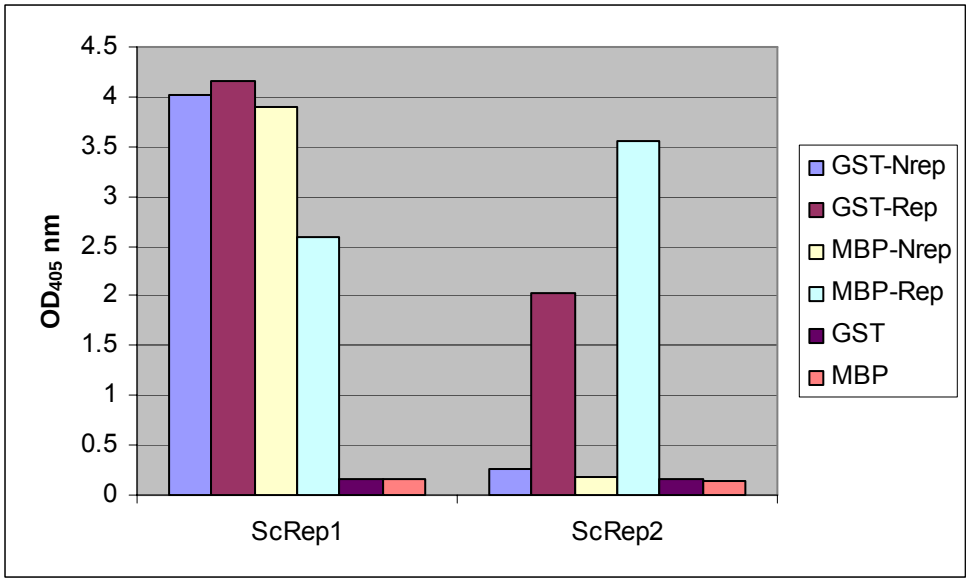


Figure 16 : Direct ELISA of the scFv-ScRep1 and scFv-ScRep2 reactivity with different antigens.

About 100µl of 10 µg/ml of GST-NRep, GST-Rep, MBP-NRep, MBP-Rep, GST and MBP 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 9E10 monoclonal antibody and GAM polyclonal antibody conjugated to horse radish peroxidase as secondary antibody. ELISA readings (OD_{405nm}) were performed after 30 min incubation with ABTS substrate at 37°C.

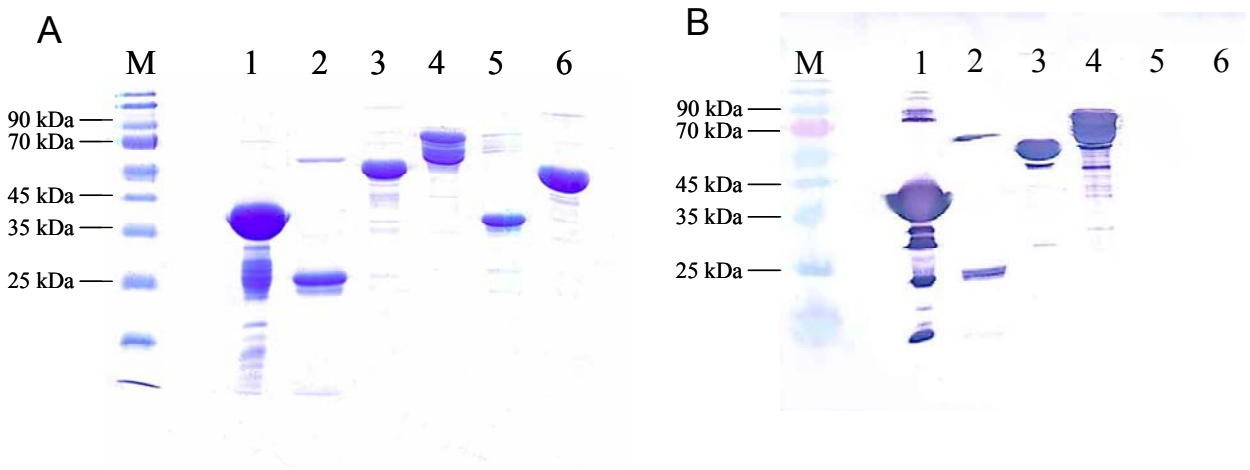


Figure 17: WB analysis of bacterially expressed scFv-ScRep1 against different Rep fusion proteins.
Purified fusion Rep proteins were separated on a 12% (w/v) SDS-PAGE gel and Coomassie-stained (A). In WB analysis (B), proteins were blotted onto a nitrocellulose membrane and bacterially expressed scFv-ScRep1 was used for detection. The banding was revealed by 9E10 monoclonal antibody and GAM^{AP} antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: GST-NRep; 2: GST-Rep; 3: MBP-NRep; 4: MBP-Rep; 5: GST-MP; 6: MBP-MP; M: Prestained protein marker

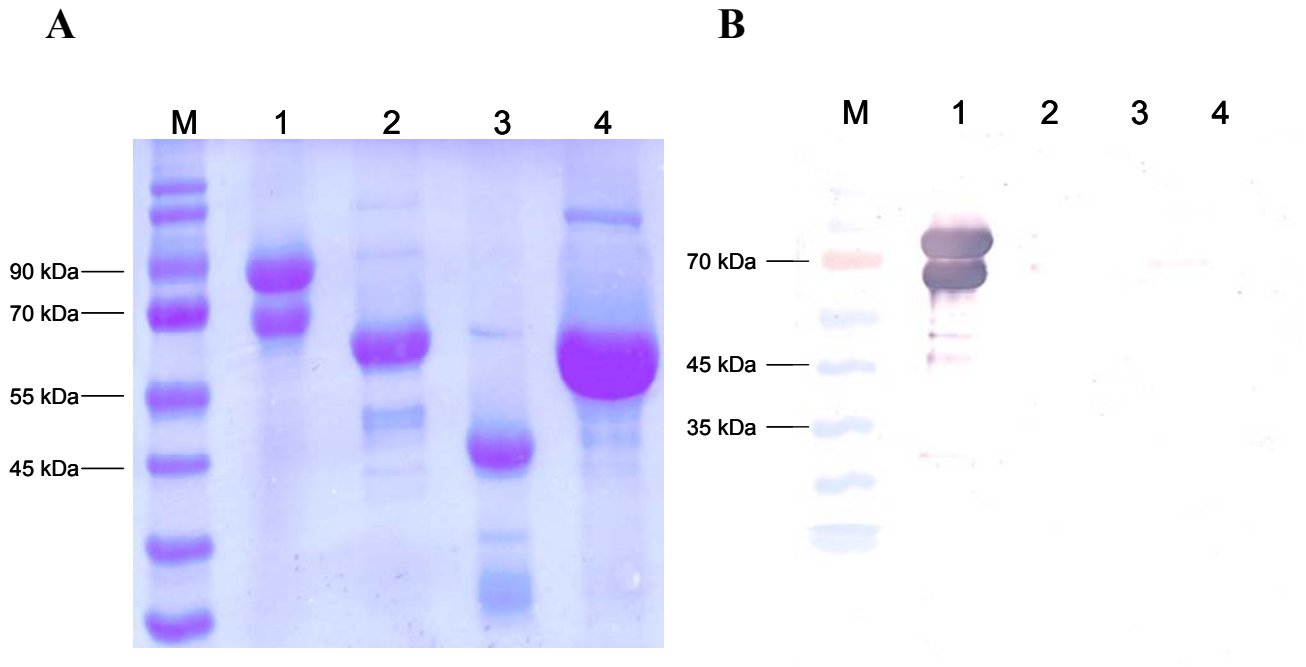


Figure 18: WB analysis of bacterially expressed scFv-ScRep2 against recombinant fusion Rep proteins.
Purified fusion Rep proteins were separated on a 12% (w/v) SDS-PAGE gel and Coomassie-stained (A). In WB analysis (B), proteins were blotted onto a nitrocellulose membrane and bacterially expressed scFv-ScRep2 was used for detection. The banding was revealed by 9E10 monoclonal antibody and GAM^{AP} antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: MBP-Rep; 2: MBP-NRep; 3: GST-NRep; 4: MBP-MP; M: Molecular weight protein marker

III.5 Cloning of scFvs against TYLCV virion from specific hybridoma cell lines

III.5.1 Purification and analysis of mouse HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 monoclonal antibodies

Four different hybridoma cell lines (kindly provided by Dr. S. Winter from DSMZ, Braunschweig, Germany) produced monoclonal antibodies HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 which could bind to TYLCV virion. These cells were propagated in tissue culture flasks and secreted MAbs from supernatants were collected and used for purification. The hybridoma culture supernatants were centrifuged to remove the cells and cell

debris before applying them to the equilibrated Protein A matrix (II.1.3). Approximately 4 mg each purified antibody was obtained. The SDS-PAGE analysis of the purified mouse MAbs showed two major bands of approximately 53 and 26 kDa corresponding to the mouse heavy and light chains, respectively. As shown in Figure 19 line 1, the heavy chain of MAb-HTYLCV1 is clearly lighter than other heavy chains.

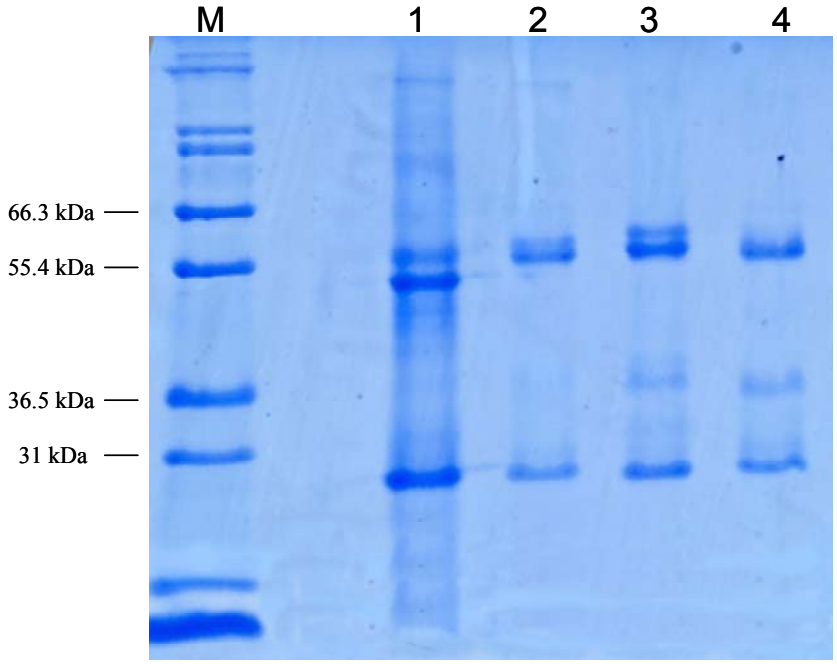


Figure 19: SDS-PAGE analysis of the affinity purified mouse MAb HAV. The mouse MAb HTYLCV1 (1), HTYLCV2 (2), HTYLCV3 (3) and HTYLCV4 (4) purified from the hybridoma culture supernatants (II.2.7.1) were separated on a 12% (w/v) SDS-PAGE gel and stained with Coomassie brilliant blue (II.2.3.2). M: Molecular weight marker.

ELISA (II.2.3.3.2) and immunoblot (II.2.3.3.1) analysis with the purified antibodies demonstrated high specificity of the HTYLCV1 antibody to recombinant GST-CP and MBP-CP but failed to react with GST-NCP and MBP-NCP carrying amino-terminal portion of CP. HTYLCV1 recognized entire CP in WB assay indicating that it probably binds to a linear epitope (Figure 20).

Immunoassays analysis showed while HTYLCV2, HTYLCV3 and HTYLCV4 monoclonal antibodies did not bind to recombinant CP, they are interacting with the infected leaf extract only (data are not shown), demonstrating that these MAbs are binding to either neotopes presented in entire virions or discontinuous epitopes in native CP.

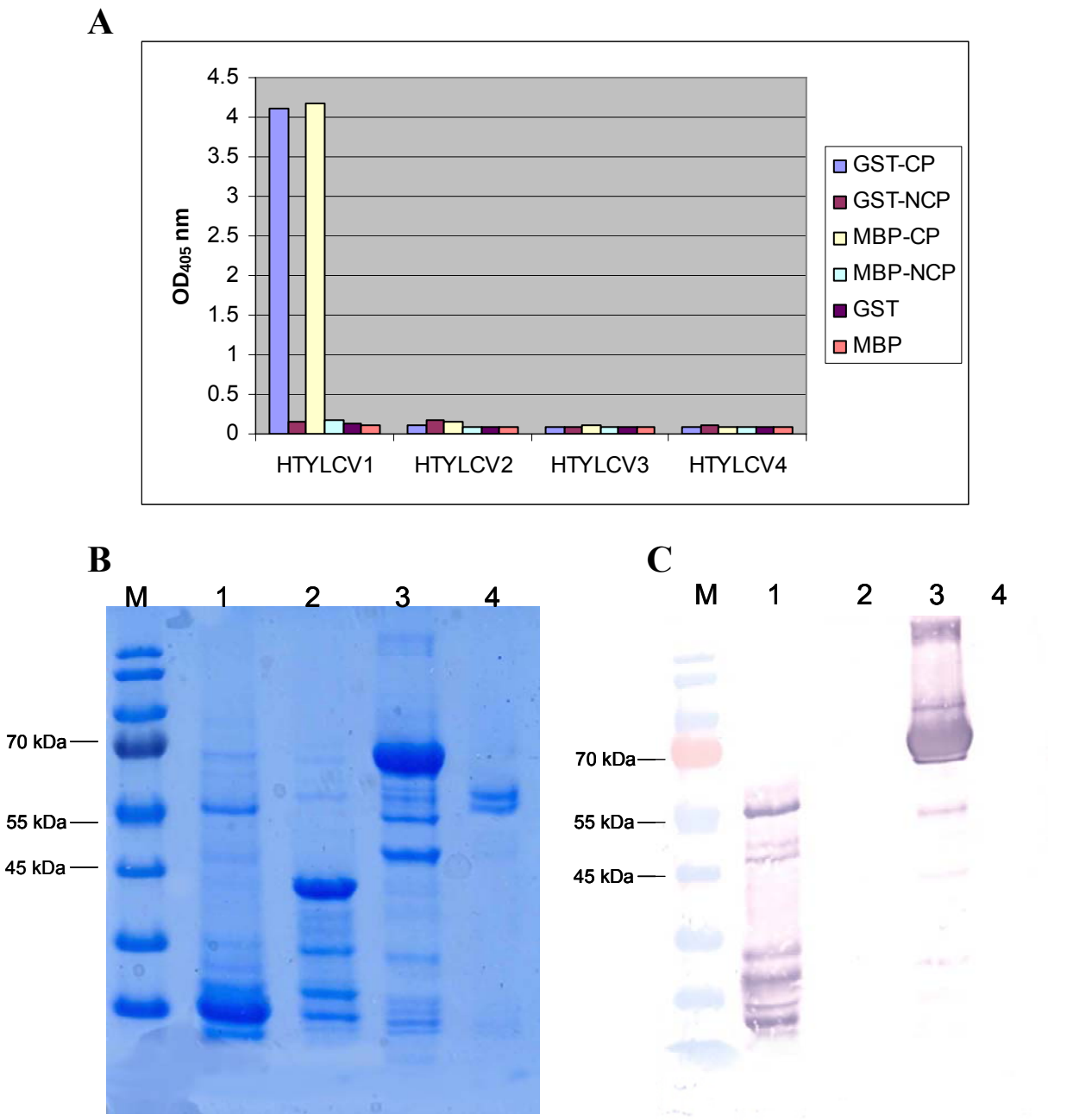


Figure 20: **Mouse monoclonal HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 binding activity tests against recombinant CP in ELISA and WB.**
A) Indirect ELISA to show reactivity of MAbs HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 to recombinant CP proteins. 100 µl of 5µg/ml purified (II.2.7.1) MAbs were applied on a MBP-CP, MBP-NCP, GST-CP, GST-NCP, MBP and GST coated ELISA plate (II.2.3.3.2). Bound MAbs were revealed by monoclonal GAM antibody conjugated to horse raddish peroxidase. ELISA readings ($OD_{405\ nm}$) were performed after 30 min incubation with ABTS substrate at 37°C. B and C) WB assay of MAb HTYLCV1 reactivity with recombinant CP. Different recombinant CP proteins were loaded into SDS-PAGE gel (B) and transferred onto nitrocellulose membrane (C) as described in (II.2.3.3.1.1). Immunodetection was carried out with alkaline phosphatase conjugated GAM antibody. Detection was performed with NBT/BCIP for 10 min at RT. M: pre-stained protein marker; 1: GST-CP; 2: GST-NCP; 3: MBP-CP and 4: MBP-NCP.

III.5.2 Amplification and cloning of the heavy and light variable domains of HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 MAbs

Indirect ELISA was used to determine the constant domain of heavy and light chains of HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 MAbs (Figure 21). All constant regions of heavy and light chains belonged to IgG2b and κ groups. Total RNA from hybridoma cell lines (II.2.7.2) were isolated (Figure 22) and reverse transcribed utilizing COH 32 (V_HIgG2a/2b) and MuPD31 (κV_L) primers with SuperScript™ II Reverse Transcriptase (RT) (Invitrogen). The corresponding V_H and V_L domains of the mouse MAbs were individually amplified by specific murine forward and reverse primers (II.1.6) from cDNAs templates and followed by digestion and cloning into pHENHI vector using same strategy utilized for the construction of the ARep phage library (II.2.5.3).

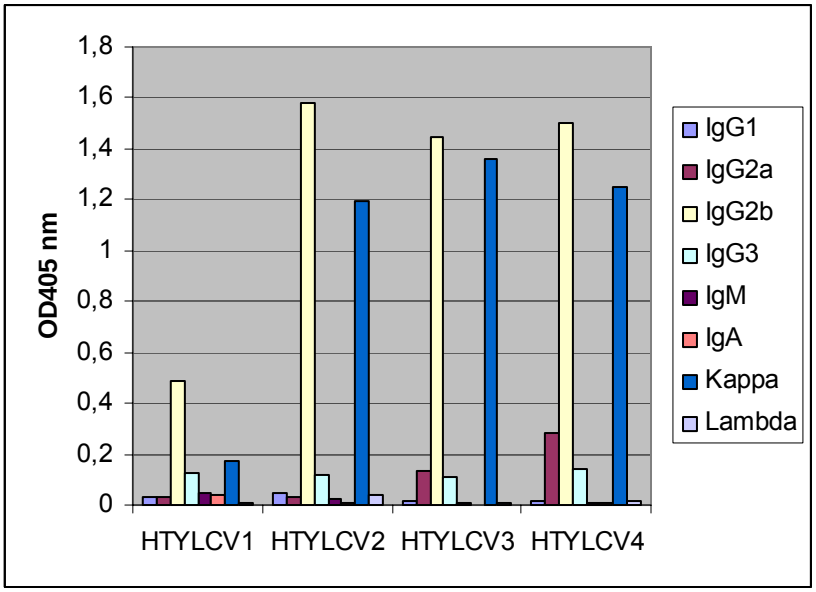


Figure 21: Indirect ELISA analysis to detect heavy and light constant regions of murine HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 MAbs.
Binding activity of HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 murine monoclonal antibodies to specific antibodies recognizing constant region of heavy chain (IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) or light chain (kappa and lambda) was studied. Detection was carried out by adding GAM conjugated to alkaline phosphatise enzyme. ELISA readings (OD_{405nm}) were performed after 30 min incubation with pNPP substrate at 37°C.

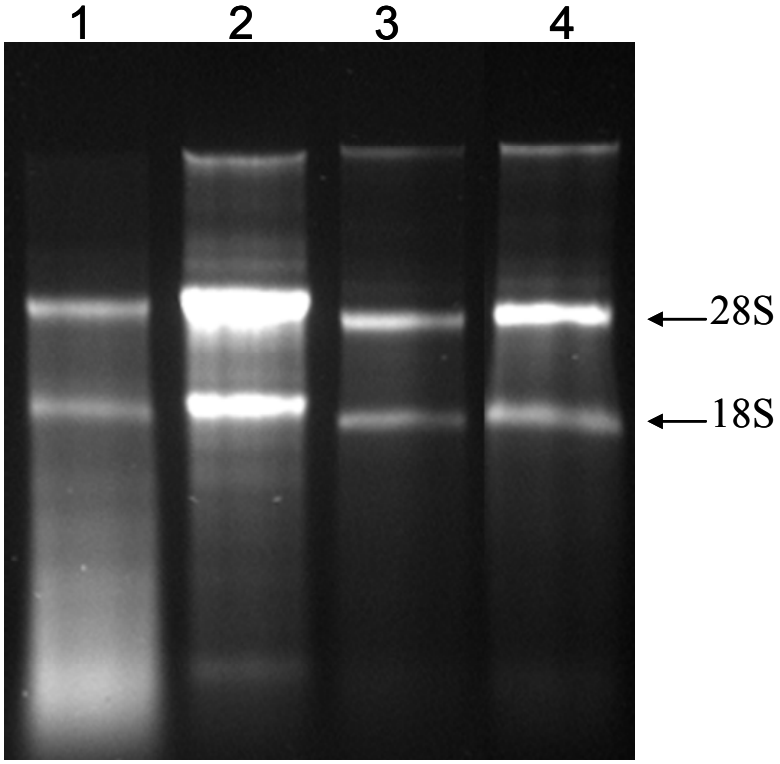
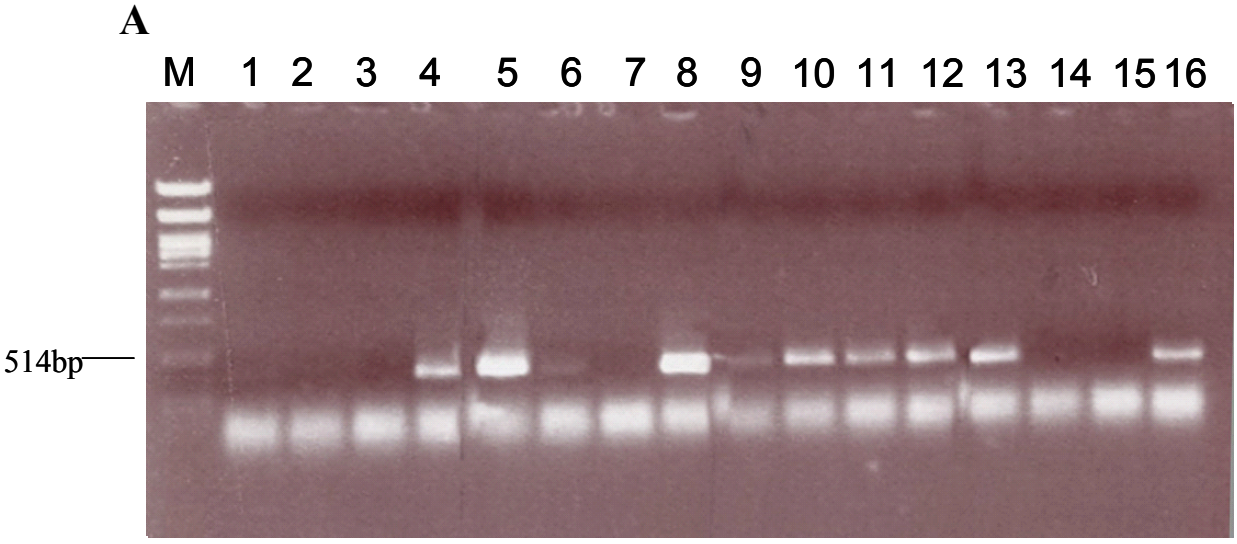


Figure 22: Analysis of total RNA extracted from mouse hybridoma cells.
Total RNAs were isolated from hybridoma cell lines secreting monoclonal HTYLCV1 (1), HTYLCV2 (2), HTYLCV3 (3) and HTYLCV4 (4) antibodies (II.2.5.1) and separated on a 1.2% (w/v) agarose gel. The major ribosomal RNA species (28S and 18S rRNA) are indicated.



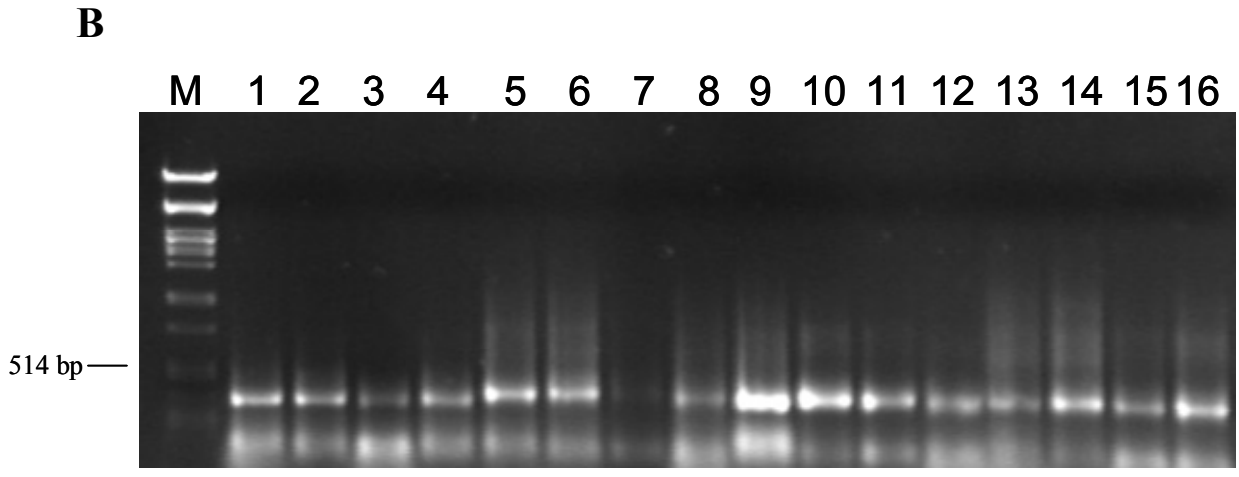


Figure 23: Amplification of variable heavy and light chain fragments from hybridoma cell lines. Nucleic acids were separated on a 1.2% (w/v) agarose gel. A and B: amplification of heavy and light chain fragments from synthesized cDNA using specific primers (II.1.6), respectively. Lines 1-4, 5-8, 9-12 and 13-16 are showing amplified variable fragments corresponding to HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 antibodies, respectively. M: λ *Pst*I DNA ladder.

III.5.3 Sequence analysis of V_H and V_L raised from mouse MAbs

Ten clones of each pHENHI-V_H and/or pHENHI-V_L sub-library made from murine MAbs were picked at random and sequenced. More than 80% of clones contained full size heavy and light chain variable domains. All V_H and V_L sequences made from same hybridoma lines were compared and belonged to the corresponding group. Alignment results revealed that all differences took place in primer binding sites lying on frame works 1 and 4 regions. Frame work regions have crucial role in correct folding of scFv fragments, and unsuitable amino acids inserted by primers may led to failure or loss in binding activity of scFv (Worn and Pluckthun, 2001). Accordingly, clones with the highest similarity to corresponding group (Table III-5) were selected and full length scFvs were constructed by inserting of V_L fragments into concerning pHENHI-V_H via *Asc*I/*Not*I digestion.

The alignment results of nucleotide sequence of V_H-HTYLCV1 with the IMGT (http://imgt.cines.fr/IMGT_vquest/) and KABAT databases and other similar V_H fragments using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) revealed a 39 bp deletion after position 281 located in framework 3 and CDR3 regions downstream the original ATG start codon in *Nco*I site. This was in consensus with SDS-PAGE analysis of HTYLCV1 (Figure 19) indicating a lighter heavy chain of HTYLCV1 in comparison to the others. These results confirmed that the amino acid sequences after deletion site were no longer similar to corresponding group and following on the same frame led to a stop codon at position 366 in polylinker region. These results indicate a possible frameshift within V_H-TYLCV1. As shown in Figure 25 two possibilities were considered for the frameshift and the reading frame was restored by deletion of one A at position 258 (at the end of frame work 3) and/or position 296 (frame work 4).

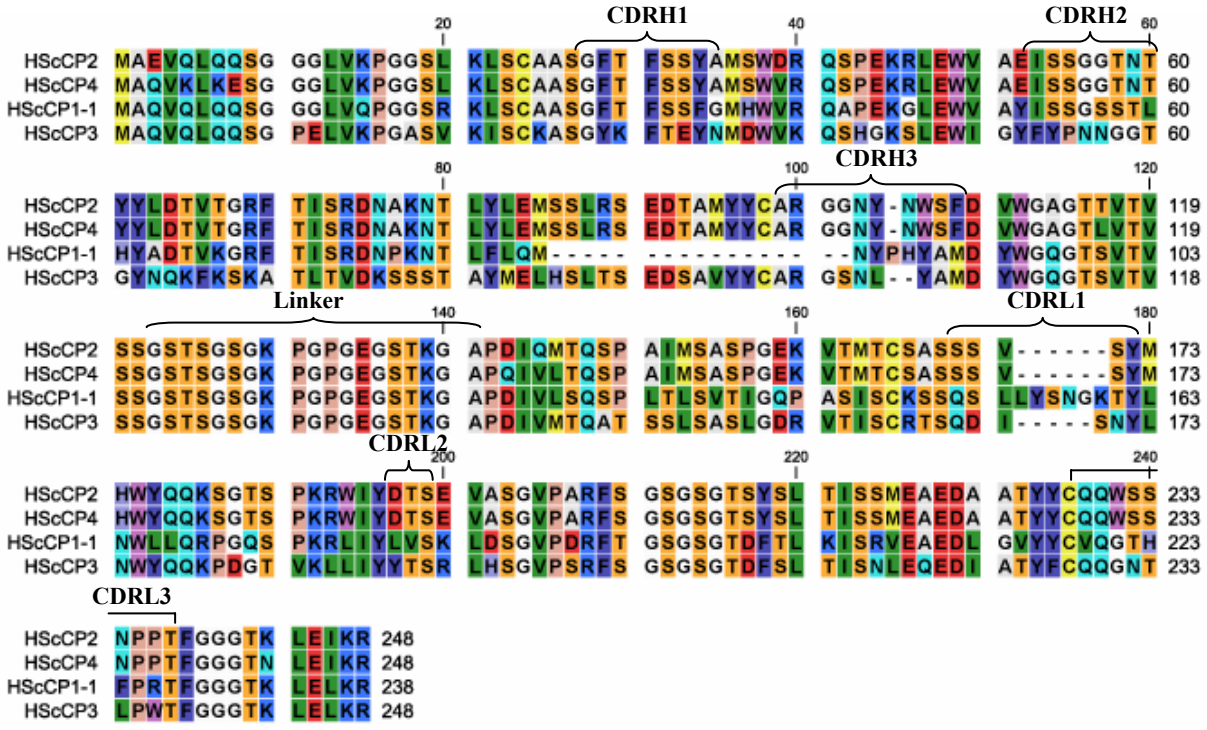


Figure 24: Amino acid sequences obtained form murine scFv fragments interacting to TYLCV (scFv-HScCP1-1, scFv-HScCP1-2, scFv-HScCP2, scFv-HScCP3 and scFv-HScCP4).
Complementary determining regions (CDRs) were determined according to IMGT databases. - : no amino acid at this position. The complementary determining regions (CDR) and polylinker have been indicated.

Table III-4: Summary of nucleotide length and deduced molecular weight of derived scFv with specificity for TYLCV virion

Designation of scFv	Nucleotide length (bp)	Deduced MW (kDa)
scFv-HScCP1-1	714 bp	25.49 kDa
scFv-HScCP2	744 bp	26.42 kDa
scFv-HScCP3	744 bp	26.84 kDa
scFv-HScCP4	744 bp	26.37 kDa

Table III-5: Comparison of amino acids in variable heavy and light chain of scFv-HScCP1-1, scFv-HScCP1-2, scFv-HScCP2, scFv-HScCP3 scFv-HScCP4 fragments with IMGT databases.

scFv	Mouse IgG Family		Closest germline gene sequences		Identity (Nucleotides)	
	V_H	V_L	V_H	V_L	V_H	V_L
scFv-HScCP1-1	IGHV5	IGKV1	AJ851868	Z72382	86.11% (248/288 nt)	97.95% (288/294 nt)
scFv-HScCP2	IGHV5	IGKV4	AF290961	AJ231234	95.83% (276/288 nt)	96.01% (265/276 nt)
scFv-HScCP3	IGHV1	IGKV10	J00488	M15520	95.13% (274/288 nt)	96.77% (270/279 nt)
cFv-HScCP4	IGHV5	IGKV4	AJ851868	AJ231234	95.83% (276/288 nt)	98.55% (272/276 nt)

III.5.4 Restoring of reading frames in V_H-HTYLCV1 sequence

III.5.4.1 Open reading frame correction in frame work 3 of V_H-HTYLCV1

Restoring of reading frame in the end of framework 3 within V_H-HTYLCV1 sequence was accomplished through one A deletion at position 258 (Figure 25). For this aim, QuickChange® II site-directed mutagenesis kit (Stratagene) and new primers (II.1.6) were used according to the manufacturer `s protocol (II.1.4).

The correct deletion of the "A" nucleotide was confirmed by sequencing. The modified V_H-HTYLCV1 was cloned into pHEN-V_L-HTYLCV1 via *Nco*I/*BstE*II and new scFv was named scFv-HScCP1-1.

III.5.4.2 Open reading frame correction in frame work 4 of V_H-HTYLCV1

As an alternative strategy, the frame of V_H-HTYLCV1 sequence was modified through one A deletion at position 296 within primer binding site at framework 4. For this purpose, entire V_H fragment was amplified again using new reverse primer (II.1.6) containing *Bst*EII (Figure 25). The amplified fragment directly cloned into pMBL cloning vector and a clone with expected sequence was selected. The modified V_H fragment was recovered by *Nco*I/*BstE*II digestion and introduced upstream the polylinker into digested pHEN-V_L-HTYLCV1. The new construct was named scFv-HScCP1-2.

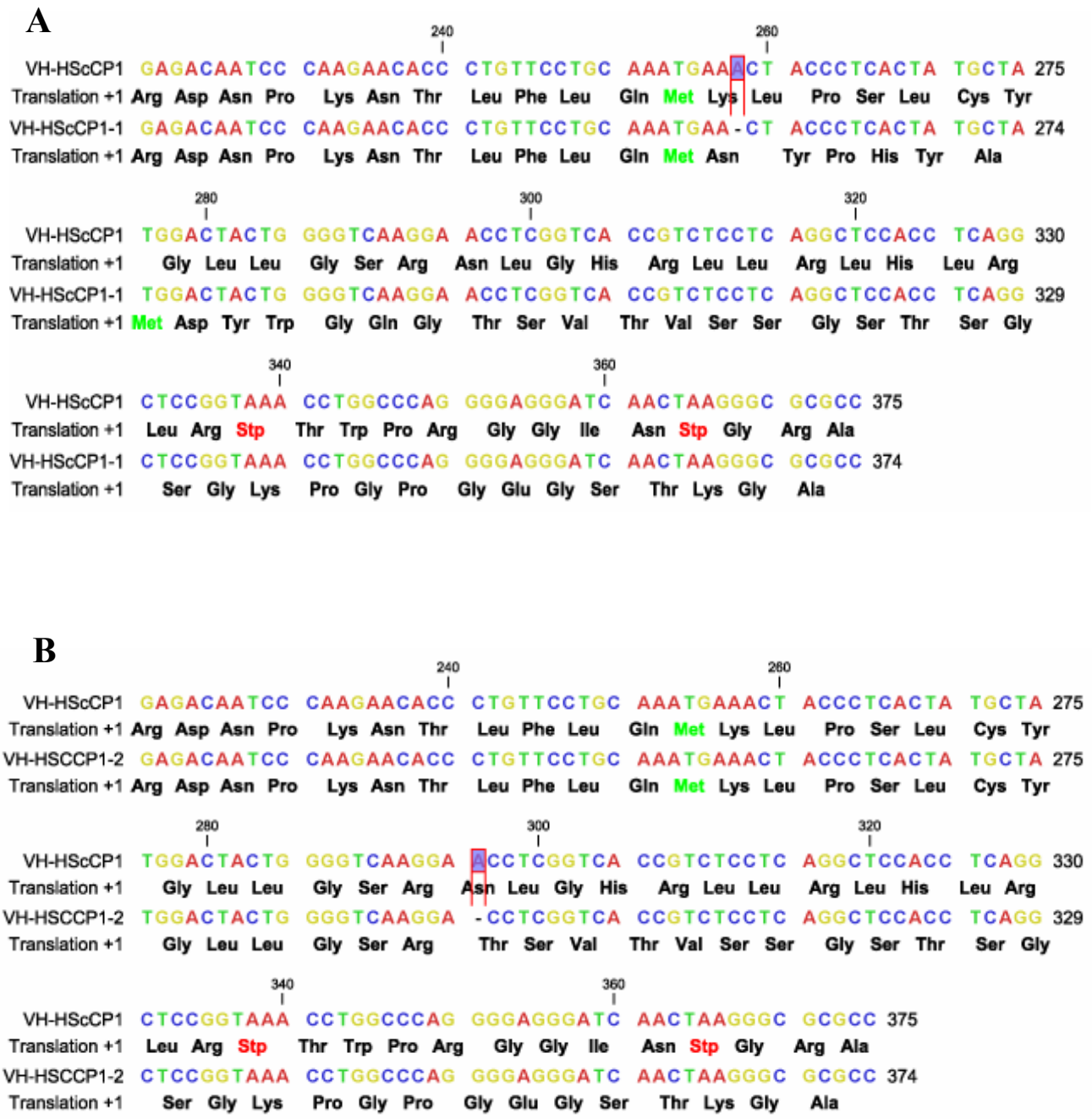


Figure 25: Modifying of reading frame in V_H part of scFv-HScCP1.

Aligning of original sequence of scFv-HScCP1 to modified scFv-HScCP1-1 (A) and scFv-HScCP1-2 (B). The frame in the original sequence was restored by deletion of nucleotide ´A` at position 258 (A) or 296 (B) downstream the original ATG stop codon located in the *Nco*I site.

III.5.4.3 Expression of scFv-HScCP1-1, scFv-HScCP1-2, scFv-HScCP2, scFv-HScCP3 and scFv-HScCP4 in bacterial culture

One colony harboring full size scFv from each pHEN-HScCP1-1, pHEN-HScCP1-2, pHEN-HScCP2, pHEN-HScCP3 and pHEN-HScCP4 clones were selected and a small-scale expression in *E. coli* strain HB2151 was carried out as described earlier (II.2.2.3.1). WB experiments showed expression of full size scFv on nitrocellulose membrane (II.1.3) (Figure 26). The WB result revealed that scFv-HScCP1-1 and scFv-HScCP1-2 are clearly smaller than scFv-HScCP2 and scFv-HScCP3 and scFv-HScCP4. ELISA results showed that scFv-HScCP3 could detect ACMV virus in infected leaves. The scFv-HScCP2, scFv-HScCP3 and scFv-HScCP4, like corresponding MAbs did not interact with recombinant CP fusion proteins. WB experiments showed low specificity of scFv-HScCP1-1 against MBP-CP and GST-CP (data are not shown) while scFv-HScCP1-2 failed to bind against recombinant CP.

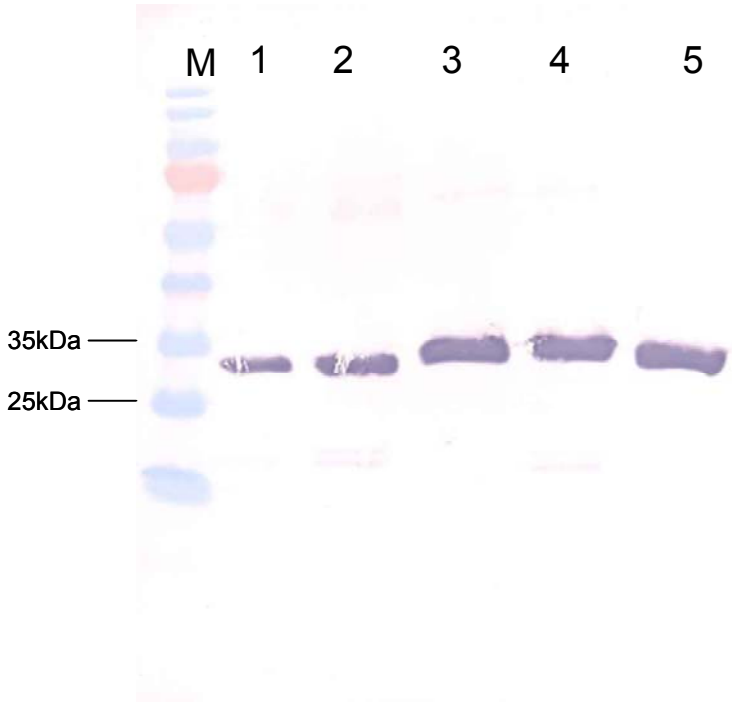


Figure 26: Immuno-blot analysis of bacterially expressed scFv-HScCP1-1, scFv-HScCP1-2, scFv-HScCP2, scFv-HScCP3 and scFv-HScCP4.

Periplasmicly expressed scFvs were separated on a 12% (w/v) SDS-PAGE gel and blotted onto nitrocellulose membrane. The blotted scFvs were revealed by 9E10 monoclonal antibody and GAM^{AP} antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: scFv-HScCP1-1; 2: scFv-HScCP1-2; 3: scFv-HScCP2; 4: scFv-HScCP3; 5: scFv-HScCP4; M: Molecular weight protein marker.

III.6 Expression and characterization of recombinant proteins in transiently transformed plants

III.6.1 Cloning of C1 and scFvs genes in pTRAkt plant expression vectors

III.6.1.1 Cloning of C1 gene as fusion with DsRed protein

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; Matz et al., 1999). It emits at a wavelength (600-620nm) clearly distinguished 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).

For cloning of C1 gene into pTRAkt-DsRed vector, C1 was amplified using specific primers (II.1.6) harbouring *Afl*III restriction enzyme site to introduce at the 5‘ and 3‘ ends. PCR product was then directly cloned into pMBL cloning vector (II.1.10). The correct clone was selected by sequencing and C1 coding gene was recovered by *Afl*III digestion and introduced upstream the DsRed coding region into *Nco*I digested pTRAkt-DsRed. The new construct was called pTRAkt-C1-DsRed (Figure 27).

The constructs pTRAkt-DsRed and pTRAkt-C1-DsRed were used for transformation into *Agrobacterium* by electroporation (II.2.1.13.6). Ten independent recombinant colonies from each transformation were screened for the presence of insert by colony check PCR (II.2.1.2) using pSS specific primers (II.1.6) and no positive clone containing pTRAkt-C1-DsRed vector was identified. Transformation of pTRAkt-C1-DsRed construct into *Agrobacterium* was repeated several times, but no transformed clone was recovered. This may indicate toxic activities of Rep-DsRed fusion protein in agrobacteria cells. To overcome this problem, one deletion (98 aa) was carried out within the part of Rep which is responsible for ATPase activity (Figure 1B). For this aim, 294 nucleotides placed between 629-926 positions (designed as Δ region in Figure 1B) were removed by *Eco*NI/*Sfi*I digestion. To restore the correct frame after digestion site, the restricted pTRAkt-C1-DsRed was treated with Klenow fragment DNA polymerase following blunt end ligation by T4 DNA ligase. The new construct was named pTRAkt-DC1-DsRed and transformed successfully into *Agrobacterium* by electeroporation (II.2.1.13.6). Single colony of recombinant *Agrobacterium* cultures harbouring the expected construct was used for transient expression (II.2.8.3.1).

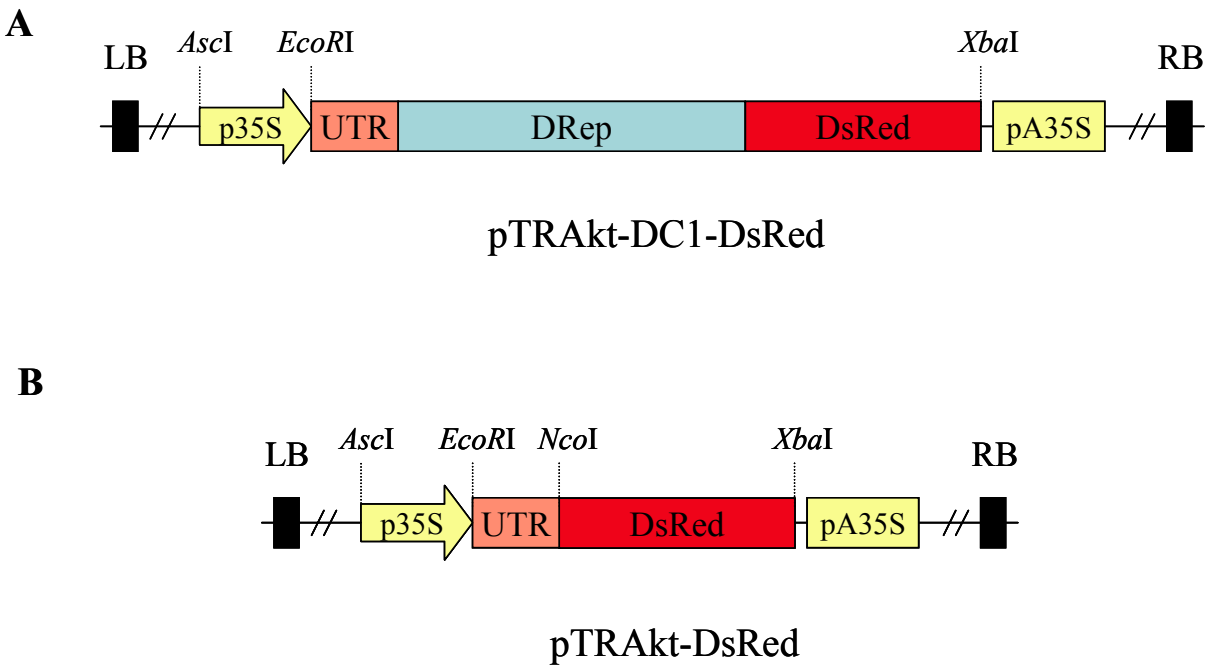


Figure 27: Schematic presentation of the constructs used for DRep-DsRed (A) and DsRed (B) transient expression in plant.

p35SS: 35S promoter from Cauliflower Mosaic Virus (CaMV) with duplicated enhancer, UTR: 5` un-translated region of *Tobacco etch virus*, pA35: polyadenylation signal. The cloning sites are depicted.

III.6.1.2 Cloning of scFv genes into pTRAkt plant expression vector

III.6.1.2.1 Cloning of scFvs genes into pTRAkt plant expression vector targeted to cytosol and nucleus

For scFv expression and localization within the cytosol, scFv-RWAV (generated and characterized by A. Zakri, Institute of Molecular Biotechnology, RWTH-Aachen) was amplified by PCR from pHEN-scFv-RWAV plasmid using specific primers containing *c-myc*, His6 tag and *Nco*I/*Xba*I restriction sites (II.1.6). The amplified scFv-RWAV was initially introduced into pMBL cloning vector and followed by cloning into pTRAkt vector via *Nco*I/*Xba*I digestion and the new construct was named pTRAkt-RWAV (Figure 27). Since no leader peptide or tag has been added before or after scFv gene, this construct will be used for cytosolic expression of scFv fragment in plant cells. The successful cloning of pTRAkt-RWAV was verified by test digestion and sequencing. Next, individual scFv-ScRep1, scFv-ScRep2, scFv-HTYLCV2, scFv-HTYLCV3 and scFv-HTYLCV4 constructs were exchanged with scFv-RWAV in pTRAkt-RWAV via *Nco*I/*Not*I digestion. The resulting plasmids were

designated pTRAkt-ScRep1, pTRAkt-ScRep2, pTRAkt-HScCP2, pTRAkt-HScCP3 and pTRAkt-HScCP4. In the case of scFv-HScCP3, due to an additional *Nco*I sites located in the middle part of corresponding V_L sequence, a ‘‘partial digestion’’ strategy was used.
Additionally, two scFvs (scFv-ScRep1 and scFv-RWAV) were targeted to the nucleus by introducing the “SV40 T antigen” nuclear localization signal (NLS) at the N-terminus of the scFvs. For this purpose, the coding region of scFv-RWAV gene was amplified and cloned into pTRAkt in same way as for obtaining cytosolic expression except for using the forward primer containing NLS coding sequence (II.1.6). The resulting plasmids are named pTRAkt-NLS-ScRep1 and pTRAkt- NLS-RWAV.
All new constructs were transformed into *Agrobacterium* by electroporation (II.2.1.13.6) Ten independent recombinant colonies from each transformation were screened for the presence of insert by colony PCR (II.2.1.2) using pSS specific primers (II.1.6).

III.6.1.2.2 Cloning of scFv genes in pTRAkt plant expression vector as N-terminal fusions with GFP and targeted to cytosol and nucleus

The pTRAkt-GFP is a plant expression vector derived from pTRAkt (II.1.10) by insertion of the green fluorescent protein (GFP) coding sequence. For scFvs expression and localization in the cytosol and nucleus as N-terminal fusion with GFP, scFv-ScRep1 and scFv-ScRep2 were cloned into pTRAkt-scFv-RW-AV-GFP and pT-NLS-scFv-SCR-AC-GFP (kindly provided by A. Zakri, Institute of Molecular Biotechnology, RWTH-Aachen) via *Nco*I/*Not*I digestion and subsequent ligation and transformation into DH5α strain *E. coli.* The scFv fragments are located upstream the GFP coding sequence. The resulting plasmids, pTRAkt-ScRep1-GFP, pTRAkt-ScRep2-GFP and pTRAkt-NLS-ScRep1-GFP, were transformed into *Agrobacterium* by electroporation (II.2.1.13.6). Ten independent recombinant colonies from each transformation were screened for the presence of insert by colony check PCR (II.2.1.2) using pSS specific primers (II.1.6).

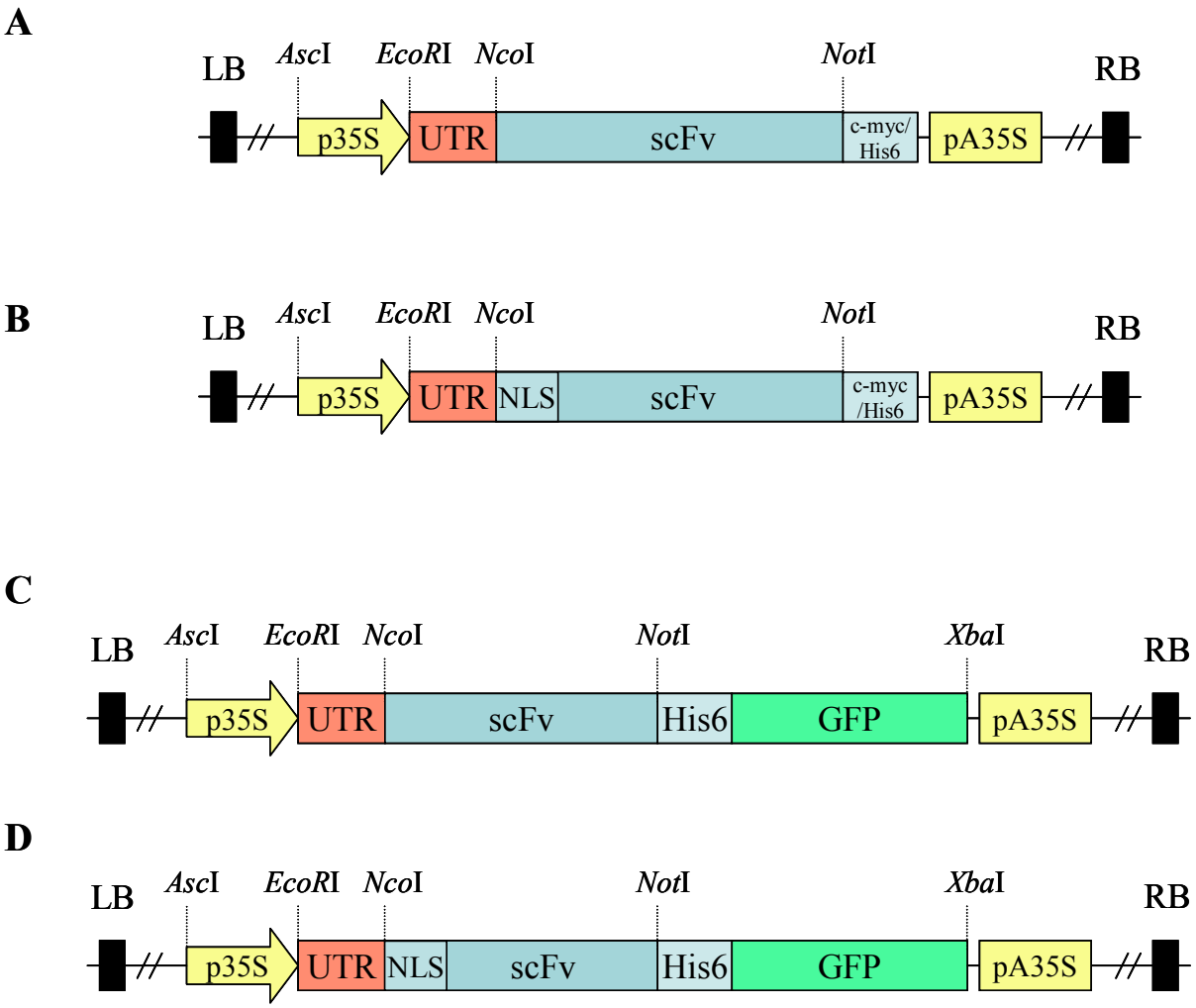


Figure 28: Schematic presentation of the constructs used for scFvs expression in plant.

The vector pTRAkt was used for expression of scFv in tobacco leaves. A: the constructs used for expression in the cytosol, B: the constructs used for expression in the nucleus. C: the constructs used for expression in the cytosol as a fusion to GFP protein. D: the constructs used for expression in the nucleus as a fusion to GFP protein. p35SS: 35S promoter from *Cauliflower Mosaic Virus* (CaMV) with duplicated enhancer; UTR: 5` untranslated region of *Tobacco etch virus;* pA35: polyadenylation signal; NLS: SV40 T antigen nuclear localization signal; *c-myc*: myc epitope tag for detection; His6: six repeated histidin for detection and purification of recombinant protein . The cloning sites are shown**.**

III.6.2 Transient transformation of tobacco plants

III.6.2.1 Transient expression of Rep and scFvs in plant cells

To study scFvs and Rep expression in tobacco plants, scFv-ScRep1, scFv-ScRep2, scFv-RWAV, scFv-HScCP2, scFv-HScCP3, scFv-HScCP4, scFv-NLS-ScRep1, scFv-Cyt-ScRep1-GFP, scFv-Cyt-ScRep2-GFP, scFv-NLS-ScRep1-GFP and DRep-DsRed constructs were transiently transformed either by injection of the induced recombinant *Agrobacterium* to the underside side of a *N. tabacum* leaf using a syringe without a needle and/or by vacuum infiltration (II.2.8.3.1).

Infiltration of *N. tabacum* leaves with DsRed-DRep caused distinct necrotic local lesion within 3 days (Figure 29).

An Olympus BX41 fluorescent microscope was used to monitor the intracellular localization of GFP and DsRed fusion constructs. As shown in Figure 30-B, DRep-DsRed fusion protein is predominantly localized within the nucleus and some fluorescence is observed in the cytosol compartment. In contrast, DsRed fluorescence in its native form was not particularly restricted to the nuclei of cells, but occurred throughout the cytoplasm (Figure 30-A).

The GFP fusion proteins, including scFv-Cyt-ScRep1-GFP, scFv-NLS-ScRep1-GFP and scFv-Cyt-ScRep2-GFP, are accumulated in both the cytoplasm and nucleus (Figure 31). Individual GFP and DsRed proteins expressed from pTRAkt-GFP and pTRAkt-DsRed plasmids are visible in both the cytoplasm and nucleoplasm areas.

Figure 29: Effect of DRep-DsRed protein on *N. tabacum* transiently expressing fusion protein.
Tobacco leaves agro-infiltrated with plasmid harbouring DRep-DsRed (A) and DsRed (B) proteins (III.6.1.1) by injection onto fully expanded leaves. After 4 days, necrotic lesions appeared on infiltrated leaves with pTRAkt-DRep-DsRed plasmid.

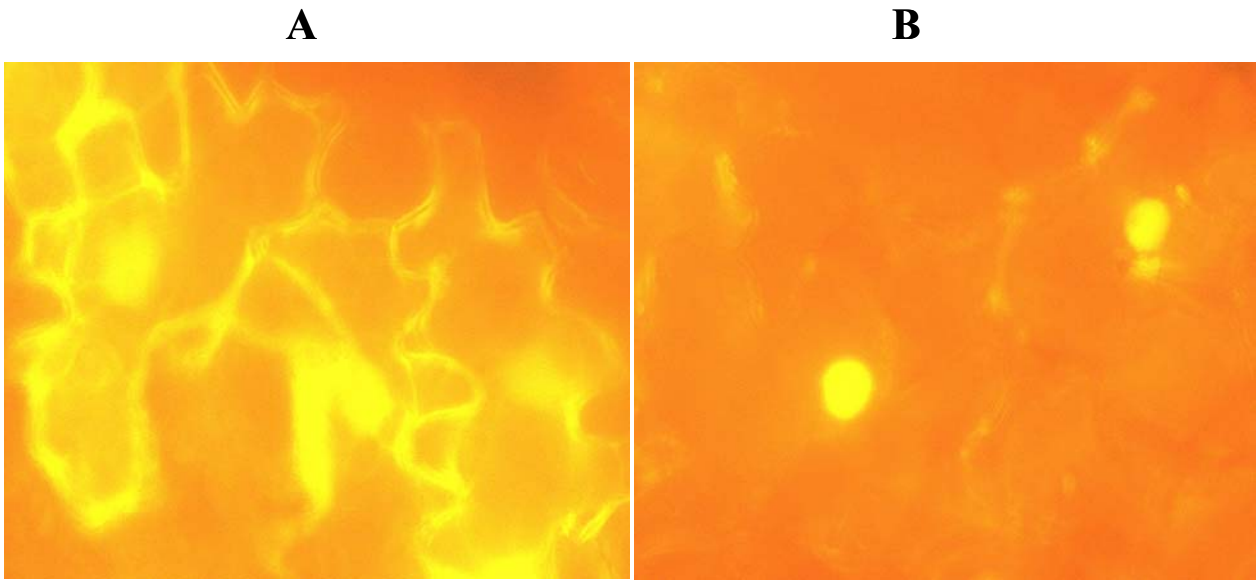


Figure 30: Transient expression of DRep-DsRed and DsRed in *N. tabacum* leaves.

Tobacco leaves were infiltrated (II.2.8.3.1) with recombinant agrobacteria harbouring the constructs pTRAkt-DsRed and pTRAkt-DRep-DsRed. After 4 days incubation, DsRed (A) and DRep-DsRed (B) expression and localization were analyzed using fluorescent microscope. DsRed was excited at 568 nm and emissions collected at 600 to 620 nm.

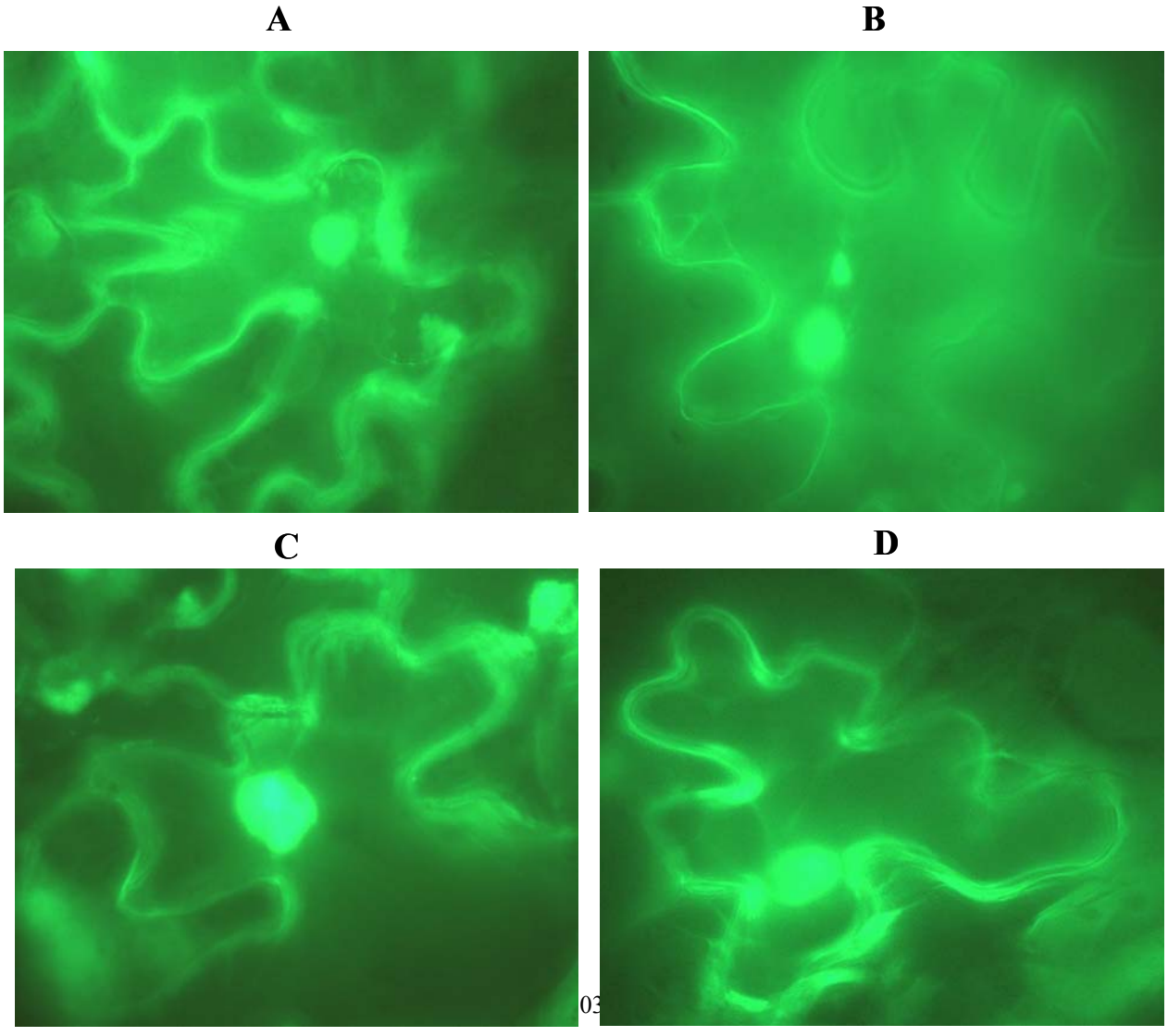


Figure 31: scFvs expression as fusion with GFP in tobacco leaves.

N. tabacum leaves were transiently transformed by agroinfiltration (II.2.8.3.1) with plasmid carrying GFP alone (A), scFv-Cyt-ScRep1-GFP (B), scFv-NLS-ScRep1-GFP (C) and scFv-Cyt-ScRep2-GFP (D). GFP fusion expression and localization was analyzed using fluorescent microscope. GFP was excited at 488 nm and emissions collected at 500 to 515 nm.

The western blot analyses of the total soluble protein extracted from infiltrated leaves showed a distinct band of approximately 30 kDa for scFv-ScRep1, scFv-ScRep2, scFv-NLS-ScRep1 (Figure 32). Expression levels of cytosolic scFv-ScRep1 construct was higher than scFv-ScRep2 and scFv-NLS-ScRep1. Other scFv fragments constructs including scFv-RWAV, scFv-HScCP2, scFv-HScCP3, scFv-HScCP4, scFv-Cyt-ScRep1-GFP, scFv-Cyt-ScRep2-GFP, scFv-Cyt-RWAV-GFP, scFv-NLS-ScRep1-GFP could not be detected by immunoblot analysis (data not shown).

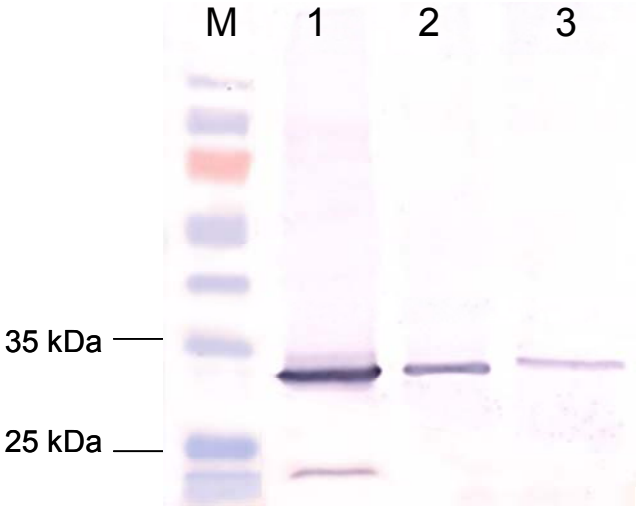


Figure 32: Immunoblot detection of transiently plant expressed scFv.

Approximately 15µl of crude extract of tobacco plants transiently expressing scFvs was used for separation on 12% (w/v) SDS-PAGE gel and blotted onto nitrocellulose membrane. The blotted scFvs were revealed by 9E10 monoclonal antibody and GAM antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: scFv-ScRep1; 2: scFv-NLS-ScRep1; 3: scFv-ScRep2; M: Pre-stained molecular weight protein marker.

III.6.2.2 Reactivity of plant extracted scFvs against recombinant proteins

To determine the activity of plant expressed scFv fragments, crude extracts of tobacco plants transiently expressing scFv-ScRep1, scFv-ScRep2 and scFv-NLS-ScRep1, scFv-Cyt-ScRep1-GFP and scFv-Cyt-ScRep2-GFP were extracted (II.2.8.5) and subjected for detection of recombinant Rep proteins in ELISA and western blot analysis. These experiments revealed that scFv-ScRep1, scFv-ScRep2 and scFv-NLS-ScRep1 are presenting high binding activity to recombinant Rep proteins indicating correct folding of these (Figures 33, 34 and 35).

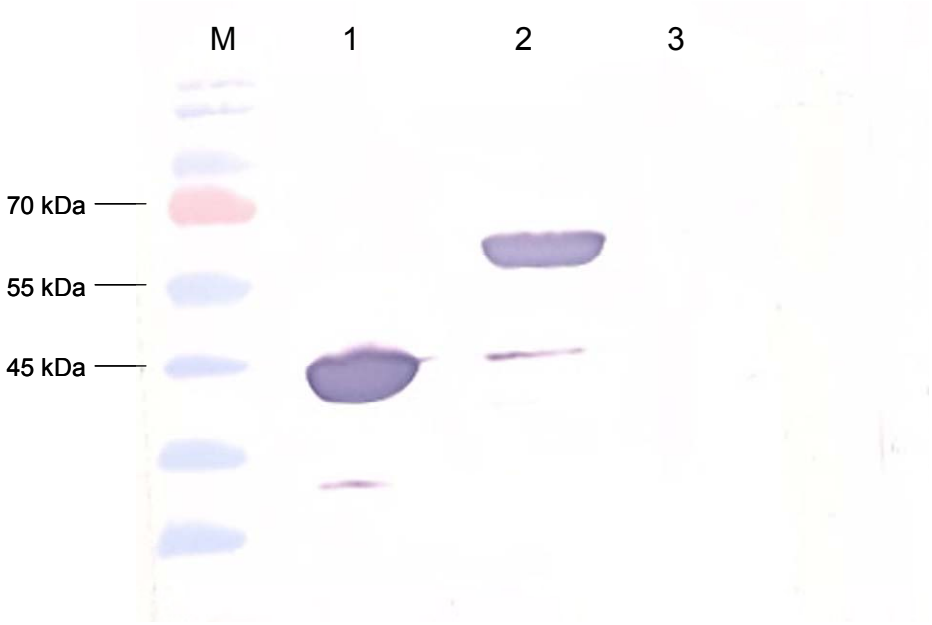


Figure 33: WB analysis of bacterially expressed scFv-ScRep1 against fusion Rep proteins.

Purified fusion Rep proteins were separated on a 12% (w/v) SDS-PAGE gel and blotted onto nitrocellulose membrane. Crude extract of plant transiently expressed scFv-ScRep1 was used for detection. The interactions were revealed by 9E10 monoclonal antibody and GAM antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: GST-NRep; 2: MBP-NRep; 3: MBP-MP; M: Pre-stained molecular weight protein marker.

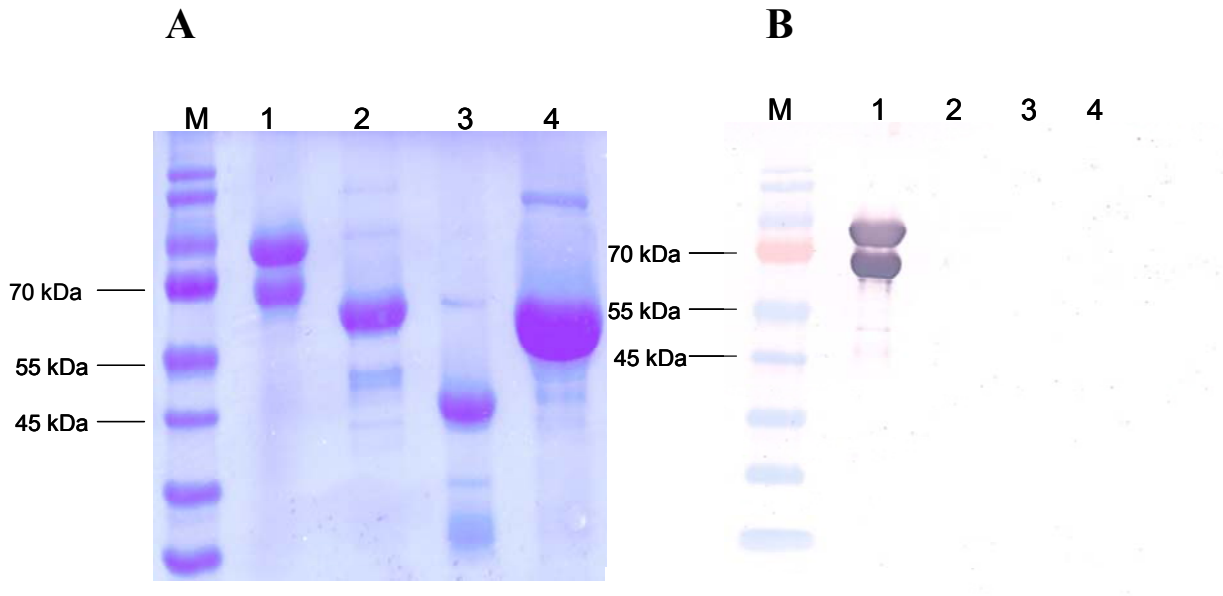


Figure 34: WB analysis of plant extracted scFv-ScRep2 against Rep fusion proteins.
Purified fusion Rep proteins were separated on a 12% (w/v) SDS-PAGE gel and Coomassie-stained (A). In WB analysis (B), fusion Rep proteins were blotted onto nitrocellulose membrane and crude extract of plant transiently expressed scFv-ScRep2 was used for detection. The interactions were revealed by 9E10 monoclonal antibody and GAM antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: MBP-Rep; 2: MBP-NRep; 3: GST-NRep; 4: MBP-MP; M: Molecular weight protein marker

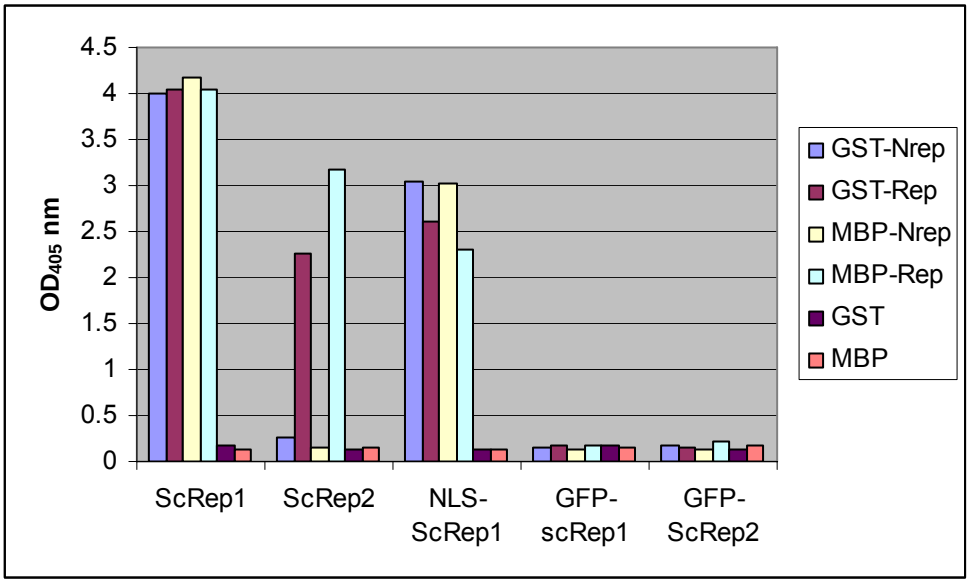


Figure 35: Direct ELISA of plant expressed scFv fragments reactivity with different antigens.

100 µl of 20 µg/ml of GST-Rep, GST-NRep, MBP-Rep, MBP-NRep, MBP and GST fusion proteins were coated on microtiter plates. 100µl of the overnight induced bacterial supernatant was added and bound scFvs were detected by addition of 9E10 monoclonal antibody and GAM polyclonal antibody conjugated to horse raddish peroxidase as secondary antibody. ELISA readings (OD_{405nm}) were performed after 30 min incubation with ABTS substrate at 37°C.

III.6.2.3 Rep-specific scFvs binding activities *in vivo*

To investigate *in vivo* binding activities of scFv-ScRep1 and scFv-ScRep2 against Rep, scFv-Cyt-ScRep1-GFP and scfv-Cyt-ScRep2-GFP (III.6.1.2.2) were each co-expressed with DRep-DsRed (III.6.1.1) in tobacco leaves using agroinfiltration by means of a syringe (II.2.8.3.1) 3-7 days after infiltration, the DsRed and GFP fluorescence were monitored by fluorescent microscopy. Identically to individual infiltration, co-infiltration results revealed localization of scFv fragments GFP fusions and DRep-DsRed at both the cytoplasm and nucleus (Figure 36 and 37). In some cells, co-localization of DRep-DsRed and scFv-GFP fusion proteins was observed (Figure 36-I and 37-I).

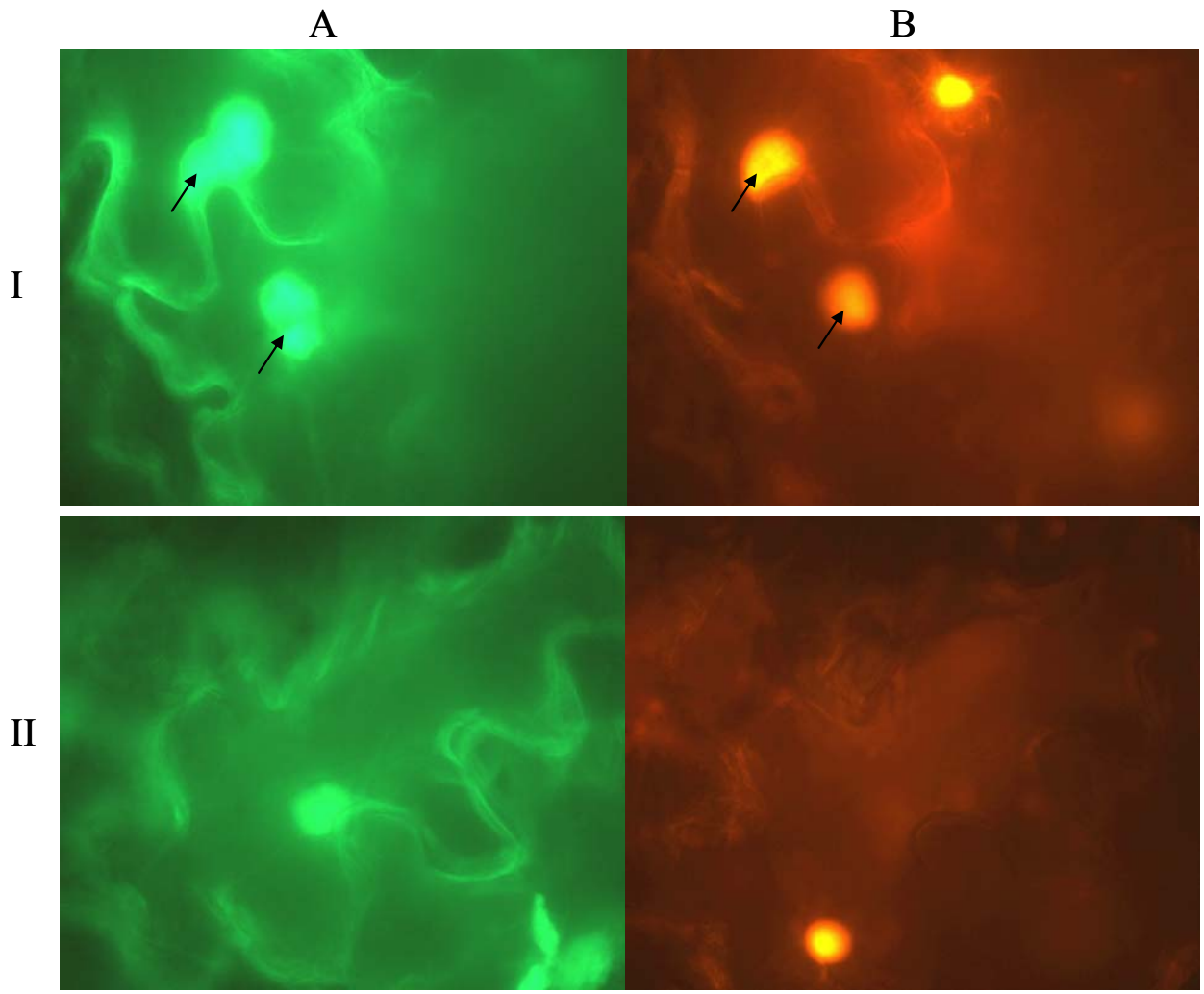


Figure 36: scFv-ScRep1 binding activity *in vivo*.

Rep specific scFv-Cyt-ScRep1-GFP was co-expressed in tobacco leaves (II.2.8.3.1) with DRep-DsRed through agroinfiltration. Their interactions and localizations were analyzed using fluorescent microscope (II.1.11). The columns A and B show the GFP and DsRed imaging, respectively. 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.

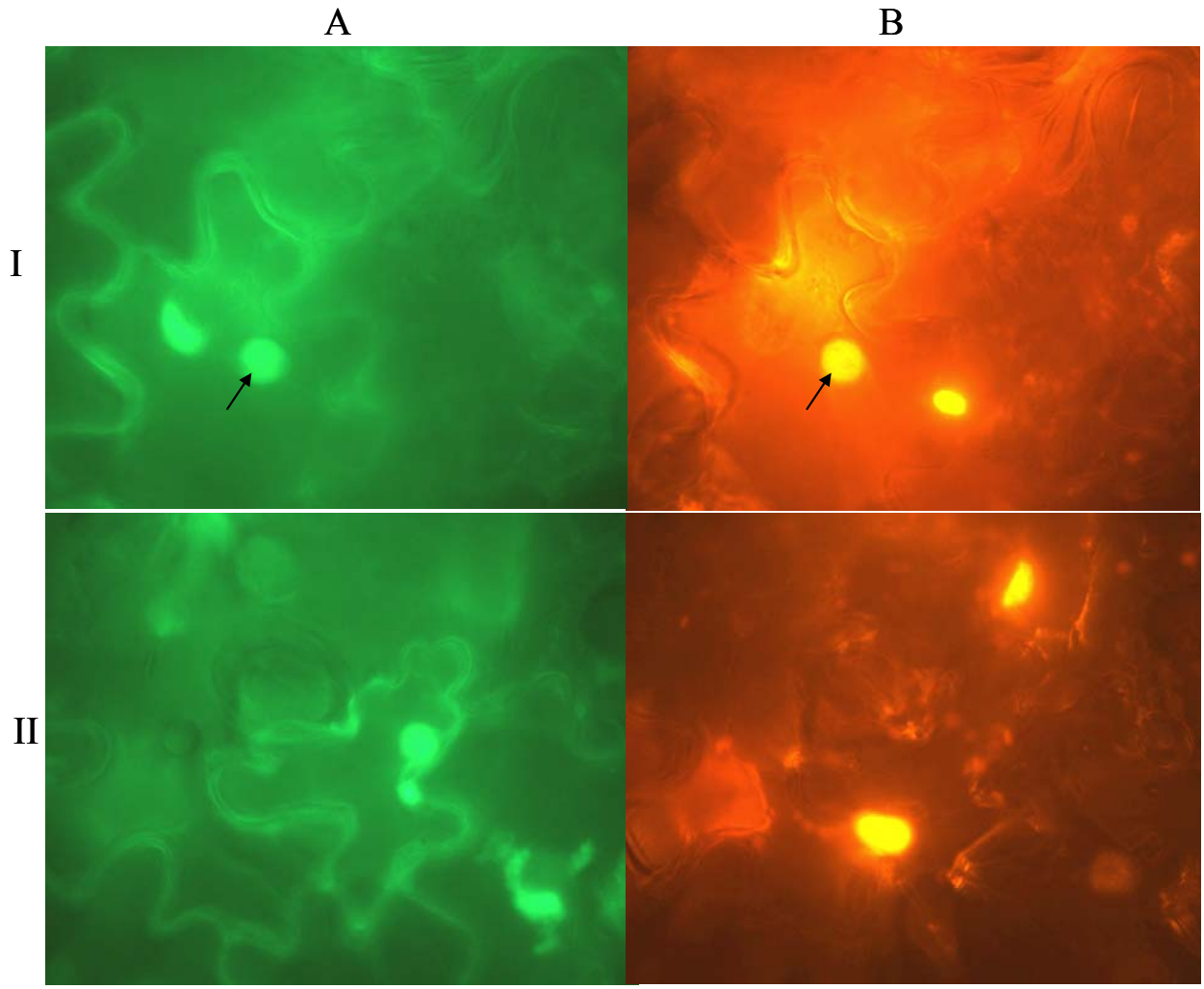


Figure 37: scFv-ScRep2 binding activity *in vivo*.

Rep specific scFv-Cyt-ScRep2-GFP was co-expressed in tobacco leaves (II.2.8.3.1) with DRep-DsRed using agroinfiltration. Interaction and localization were analyzed by fluorescent microscope (II.1.11). The columns A and B show the GFP and DsRed imaging, respectively. 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.

III.7 Generation and characterization of stable transformed plants over-expressing scFvs

III.7.1 Stable transformation of *N. benthamiana* plants over-expressing scFvs

Following the transient expression, *N. benthamiana* stable transformation was performed through leaf disc method (II.2.8.3.2.1) with the *A. tumefaciens* harbouring pTRAkt-ScRep1, pTRAkt-NLS-ScRep1, pTRAkt-RWAV, pTRAkt-HScCP2, pTRAkt-HScCP3, pTRAkt-HScCP4 and pTRAkt-ScRep1-GFP constructs. The kanamycin resistant shoots were sub cultured in the tobacco root-induction medium at intervals of 14 days. Subsequently, individual transformed rooted plants containing the relative construct were moved into the soil and kept in greenhouse under high humidity and assigned as T0 lines. The numbers of independent putative transformants are presented in table III-6. The concerning regenerated tobacco plants expressing ScRep1, NLS-ScRep1, RWAV, HScCP2, HScCP3 and HScCP4 proteins were briefly named SR, NSR, RW, HSC2, HSC3 and HSC4, respectively. Most of regenerated SR, NSR, RW and HSC2 tobacco lines in comparison to wild type plants had normal growth in the soil and did not show unusual growth characteristics. However, most of the rooted T0 HSC3 (23 out of 30 plants) and HSC4 (26 out of 30 plants) lines failed to grow normally in soil and died 1-2 weeks after transplantation. In 2-3 weeks, well grown T0 progenies producing different transgenes were self pollinated for establishment of next generation (T1).

To assess expression of scFv genes, leaf tissue from putative T0 transgenic plants were analyzed for accumulation of recombinant proteins by immunobloting analysis (II.2.3.3.1) of total soluble protein crude extracts (II.2.8.5). These results revealed that 10 out of 16 analyzed T0 SR progenies accumulated detectable amounts of scFv-ScRep1 protein in the cytosol. Further, Western blot analysis indicated these lines produced a polypeptide with the predicted size of 30 kDa. As shown in figure 38, the SR27 line showed the strongest reaction with anti-Flag antibody. In addition, these results indicated that SR15 and SR22 lines accumulate relatively high amount of ScRep1 within the cytosol. Other SR lines produced less amount of transgene varying from non-detectable level (SR6) to faint band (SR17) corresponding to a 30 kDa polypeptide.

In contrast, similar experiments showed that transgenic T0 tobacco plants expressing NLS-ScRep1, RWAV, ScRep1-GFP, HScCP2, HScCP3 and HScCP4 failed to accumulate detectable amount of recombinant proteins within the cells, although complementary PCR analysis using genomic DNA confirmed presence of transgenes in these plants.

The transgenic tobacco plants (SRG lines) producing ScRep1-GFP protein were screened by fluorescent microscopy and those expressing transgene protein were selected for next experiments.

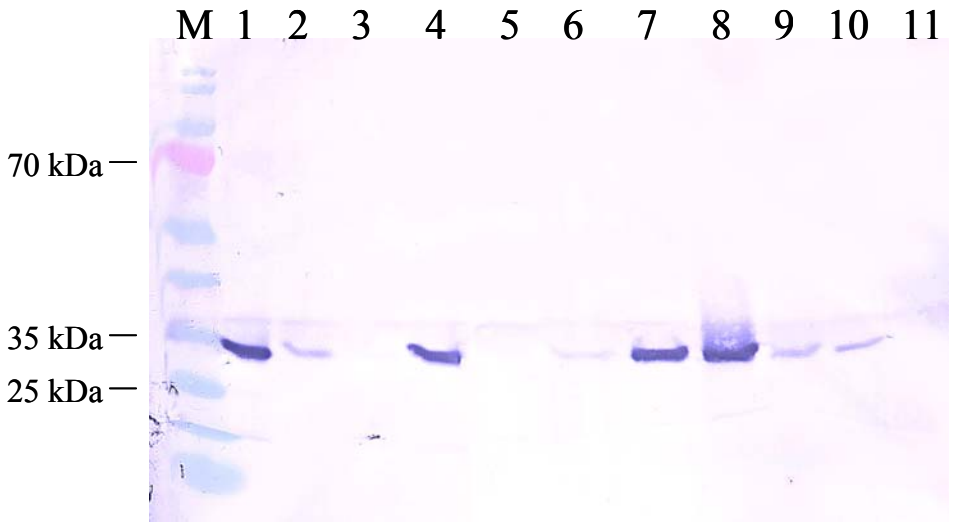


Figure 38: Western blot analysis of T0 transgenic SR plants expressing scFv-ScRep1

Approximately 15µl of crude extracts of transgenic SR tobacco plants expressing scFv-ScRep1 were used for separation on 12% (w/v) SDS-PAGE gel (II-2.3.2) and blotted onto nitrocellulose membrane (II.2.3.3.1.1). The blotted scFvs were revealed by 9E10 monoclonal antibody and GAM antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: positive control (scFv-ScRep1 expressed by transiently transformed tobacco); 2: SR4; 3: SR6; 4: SR15; 5: SR16; 6: SR20; 7: SR22; 8: SR27; 9: SR32; 10: SR17; 11: negative control; M: Pre-stained molecular weight protein marker

III.7.2 TYLCV virus resistance assay on T0 transgenic tobacco plants

Since putative side effect of TYLCV infection on plant reproduction processes, individual T0 progenies were initially allowed to grow till mature seeds were harvested. To determine protective ability of transgenes, independent T0 progenies expressing different scFv proteins were challenged with agroinfectious clone harbouring the pBIN19-2TYLCV-Ir construct. The agroinoculation was accomplished by injection of 10 µl suspension of agrobacterium culture with OD_{600nm} ~ 0.05 into the stem and petioles. Negative and positive controls were inoculated in same way by injection of water and same suspension into the non-transgenic tobacco plants. Early symptoms were appeared 3-4 weeks post inoculation (wpi) on non-

transgenic wild type and sensitive transgenic plants. The main symptoms included leaf curling and reducing of size of new emerged leaves (Figure 39-B and 39-D). Symptom developments were scored for 5 weeks and those with no clear symptoms were selected for next generation.
Initial symptom observation indicates some protection against viral systemic spread occurred within the inoculated T0 transgenic plants (Table III-6). Compared to the non-transgenic and sensitive plants that developed distinct leaf curling, these plants remained symptomless or showed a significant reduction of symptom development (Figure 39-C). These plants were selected to undergo subsequent analysis.
The presence of viral DNA in the infected plants was verified by PCR analysis using V2 viral gene (MP) specific primers (II.1.6.1). These results confirmed feasibility of PCR for detection of viral DNA within inoculated tobacco plants using either crude leaf extract or purified total DNA, it also proved its ability for detection of virus in the asymptomatic plants. To study presence of infectious clone DNA within leaves of agroinoculated plants, a complementary PCR with another primer set specific for vector sequences (II.1.6.9) was carried out. This analysis confirmed that only viral DNA is systemically dispersed in the infected plant while pBin19 vector DNA is exclusively confined within the inoculated site (figure 41).
Southern hybridization analysis was carried out to study TYLCV replication and the accumulation of its DNA within inoculated plants. For this aim, around 1 µg of total DNA extracted from tobacco plants was separated by agarose gel electrophoresis. The bands were blotted onto positively charged membrane (II.1.3) and hybridized with digoxigenin-labeled DNA probe. The specific viral DNA bands were immunoenzymatically revealed by anti-digoxigenin AP conjugated Fab fragments with luminol-based chemiluminescence detection system (Lumi-Imager F1^{TM}- Roche. The Southern blotting assay confirmed that T0 asymptomatic plants accumulate non-detectable amount of viral (figure 42).
Alternatively, TAS-ELISA analysis was performed for detection of TYLCV particles within the infected plants. These results indicated that its application is restricted for detection of virion particles within the old infected plants showing severe symptoms and it failed to distinguish healthy plants from infected tobacco with mild symptom containing low virus titre (data not shown).
Table III-6 represents the results obtained from T0 progenies virus resistant assays. The plants without clear symptoms, 5 weeks after inoculation, with low or no of viral DNA accumulation assigned as resistant plants. These results indicated that all inoculated NSR, HSC2, HSC3 and HSC4 lines as well as wild type plants showed clear symptoms and represent no resistance

against TYLCV challenge. However, SRG, SR and RW lines revealed varying degree of resistance from 8-28 percent.

The individual T0 tobacco lines showing protection against TYLCV infection (table III-6) (e.g. tobacco lines G28, R14) were subjected for next generation establishment.

Table III-6: Analysis of wild type and transgenic T0 *N. benthamiana* plants challenged with TYLCV agroinfectious clone

Transgenic line (T0)	**Total Nr. Of regenerated plants**	**No. of uninfected plants / total No. of inoculated plants (5 wpi)**	**Resistance (%)**
Wild Type	—	0/15	0 [a]
SR	25	2/25	8
NSR	25	0/31	0
RW	31	2/25	8
HSC2	26	0/15	0
HSC3	7	0/7	0
HSC4	4	0/4	0
SRG	29	**7/25**	**28**

[a] Percentage of plants not infectd 5 weeks post inoculation/ total number of plants inoculated, Previously selected on kanamycin

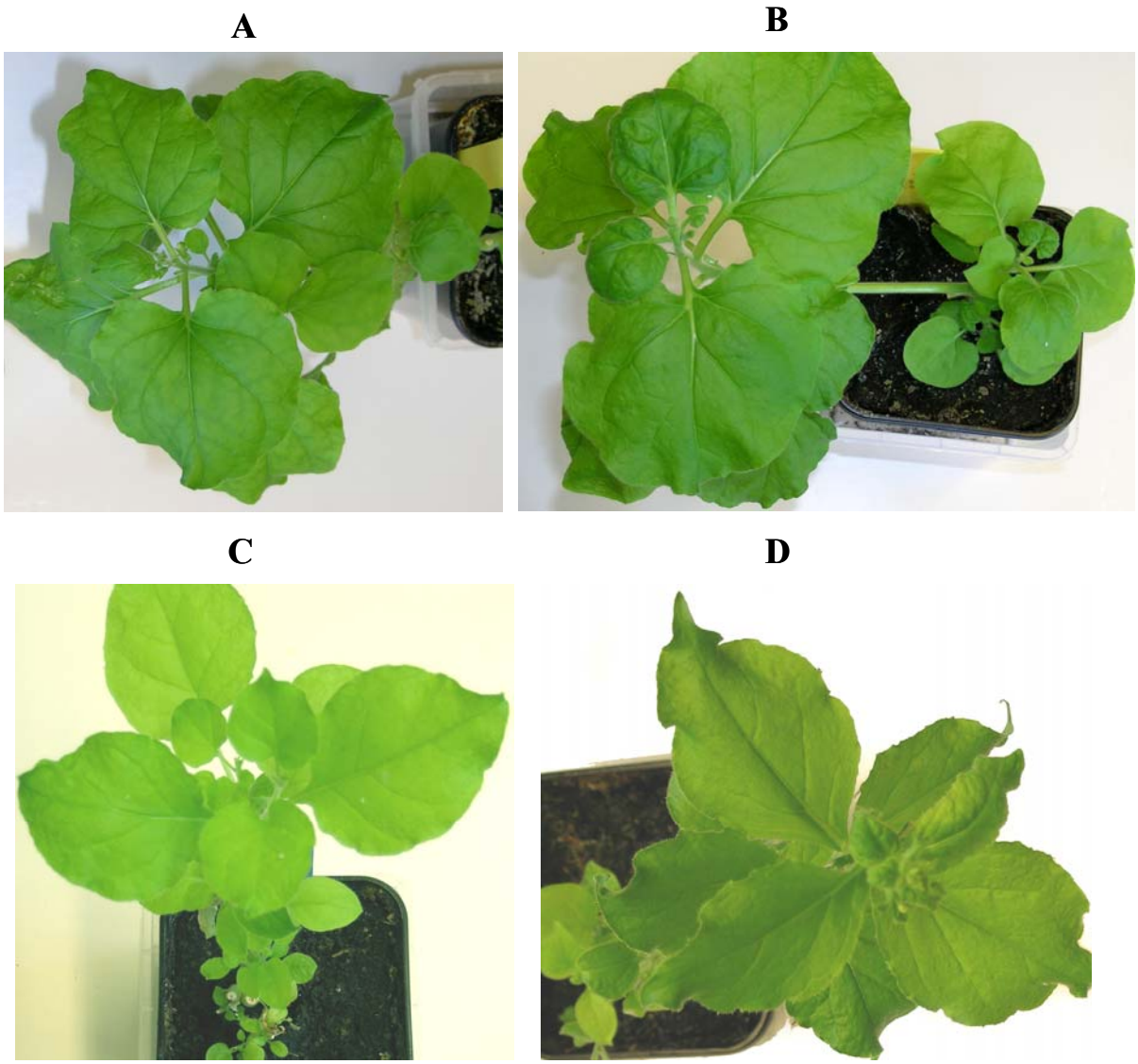


Figure 39: Resistance of T0 transgenic plants (*N. benthamiana*) to TYLCV infection.

Wild type and transgenic T0 tobacco plants expressing specific scFv fragments were challenged by TYLCV agroinfectous clone. Non-transformed plants (B) and susceptible transgenic plants (D) showed typical disease symptoms of virus infection, including curling of new emerged leaves; whereas the resistant transgenic plants (C) are free of disease symptoms. The figures show the symptoms at about 5 weeks after inoculation. A: wild type non-transgenic tobacco plant injected by virus free suspension; B: Inoculated wild type non transgenic tobacco plant as a positive control; C: Resistant transgenic plant from SRG28 line; D: Susceptible transgenic plant from SRG17 line.

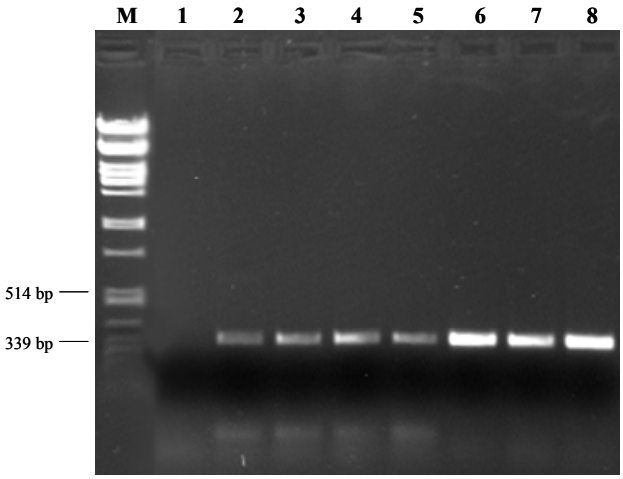


Figure 40: Detection of viral DNA sequence within the inoculated tobacco.

The PCR analysis was carried out using TYLCV V2 gene specific primer (MP) with total DNA purified (II.8.7) from tobacco plants 4 weeks after inoculation. The PCR product was subjected on 1.2% agarose gel electrophoresis. 1: negative control (wild type tobacco not-inoculated); 2: SRG28 line; 3: SRG36, 4: SRG18; SR27; 6: RW14; 7: SRG2; 8: positive control (wild type tobacco inoculated by TYLCV); M: λ DNA marker.

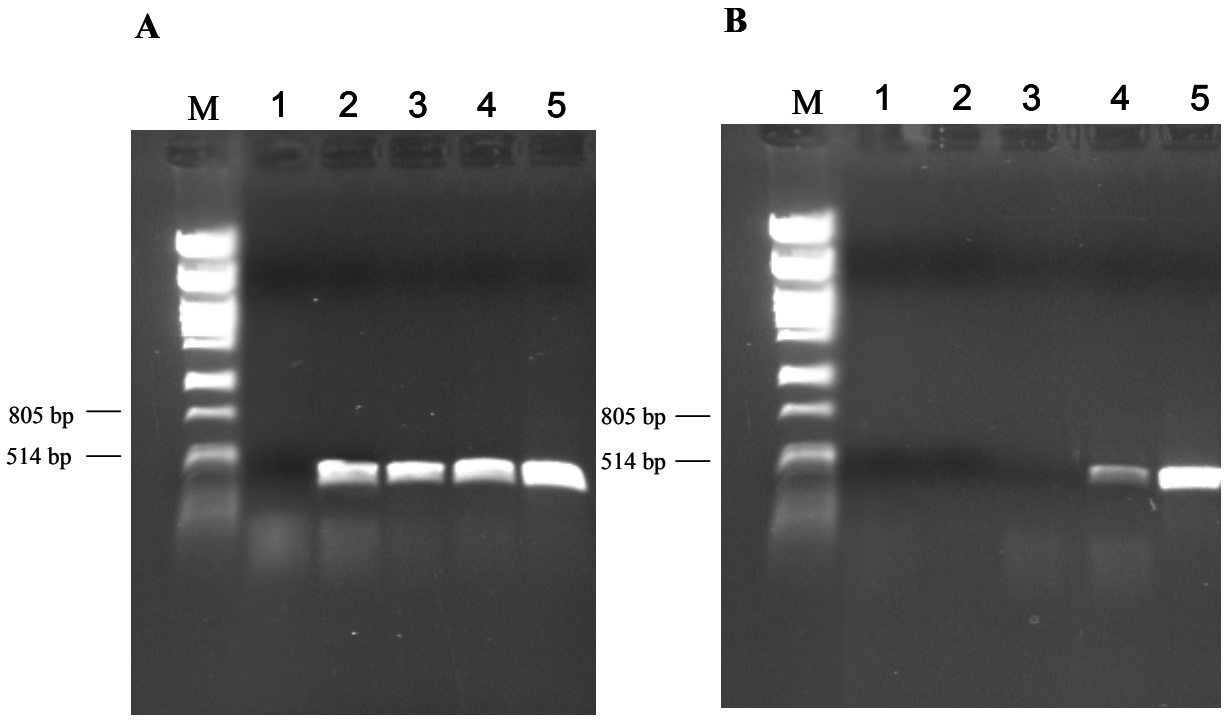


Figure 41: Tracing of viral and agroinfectious clone DNA within the agroinoculated tobacco plant.

A wild type nontransgenic *N. benthamiana* plant was inoculated by leaf infiltration (II.2.8.3.1) with agrobacterium harbouring pBIN19-2TYLCV infectious clone. The plant kept for 4 weeks and distribution of viral and vector DNA in the plant was studied by PCR analysis using specific primers.

The figure A shows the presence of viral DNA within different leaves of entire infected plant using TYLCV V2 gene specific primer, whereas in the same leaves, figure B reveal presence of pBIN19 sequence by specific primer set bracketing 401 bp of T-DNA. The PCR product was subjected on 1.2% agarose gel electrophoresis. 1: negative control (wild type tobacco not-inoculated); 2 and 3: symptomatic leaves emerged after agroinoculation; 4: leaf tissue around the inoculation site; 5: positive control (pBIN19-2TYLCV DNA); M: λ DNA marker.

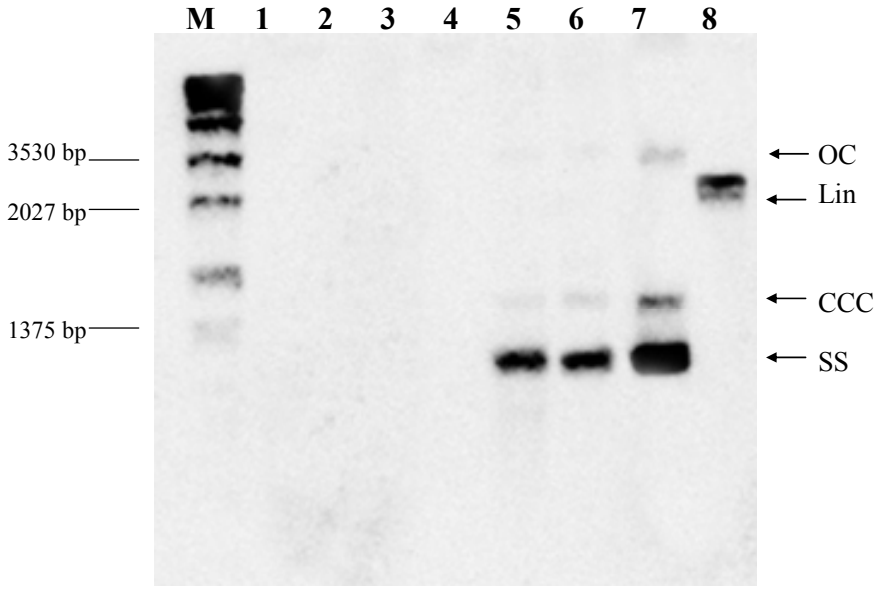


Figure 42: Hybridization assay to determine TYLCV DNA accumulation in inoculated T0 transgenic tobacco plants.

Southern blot of DNA isolated from transgenic plants (II.8.8) expressing scFv was performed. Total DNA from tobacco plants was extracted (II.8.7) 5 weeks after inoculation with TYLCV agroinfectious clone. About 1 µg of total DNA were separated on 1% agarose gele, transferred to Hybond-N positively charged membrane and hybridized with digoxigenin labeled TYLCV specific DNA probe. 1: negative control (DNA purified from not-inoculated plant); 2, 3 and 4: total DNA extracted from asymptomatic resistant lines RW14, SR27 and SRG28, respectively. Lanes 5 and 6 represent viral DNA accumulation in the susceptible transgenic lines SRG32 and SR15. 7: accumulation of viral DNA in wild type nontransgenic plant. 8: linearized TYLCV DNA by *Nco*I digestion of pBIN19-2TYLCV. M: Dig-labled, DNA molecular weight marker III. The replicative forms of TYLCV are marked by; OC: open circular dsDNA; Lin: linear dsDNA; CCC: covalently closed circular dsDNA; SS: single stranded DNA

III.7.3 Challenging of T1 transgenic plants with TYLCV

To obtain T1 putative independent transgenic plants, seeds derived from self-pollination of the resistant T0 lines were grown on MS medium supplemented with kanamycin. Non-transgenic seedlings were screened out by analysing of T0 seeds for tolerance to the kanamycin. Due to presence of *nptII* gene within the integrated T-DNA, non-transgenic T1 plants were screened for kanamycin resistance. Subsequently, resistant seedlings were transplanted to soil and kept in greenhouse. Alternatively, the SRG transgenic T1 progenies expressing ScRep1-GFP were selected base of fluorescence emission ability (II.2.8.4).

The total number of kanamycin resistant tobacco plants per cultured seeds were calculated (Table III-7). Normally, they showed varying kanamycin resistance from 60 to 90 percent. Interestingly, all seeds raised from T0 SR22 line germinated poorly in kanamycin medium and obtained seedlings were too weak for transplantation to the soil and further assays.

Of each T0 line, fifteen independent putative T1 transgenic progenies in 5-8 leaf stage were selected and tested for their ability to resist TYLCV infection. For this aim, a fresh agrobacterium culture harbouring a dimer of a fully infectious copy of the TYLCV-Ir genome, was made on petri dish containing YEB medium and relative antibiotics (II.2.1.12.2). To inoculate tobacco plants, the apical part of plant was removed and a pipet tip contaminated with agrobacterium culture was rubbed on the wounded surface. The excised apices were stored for subsequent molecular analysis.

Hereafter, plants were weekly observed for disease symptoms and assayed for presence of TYLCV DNA through molecular hybridization method (II.8.8).

Early symptoms in non-transgenic and sensitive plants appeared 2-3 wpi and developed during next weeks. Resistance response was evaluated at 4-5 wpi bases of symptom observation and DNA hybridization assays. Routinely, four weeks after inoculation, a young, fully expanded leaf near the apex was excised from individual plants and subjected for total DNA extraction and subsequent DNA hybridization analysis. This assay repeated later for plants in which TYLCV DNA was not detected.

A

B

C

D

E

Figure 43: Resistance of T1 transgenic plants (*N. benthamiana*) to TYLCV infection.
Wild type and transgenic T1 tobacco plants expressing specific scFv fragments were challenged with TYLCV agroinfectious clone. Inoculation was accomplished on decapitated tobacco plants at 5-8 leaf stage. Non-transformed plants (D) and susceptible transgenic plants (E) showed typical disease symptoms of virus infection, including curling in new emerged leaves; whereas the resistant transgenic plants (B) are free of disease symptoms. An intermediate state could be observed with mild symptom on some transgenic plant (C). The figures show the symptoms at about 4 weeks after inoculation. A: wild type non-transgenic tobacco plant injected by virus free suspension; B: Resistant transgenic plant from SRG28-12 line; C: Resistant plant with moderate symptoms within the new emerged leaves in SRG28-5 line; D: Inoculated wild type with severe symptoms on non transgenic tobacco plant as a positive control; E: Susceptible transgenic plant from SRG28-3 line.

A heterogeneous response was obtained within inoculated T1 progenies. Upon infection, wild type and sensitive transgenic plants showed sever symptoms including curling of the leaves and their size reduction (figure 43D and 43-E). The initial inoculation results revealed that SRG T1 transgenic plants expressing ScRep1-GFP recombinant protein showed a spectrum of symptoms ranging from a sever disease to a mild one and virtual absence of any symptoms. Figure 43 illustrates different symptom statues on inoculated T1 transgenic plants in comparison to tobacco wild type in 4 weeks after inoculation. Accumulation of viral DNA moviruses, it has been shown that Rep can interact with other viral proteins like REn. This interaction has not been seen In vivo, although in Replication of TGMV DNA molecules ation of viral DNA in infected plants, i.e. plants with sever symptoms also showed accumulation of TYLCV DNA in an amount comparable to that of untransformed plants. In contrast, plants lacking disease symptoms contained no or very little accumulation of TYLCV DNA. Complimentary analysis with S1 nuclease treatment was used to distinguish viral ssDNA structure from dsDNA intermediates (figure 44, lanes 10-11). Four weeks after inoculation, some plants with milder symptoms were also obtained as they accumulated a small and variable amount of viral DNA in comparison to sensitive non-transgenic plants (G28-3). In addition, table III-7 indicates that all T1 progenies raised from RW14, RW22 and SR27, in contrast to corresponding T0 precursor showed typical TYLCV symptoms; yellowing and curling of young leaved and stunting.

Table III-7: Analysis of wild type and transgenic T1 progenies *N. benthamiana* plants challenged with TYLCV agroinfectious clone

T1 transgenic progeny	Kanamycin resistance (%)	No. of uninfected plants/ inoculated plants			Virus resistance%
		2 wpi	3 wpi	4 wpi	
Wild type	——	12/15	0/15	0/15	0[b]
SR22	0 (0/45)[a]	——	——	——	——
SR27	76.9 (30/39)	11/15	3/15	0/15	0
RW14	82.2 (37/45)	10/15	315	0/15	0
RW22	80 (32/40)	11/15	0/15	0/15	0
SRG4	90 (18/20)	13/15	4/15	2/15	13.3
SRG18	**90.4 (19/21)**	**12/15**	**8/15**	**5/15**	**33.3**
SRG27	77.7 (21/27)	13/15	5/15	3/15	20
SRG28	**85 (17/20)**	**13/15**	**9/15**	**6/15**	**40**
SRG34	86.9 (20/23)	14/15	3/15	2/15	13.3
SRG36	60.7 (17/28)	13/15	2/15	1/15	6.6
SRG42	65 (13/20)	13/15	5/15	3/15	20

[a] Number of T1 seedlings resistant to kanamycin / total number of cultured seeds
[b] Percentage of plants not infected 4 weeks post inoculation/total analyzed plants

The resistance phenotype was determined by absence or remarkable reducing of disease symptoms and a concomitant substantial reduction or complete suppression of viral DNA replication. As shown in table III-7, T1 plants developed from SRG28 and SRG18 lines revealed highest resistance.

Similar results were obtained when resistance assays were repeated. Together these results indicated that the resistance is inherited and even improved through the T1 generation (28% in T0 SRG line in comparison to 40% in T1 SRG28). The best performing lines were further analyzed.

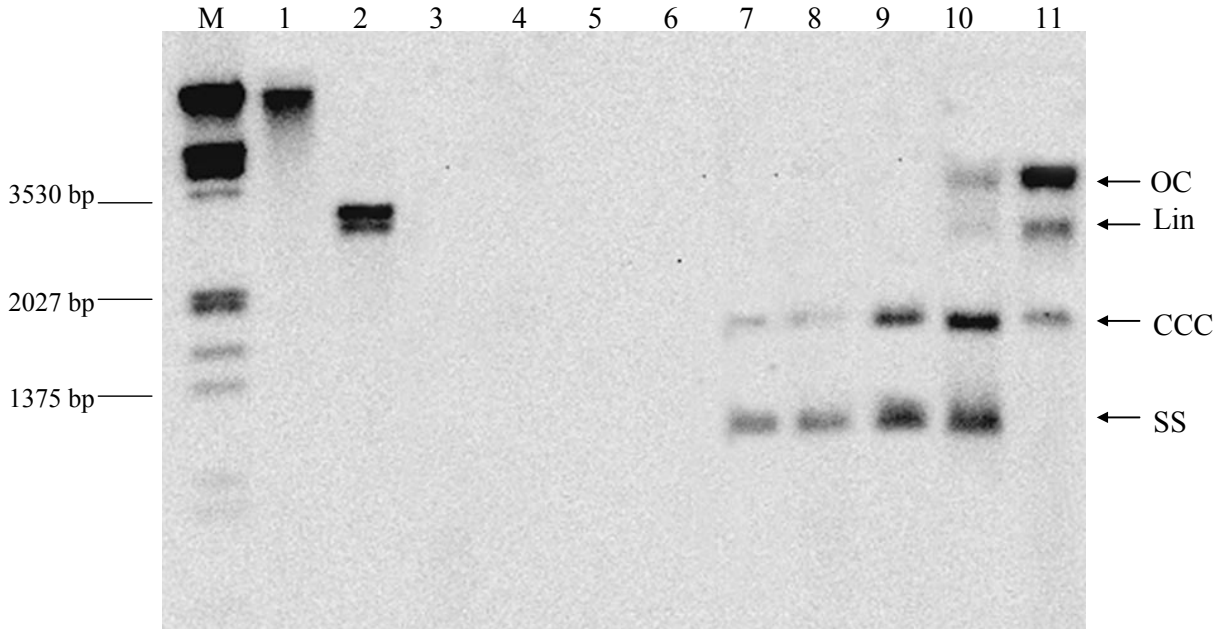


Figure 44: Hybridization assay to determine TYLCV DNA accumulation in T1 transgenic tobacco plants.

Southern blot of DNA (II.8.8) isolated from transgenic plants expressing scFv 4 weeks after inoculation with TYLCV agroinfectious clone. Total DNA from tobacco plants was extracted (II.8.7) and about 1 µg of it was separated on 1% agarose gel, transferred to Hybond-N positively charged membrane and hybridized with digoxigenin labeled TYLCV specific DNA probe. The pBIN19-2TYLCV purified DNA and linearized TYLCV released by *Nco*I digestion are shown in lanes 1 and 2, respectively. Lane 3 shows the negative control (DNA purified from not infected plant). Lanes 4-6 indicate lack of viral DNA in the resistant transgenic lines SRG28-4, SRG28-5 and SRG28-12. Lanes 7 and 8 show intermediate viral DNA accumulation within the resistant transgenic lines SRG28-7 and SRG28-9. Lanes 9 and 10 present results obtained from sensitive transgenic line SRG28-3 and wild type non-transgenic plants, respectively. The effect of S1 nuclease to remove ssDNA molecules in total DNA purified from infected plant is shown at lane 11. M: Dig-labled, DNA molecular weight marker III. The replicative forms of TYLCV are marked by; OC: open circular dsDNA; Lin: linear dsDNA; CCC: covalently closed circular dsDNA; SS: single stranded DNA

III.7.4 Comparative analysis of transgenic lines expressing ScRep1-GFP protein

III.7.4.1 Quantitative PCR analysis

The expression of the introduced ScRep1-GFP gene in T1 transgenic plants was analyzed by real-time quantitative PCR (II.2.8.6.2). It was performed using fluorescent intercalating dye SYBERgreen assay on the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Bioscience). This dye gives a specific fluorescent signal when bound to double standard

DNA. Since the dye could bind equally to aspecific PCR amplification product, the specificity of the amplification was confirmed on an agarose gele. Total RNA from wild type tobacco (as a negative control) and SRG transgenic plants was separated and subjected to cDNA synthesis. About 1 µg of total RNA was digested with DNase enzyme and used for reverse transcription reaction with pA35S specific reverse primer (II.1.6.6). The real time PCR was accomplished using specific primers located in the 120 bp of 3´ untranslated region (pA35S) of the T-DNA insert. To confirm accuracy and reproducibility of real time PCR, the assay precision was determined in three repeats within one light cycler run. The figure 45 illustrates Q-PCR results obtained from some SRG transgenic plants.

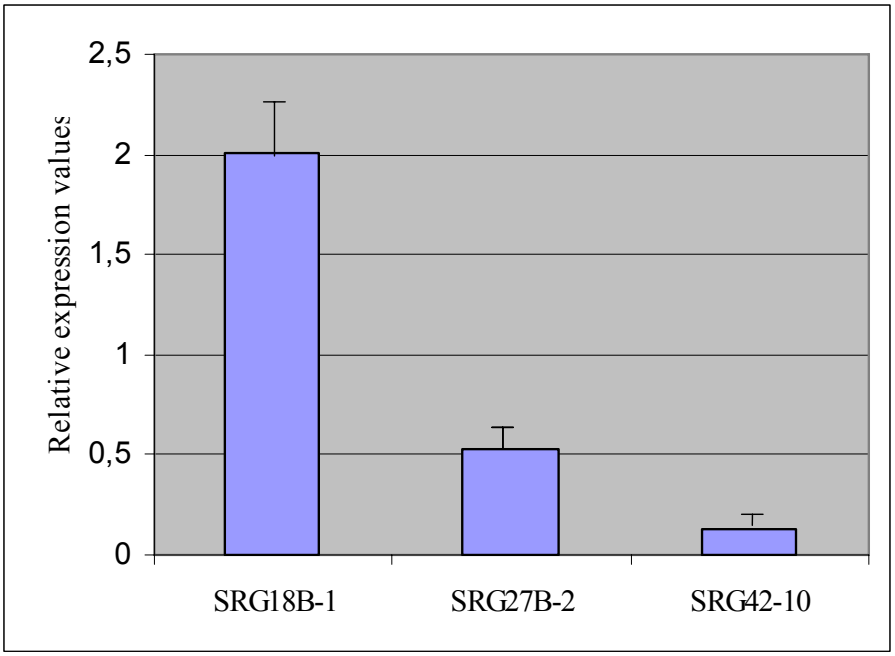


Figure 45: SYBR-Green RT-PCR analysis of ScRep1-GFP gene expression in independent SRG lines. Relative expression of ScRep1-GFP gene measured in different SRG lines. Total RNA was reverse transcribed, and aliquots were amplified using primer pairs specific for pA35S region. Data presented are means and standard deviations of three independent replicated experiments.

III.7.4.2 Fluorescence emission intensity

The comparative analysis to determine fluorescence emission ability among independent T1 SRG transgenic plants was carried out. These results revealed that individual SRG transgenic plants emit varying intensity of fluorescence under excitation by UV light (figure 46). Generally, plants expressing higher amounts of transgene have higher fluorescence emission. Together comparative quantitative RT-PCR and fluorescence intensity analysis with those obtained from virus resistant assay indicated that mostly plants with an elevated transcripts level consistently exhibited a higher degree of virus resistance.

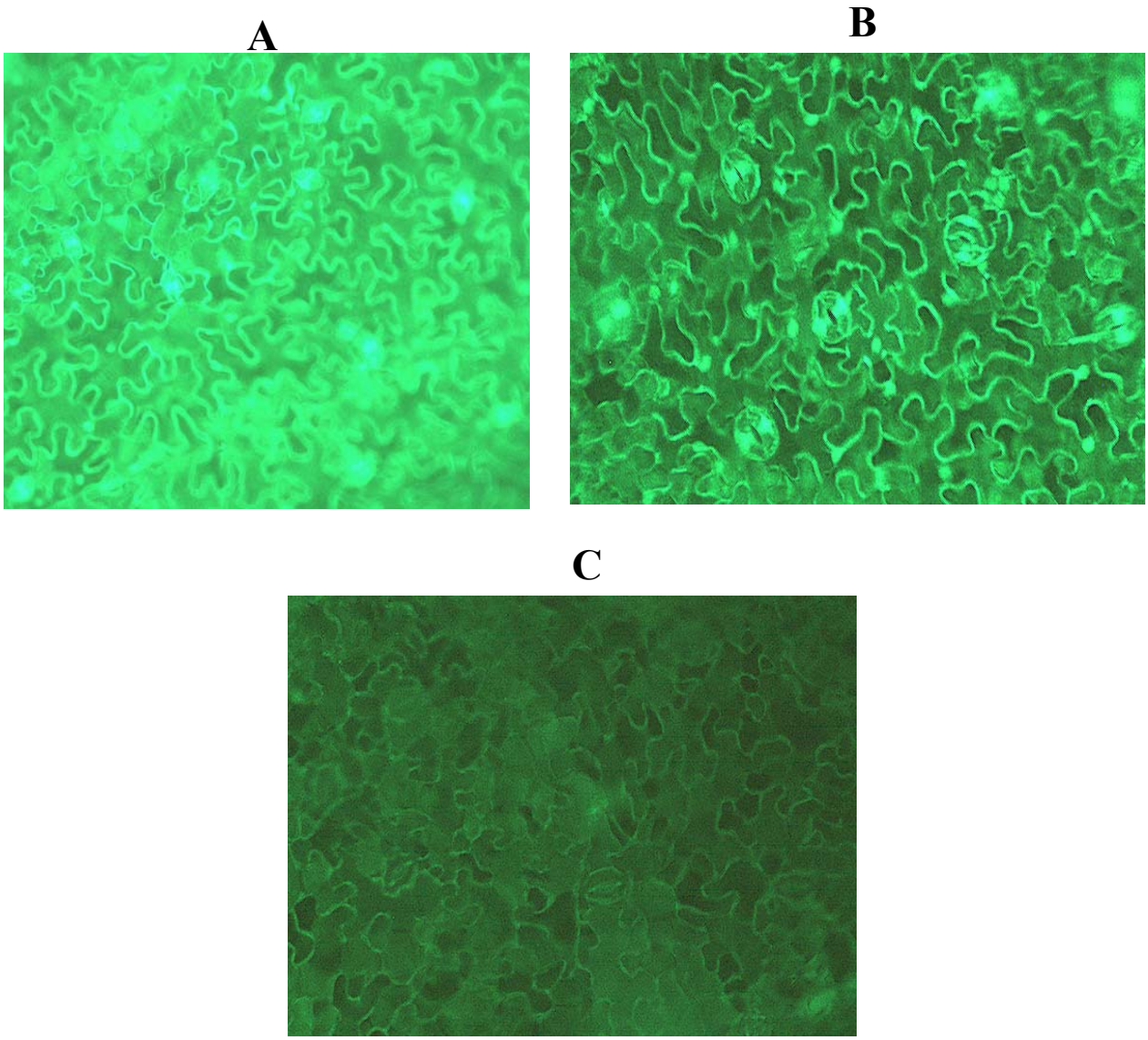


Figure 46: Comparative analysis for fluorescence emission intensity among independent transgenic plants expressing ScRep1-GFP protein.

Fluorescence emission ability in the individual T1 transgenic tobacco plants expressing ScRep1-GFP was compared. The forth leaf from top of SRG transgenic tobacco plants at 5 leaf stage was studied. Figures A, B and C represent descending rate of fluorescent emission in the SRG18B-7, SRG18B-10 and SRG27B-6 lines, respectively. GFP was excited at 488 nm and emissions collected at 500 to 515 nm.

Furthermore, similar analysis was performed in different leaves of independent SRG plants at 4-5 leaf stage. These results revealed ascending rate of fluorescence emission from leaves positioned in direction of top to beneath ones (Figure 47).

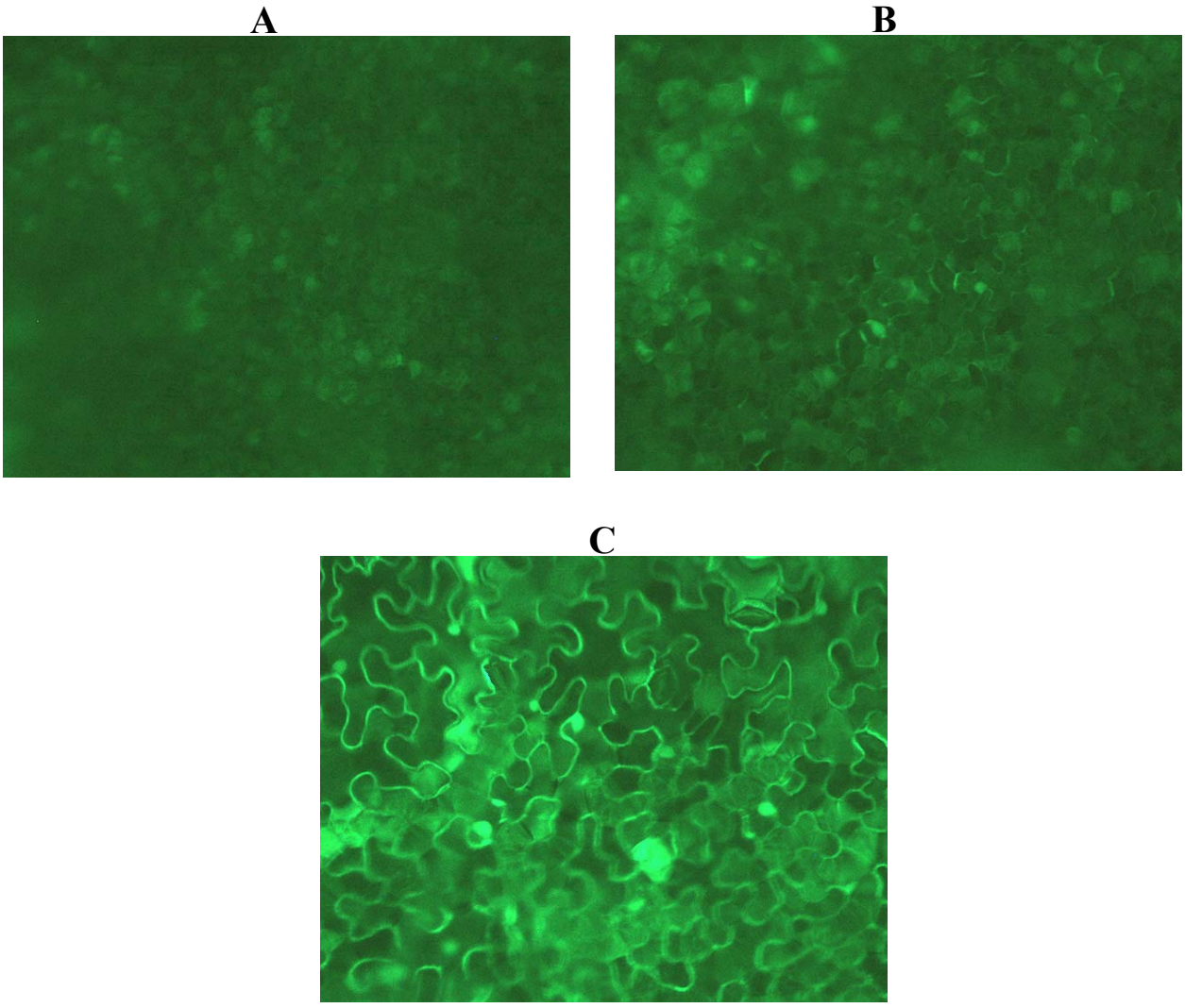


Figure 47: Fluorescence emission intensity within the leaves of individual transgenic plants.

Accumulation of ScRep1-GFP fusion protein in single leaves detached from plant line SRG28C-10 examined at 4 leaf stage. Three full expanded leaves were excised and their fluorescence emission ability was analyzed by fluorescent microscopy. A, B and C figures represent fluorescence abilities of leaves positioned at Top, middle and bottom of the main stem, respectively. GFP was excited at 488 nm and emissions collected at 500 to 515 nm.

In spite of using constitutive promoter p35S for expression of transgene in the SRG transgenic tobacco plants, in some cases, cells within the same leaf showed different fluorescence emission ability (Figure 48).

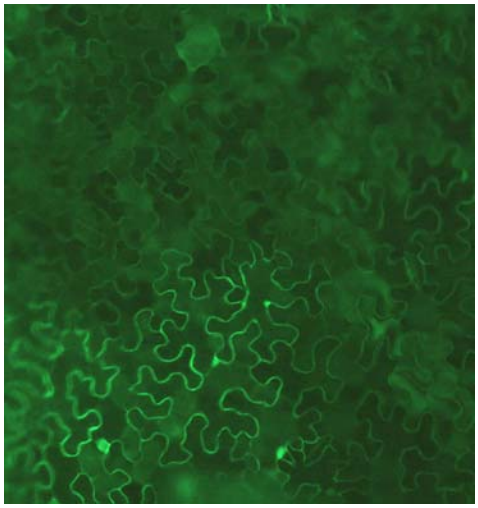


Figure 48: Non-constitutive expression of transgene within the stable transformed tobacco plant. Non-constitutive expression behavior of transgene was studied in the stable transformed tobacco plants expressing ScRep1-GFP protein. Fluorescent microscopy shows that individual cells have different fluorescence emission ability. GFP was excited at 488 nm and emissions collected at 500 to 515 nm

IV Discussion

Plant viruses are one of the most important plant pathogens, causing great economic problems in several crops throughout the word. To control these agents, conventional methods like crop rotation, early detection and subsequent removal of inoculum source, cross protection and chemical control of their vectors have been applied for several decades. Nowadays, molecular biotechnology has provided powerful new measures, such as the development of transgenic plants expressing antiviral elements, pathogen related proteins or antisense RNAs that block pathogenesis. Antibody mediated resistance is a novel approach to create plants resistant to viruses, based upon the expression of recombinant antibodies binding to viral proteins to inhibit the virus's replication in plants.

The purpose of this study was to generate and characterize specific scFv fragments acting against TYLCV functional proteins and develop transgenic plants which produce these recombinant antibody fragments. Described in this study is the development of seven different transgenic plants. Subsequent analysis included challenging of transgenic plants with TYLCV agroinfectious clone and study of viral replication in these plants.

Briefly summarized, the C1, V1 and V2 genes encoding Rep, CP and MP, respectively, were amplified by specific primers using the pBIN19-2TYLCV-Ir construct as a template. The PCR products were directly cloned into the TOPO vector (III.1.1) and successful clones were selected. Next, viral genes were sub-cloned into pGEX-5x3 (II.2.2.1) and pMALc2x (II.2.2.2) expression vectors and recombinant proteins were expressed in *E. coli* as C-terminal fusion with GST and MBP. In addition, the amino terminal part of CP and Rep proteins were also cloned and expressed as C-terminal fusions with GST and MBP.

Using phage display technology one scFv against Rep were isolated through panning of naïve Tomlinson I scFv phage library. In addition, an ARep phage display library (III.3) constructed from spleen total RNA of a mouse immunized with MBP-Rep was panned and another scFv were screened and characterized. Four more scFv were developed from total RNA of murine hybridoma cells secreting specific MAbs against TYLCV virions.

All scFv were cloned into a plant expression vector, and to perform an initial characterization were transiently transformed into tobacco leaves. Additionally, the Rep specific scFv fragments were cloned into the plant expression vector as N-terminal fusions with GFP. Expression of scFv fragment constructs and their functionality within plant cells were analysed. Next, these constructs were used to generate stable transformations in entire tobacco plants. Accumulations of scFv transcripts in plants presenting susceptible and resistant

phenotypes were investigated. Stable transformed tobacco plants were agroinoculated and protective ability of T0 and T1 transgenic progenies expressing scFv fragments was evaluated.

IV.1 Expression and purification of viral recombinant proteins

Heterologous gene expression in *E. coli* is the most frequently used expression system for high level production of recombinant proteins in molecular biology (Fani et al., 1998). Purified recombinant viral proteins were mainly used for immunization (II.2.4), panning of scFv phage library (II.2.6) and scFv characterization (II.2.7.4). To obtain TYLCV CP, MP and Rep proteins, the viral genes V1, V2 and C1 were amplified using specific primers and cloned into the TOPO vector (III.1.1). The viral genes were then cloned into pGEX5x3 (III.1.2) and pMALc2x (III.1.3) expression vectors to produce recombinant proteins fused to the carboxyl-terminal of GST and MBP. The Glutathion S-transferase (GST) and Maltose binding protein (MBP) gene fusion systems are versatile systems for expression and purification of fusion proteins produced in *E. coli*. These systems are based on inducible and high level expression of genes fused to GST and MBP proteins and allows for production of fusion proteins in one-step affinity purification under native conditions. These fusion partners can lead to an increase in solubility of target proteins (Frangioni and Neel, 1993; Hannig and Makrides, 1998). The CP and Rep protein of geminiviruses are multi-functional and their amino terminals are responsible for several critical activities. The amino terminal portion of Rep (aa 1-136) and CP (aa 1-113) were individually selected and cloned downstream of GST and MBP, as C-terminal fused proteins, in corresponding vectors. For this purpose, the amino terminals of Rep and CP in pGEX-C1 and pGEX-V1 vectors were kept and the remainder of the gene in both cases was removed by restriction and subsequent ligation (III.1.2). The amino-terminal fragment selected from Rep contains the cleavage, linkage and DNA binding domains activities while for CP this part is responsible for nuclear localization.

Over-expression of CP, NCP and Rep proteins in both expression systems suggested that a high proportion of unfolded and insoluble fusion proteins aggregated within inclusion bodies that were mostly removed with cell debris. To improve yield of soluble and correctly folded proteins, the culturing and induction conditions had to be optimized. To this aim, several modifications, such as decreasing the IPTG concentration to 0.25 mM, increasing induction time up to 6 hours and reducing the temperature to 22°C after induction, were applied. These optimizations resulted a decrease in protein expression and the produced fusion proteins were allowed sufficient time to fold correctly, therefore limiting accumulation inactive protein

aggregates, i.e. inclusion bodies, without reducing the final yield of the target protein. The positive effect of low temperature to reduce inclusion body formation has been demonstrated in many studies (Bishai et al., 1987; Schein, 1989). While low temperatures between 15-20°C have a slight effect on cell folding machinery, they significantly decrease the level of transcription and translation (Betton et al., 2002; Hunke and Betton, 2003) and as a result most of translated proteins are correctly folded.

SDS-PAGE and western blot analysis showed high integrity for GST/MBP-MP, but the majority of the purified GST-CP and GST-Rep were present as degraded proteins (Figure 3). Several attempts were carried out to optimize expression and purification conditions but they were unable to prevent this loss of integrity. Degradation of recombinant proteins is typically caused by proteolitic activities of bacterial enzymes within the cell (Baneyx and Mujacic, 2004) or the effect of rare codons and premature termination of heterologous gene expression (Ivanov et al., 1997). Proteolysis is a regulated process that is involved in several metabolic activities, such as the removal of incorrectly folded and abnormal proteins (Hannig and Makrides, 1998; Baneyx and Mujacic, 2004). Heterologous gene containing high levels of rare codons that are used at low frequency in *E. coli* and often leads to low levels heterologous protein expression or truncated products because of premature stops in protein translation (Ejdeback et al., 1997; Ivanov et al., 1997). In MBP fusion system, purified MBP-Rep was visualized as two major bands in SDS-PAGE analysis (Figure 4, line 4), suggesting a cleavage site located downstream from the oligomerization domain of Rep, which is likely a target for enzyme mediated proteolysis (Gronenborn, unpublished data).

Because of degradation and low level expression occurring in production of entire CP and Rep fusion proteins, constructs for expression of N-terminal parts of CP and Rep were used. As a result, a higher efficiency in purification of NCP and NRep fusion proteins was obtained. While purified GST/MBP-NRep were visualized as unique bands of the expected size on SDS-PAGE, some degradations were still present in purified GST/MBP-NCP (Figures 3 and 4).

IV.2 Mice immunization

To obtain specific antibody from a cloned immunological repertoire, a large and diverse library as well as an efficient selection procedure are required. The key to achieve this goal is the generation of a good immune response, isolation of high quality RNA and efficient cDNA synthesis from which the library constructed.

To elicit a strong immune response against viral Rep protein, three mice were immunized with MBP-Rep fusion protein by repeated injection of small doses of antigen (50 µg). After each injection the antiserum titre was measured by indirect ELISA. A high antiserum titre against the injected antigen usually indicates enrichment of an antigen-specific cell clones. The boosting continued until antiserum titre reached over 1:150,000 (III.2). To confirm the presence of antibodies against the viral antigen, Rep fusions (like GST-Rep) different from those used for immunization was also used for titration of serum polyclonal antibody.

IV.3 Phage displayed scFv fragments selection

To select specific scFv, two naïve Tomlinson scFv-phage display libraries, I and J, as well as an immunized ARep scFv library were panned. Phage display is a powerful means for obtaining specific peptides with high binding properties from a huge number of variants. There is a direct link between DNA sequences and protein function, and so from the single experimental setup the specific single clones can be rapidly selected from vast pools (Winter et al., 1994; Conrad and Scheller, 2005).

MP, CP and Rep recombinant fusion proteins were used to select specific binders with high affinity from the Tomlinson I and J naïve libraries. The size of Tomlinson libraries were approximately 10^8 clones. To drive highly specific antibody fragments to a wide range of different antigens like polypeptides, polysaccharides, toxins and other small molecular compounds, many naïve phage display libraries have been successfully established. To create these naïve phage display libraries, variable gene sequences that have undergone some in vivo rearrangement are derived from the IgM mRNA of un-immunised animals (Willats, 2002). Fusion proteins could immobilize as antigens onto plastic immuno-tubes, and apply for selection of specific binders (Vaughan et al., 1996). To exclude false specific binders against fusion partners (rather than the proteins themselves), MBP fusion proteins were used in the 1st and 3rd round of panning while in the 2nd round GST fusion proteins were applied.

Having highly purified target proteins or molecules facilitates selection of specific binders from a diverse phage library. Limited amounts of target antigens presented in the impure mixture increases trapping of non-specific phages (Hoogenboom et al., 1998). The enrichment of specific scFv fragments was accompanied by monitoring the amount of input and output phage in each cycle (Table III.1). In the third round of panning against immobilized Rep, the number of eluted phages was increased several folds in comparison to the number of phages eluted in the first round. This indicates population of specific binders has been raised during panning rounds. After the third round of selection, more than 70% of the analyzed clones

showed specific activity with the Rep protein (Figure 11). Some Rep binding clones were selected and amplified by specific primers and subjected to *Bst*NI digestion. RFLP fingerprinting showed similar restriction patterns for all clones (Figure 12). Sequencing results confirmed identical DNA sequence for all Rep specific clones which were named scFv-ScRep1. Phage display technique was also used to select specific binders to MP and CP recombinant proteins through panning of both naïve scFv phage Tomlinson display libraries. Despite the high purity of the target proteins used in the process and repeated attempts, no specific binder was obtained after three rounds of panning.

The ARep scFv library, unlike the naïve libraries, was generated from mice immunized against MBP-Rep protein. Immunized libraries containing a large population of specific binders against corresponding antigen are usually used to select antibodies with higher affinities from an equivalent library size (Bradbury and Marks, 2004). To construct such a library, total RNA was extracted from spleen cells of an immunized mouse and used to generate cDNA from the rearranged immunoglobulin transcripts with oligo dT primers. The degenerate sets of primers annealing to conserved domains of framework regions 1 and 4 of variable fragments were used to amplify V_H and V_L regions of immunoglobulin repertories. To clone these fragments, suitable restriction enzyme sites were incorporated at the end of amplified genes through PCR amplification. These restriction sites, including *Sfi*I, *Bst*EII, *Asc*I and *Not*I, are rarely found in the antibody sequences, limiting the possibility of potentially interesting sequences being removed through the internal digestion. The scFv phage library was made by cloning of V_H and V_L fragments individually into the pHENHI vector (Figure 8). The colony check PCR using randomly selected clones showed that they carried full size scFv (Figure 9). The RFLP finger-printing assay (III.3.3) through *Bst*NI digestion indicated diversity around of 75% (Figure 10). Due to separation and subsequent randomly combination of productive pairs of variable heavy and light chain during the cloning process, large antibody libraries are needed to guarantee that most original specific V_H and V_L pairs are present in the constructed library (Gherardi and Milstein, 1992; Posner et al., 1994). Further sequencing results verified that some clones contained full length scFv with the expected size but about half of them contained frameshifts and failed to produce entire scFv. Because of the use of degenerate primers for PCR amplification of antibody variable fragments, mismatches, point mutations and other errors can occur within the amplified DNA and lead to production of non-functional scFv molecules (Krebber et al., 1997). The plasmid carrying non-productive, aberrant or truncated antibody fragments encoding sequences have a growth advantage over the clones expressing entire scFv and are

often preferred by the *E. coli* host and cause problems during the enrichment of antigen-binding antibody sequences by phage display (Seehaus et al., 1992; Dziegiel et al., 1995). These events show the importance of the high stringency for washing procedures during the panning to remove scFv-free phages (Tur et al., 2001).

Affinity selection of specific scFv was performed as for the Tomlinson libraries and after the third round of panning one clone with high binding activity to Rep was selected and designated scFv-ScRep2.

Sequence analysis of specific Rep binding scFv fragments selected from naïve and immunized phage display libraries, scFv-Rep1 and scFv-ScRep2, showed that both scFv fragments were in frame with the cloning module. Sequence alignments with IMGT database (http://imgt.cines.fr/IMGT_vquest/) showed that their heavy and light chains variable domains are members of identical groups (Table III.3).

The reaction of scFv-ScRep1 and scFv-ScRep2 with corresponding antigen in two different fusion systems (GST/MBP-Rep) confirm their specificity and suggests that the antigen folding was not affected by the fusion partner. No activity with GST and MBP was observed with these scFv fragments (Figure 16).

The scFv-Rep1 reacted with both Rep and NRep recombinant fusion proteins through ELISA and Western blotting assays (Figures 16 and 17) indicating that its epitope is located in the amino-terminal portion of the Rep protein. The N-terminal of Rep contains cleavage, linkage, DNA binding and oligomerization domains which are responsible for some critical activities of Rep during viral replication (Gutierrez, 1999).

ELISA results (Figure 16) showed that the scFv-ScRep2 only reacted with entire Rep recombinant protein, and not with C-terminal truncated Rep (NRep). This indicates that scFv-ScRep2 could not bind to the amino-terminal portion of Rep. Western blot analysis revealed that it could still bind to degraded partes of C-terminal truncated MBP-Rep (Figure 18B-Line 1) which are larger than NRep and must therefore include some additional portion of intact Rep. Together, these results suggest that scFv-ScRep2 binds with the middle part of Rep, which is responsible for the oligomerization activity of Rep. Since it has been shown that some critical aspects of geminivirus replication are entirely related to the oligomerization function of Rep (Orozco et al., 2000), this scFv seems to be an interesting candidate.

IV.4 Expression and purification of scFv fragments

The pHENHI and pIT2 expression vectors were used for large scale expression of scFv fragments. An important part of production of scFv fragments in *E. coli* is their secretion to

the periplasmic space, as it permits the production of soluble and functional proteins with correctly formed disulfide bonds in oxidative conditions. Phagemid pIT2 and pHEN series vectors have been engineered to express soluble scFv fragments into the culture medium by insertion of a *pel*B leader sequence upstream of the scFv gene and an amber stop codon between the scFv and gene III. In this system, high amounts of soluble scFvs can be produced in non-suppressor *E. coli* strains and secreted into the periplasmic space. The selected clone from Tomlinson I phage library producing scFv contained pIT2-scFv-ScRep1 construct and was initially used for large scale expression and purification of scFv-ScRep1. To provide an alternative for purification, scFv-ScRep1 was cloned into pHENHI vector. The pIT2-scFv-ScRep1, pHENHI-scFv-ScRep1 and pHENHI-scFv-ScRep2 constructs were transformed into HB2151 strain of *E. coli* and soluble scFvs were produced by induction with IPTG. The secreted scFv was released from the periplasmic space by osmotic shock and subjected to IMAC purification system under native conditions. The SDS-PAGE result showed distinct bands with the expected molecular weight of about 30 kDa (Figure 15). The total yield of purified scFvs was relatively small; about 0.5 mg per litre of culture medium. Previous studies have shown that bacterial expression systems secreting scFvs into periplasmic space yields from 0.1-2 mg per litre culture medium, depending on properties of the variable domain structure and structure of scFv (Plückthun and Riesenberg, 1996). Expression and purification of scFv-ScRep1 using pHENHI rather than pIT2 vector led to higher total yield. It seems probable the discrepancy is due to different position of His_6 tag in the two constructs. The scFv expressed from pIT2 vector contains a His_6 tag between the scFv and the *c-myc* tag (Figure 45) which may account for its poor affinity binding in IMAC purification.

The scFvs were diluted from Ni-agarose matrices with buffer containing 200 mM Imidazole. To remove the Imidazole from the purified scFvs, they were dialysed with cold PBS solution. During the dialysis a lot of white precipitate was observed in the tube, which was removed by centrifugation. The SDS-PAGE analysis indicated that the precipitate was scFv. Dialysis using PBS buffer containing 15 mM Imidazole reduced precipitate formation and increased total scFv yield (Figure 15-B). It has been proven that presence of Imidazole does not interfere with scFv binding activity and can increase its solubility (Hamilton et al., 2003). In addition, old and precipitated scFv can be re-dissolved in this buffer without loss of antigen binding activity. Neither salt bridge nor cross-linking of His_6 tails mediated by metal ions leached from the column during elution are responsible for the limited solubility of the protein in the absence of Imidazole (Hamilton et al., 2003).

IV.5 Purification and characterization of MAbs against TYLCV virion

The hybridoma clones HTYLCV1, HTYLCV2, HTYLCV3 and HTYLCV4 secreting monoclonal antibody specifying TYLCV virions were provided by Dr. S. Winter from DSMZ, Braunschweig, Germany. These cells were individually cultured and their supernatants were subjected to affinity purification using Protein A matrix. Around 4mg of purified antibodies were obtained from one litre of hybridoma culture supernatant. These monoclonal antibodies were used for their binding activities against recombinant fusion CP. Western blotting and ELISA assays showed that only MAb HTYLCV1 had high specificity and reactivity with entire CP fusions, but it did not recognize NCP protein which suggests that its corresponding epitope is not present in the amino-terminal. The high binding activity of HTYLCV1 against denatured CP in Western blotting suggests the epitope in question is linear. The carboxyl half of CP in begomoviruses contains highly conserved amino acids and most variation takes place in their amino terminal (Harrison et al., 2002). This MAb could, then, present a good choice for detection of several begomoviruses in infected plant material by immunoassays. The SDS-PAGE analysis of purified MAbs showed two major bands of approximately 55 and 26 kDa corresponding to the mouse heavy and light chain, but interestingly the HTYLCV1 heavy chain was smaller than others (Figure 19).

IV.6 Cloning of specific scFv fragments from hybridoma clones

To generate scFv from the hybridoma clones, the total RNA was extracted from hybridoma cells and used to generate cDNA. The isotype of MAb heavy and light chains were initially determined by ELISA using specific antibodies recognizing different constant regions. All constant regions of heavy and light chains belonged to IgG2b and κ groups, respectively (Figure 20-A). cDNA templates were then constructed using COH32 and MuPD31 primers by reverse transcriptase. The heavy and light variable regions were amplified individually using degenerate primers from cDNA template and cloned into pHENHI vector following the same strategy used for the construction of ARep phage library. Ten clones from each cloning reaction were randomly selected and sequenced. The sequencing results indicate some variations among the variable domain sequences obtained from identical hybridoma lines. It has been shown that aberrant mRNAs transcribed from rearranged, but non-functional, heavy and light chain genes in the hybridoma cells cause major difficulties for obtaining specific sequences. Furthermore, the adventitious templates are preferentially amplified over the complete ones by specific primers for the variable regions of antibody genes and may greatly dilute the desired antibody sequences (Ostermeier and Michel, 1996). After synthesis of a

functional imuunoglobulin molecule, the rearrangement process in the corresponding loci is terminated while the second allele of the B-cells with aberrant rearrangements produces many non-binder transcripts. As such, both functional transcripts producing mature immunoglobulin chain and aberrant mRNA containing stop codons or frameshifts are obtained. Some hybridoma lines are developed by fusion of myeloma cell with more than one B-cell that produces both functional and non-functional heavy and light chains. These in-frame but non-specific sequences can not be distinguished from the binding chain by sequencing (Cabilly and Riggs, 1985; Yamanaka et al., 1995; Ostermeier and Michel, 1996; Krebber et al., 1997). Sequencing results indicate that some variation among V_H and V_L sequences raised from identical clones took place primarily in framework regions 1 and 4. Some clones showed a few nucleotide changes within the middle segments of variable regions. It seems that the former variation arises from degenerate primes used for amplification of variable domains while the latter is the result of PCR error normally occurring in amplification. These results imply each hybridoma line contains a unique template which is responsible for production of functional immunoglobulin transcript.

While many differences are present in frameworks 1 and 4, specific clones with high binding activity to corresponding antigen should be selected. To select such functional clones, experiments like antigen binding activity of produced antibody have been successfully recommended (Krebber et al., 1997). The framework regions make up the conserved β-sheet domains which are responsible for the main-chain conformation of the CDRs and the interchain interactions responsible for bringing domains together (Chothia et al., 1985). The framework residues have been demonstrated to influence the conformation of CDR loops and thereby alter binding characteristics (Foote and Winter, 1992). In many cases, PCR-induced sequence changes in the framework regions resulted in impaired antigen binding, poor production yield and decreased thermodynamic stability (de Haard et al., 1998). Since HTYLCV-1, HTYLCV-2 and HTYLCV-3 lack binding activity against recombinant CP, traditional phage display approach is unable to select specific clones. Selection of clones was carried out based off comparison to other similar sequences and to their corresponding groups and those harbouring preserved residues with highest similarity to its group were selected and used to generate scFv. Alignment results of V_H and V_L nucleotide sequence (Table III-5) showed that most similarities are in the V_H fragments, as three out of four belonged to the IGHV5 group while the V_L fragments were distributed among 3 groups.

Sequence comparison of V_H fragments raised from four different clones revealed that V_H-HTYLCV1 is considerably smaller than the other clones. This is in consensus with SDS-

PAGE results of purified MAbs (Figure 19) indicating lesser molecular weight of the HTYLCV1 heavy chain. In addition, agarose gel electrophoresis results (Figure 23) confirmed that amplified V_H fragments have a shorter V_H-TYLCV1 length. The alignments of nucleotide sequence of V_H-HTYLCV1 with IMGT (http://imgt.cines.fr/IMGT_vquest/) and KABAT (http://www.ncbi.nlm.nih.gov/BLAST/) databases revealed a 39 bp deletion took place after position 281 located within FR 3 and CDR3 downstream from the original AUG start codon in *Nco*I site. Sequencing data from different clones harbouring the V_H-TYLCV1 showed the same situation - indicating deletion was not introduced during amplification of variable regions. Furthermore, amino acid alignments indicate their sequences after the deletion site are no longer similar to other members of the group and following on the same frame led to a stop codon at position 366 in linker region (Figure 24). These results indicate a probable frameshift within V_H-HTYLCV1. To overcome this problem, based off alignments results, two strategies were attempted (Figure 24). In the first, the frame was modified by deletion of one A at position 258 within framework region 3 using site directed mutagenesis kit and the new fragment was named V_H-HTYLCV1-1. The sequence of the heavy chain variable region anti-DNA quadruplex antibody (AAC35990.1) (Brown et al., 1998) in the database, with 95% homology, had most similarity to this sequence. As an alternative, the frame was restored by deletion of one A at position 296 within the framework region 4 and it was named V_H-HTYLCV1-2. It had more similarity to the sequence of anti-activating transcription factor 1 Ig variable heavy chain (AAC40156.1) (Bosilevac et al., 1998) with 95% homology.

To generate entire scFvs, all V_H fragments were cloned into corresponding pHENHI-V_L and new constructs were named pHENHI-HTYLCV1-1, pHENHI-HTYLCV1-2, pHENHI-HTYLCV2, pHENHI-HTYLCV3 and pHENHI-HTYLCV4. These constructs were transformed into HB2151 strain of *E. coli* followed by periplasmic expression. Blotting analysis confirmed integrity of periplasmic scFv fragments expression with expected molecular weight between 27-30 kDa (Figure 26).

The binding activity of produced scFv fragments against recombinant CP was carried out by ELISA and Western blotting. These results indicated that scFv-HTYLCV2, scFv-HTYLCV3 and scFv-HTYLCV4, like their MAbs precursors, could not bind to fusion CP. Despite high binding activity of MAb HTYLCV1, the scFv-HTYLCV1-1 showed low specificity. The reason for low specificity of scFv developed from MAb-HTYLCV1 is unclear. It could be due to miss-folding of variable fragments in absence of constant regions or, probably, that the right frame has not been restored after modification procedure.

IV.7 Expression and characterization of recombinant proteins in transiently transformed plants

DsRed is a red-emitting auto-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; 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). For this purpose, the C1 gene was cloned into pTRAkt plant expression vector under control of the 35S promoter as an N-terminal fusion with the DsRed gene. The N-terminal fusion of C1 guarantees that all the fluorescence will be a result of the fusion expression and not the DsRed alone. The resulting pTRAkt-C1-DsRed was sequenced and used for transformation into GV3101 strain of *A. tumefaciens*. Ten independent recombinant colonies were screened for presence of insert by colony check PCR using pSS specific primers but no positive clone harbouring the construct was identified. The transformation was repeated several times but still no transformed clone was recovered. This may indicate toxic activities of fusion protein for agrobacterium cells. The Rep NTP binding domain (P-loop) could interfere with agrobacteria growth (Gronenborn, unpublished data). To remove amino acids within the Rep NTP binding domain, the pTRAkt-C1-DsRed construct was digested by *Eco*NI/*Sfi*I and 294 nucleotides placed between 629-926 bps were cut out followed by modification and blunt end ligation. The new construct, pTRAkt-DC1-DsRed, was used for agrobacterium transformation. The PCR check results indicated the presence of construct in the recombinant agrobacteria cells.

For cytosolic expression, the scFvs specifying Rep and/or TYLCV virion plus scFv-RWAV genes were cloned into pTRAkt vector, without any additional tags, under the 35S promoter.

To visualize produced scFvs inside the cells, the green fluorescent protein (GFP) was used as a fusion partner. The GFP recovered from the jellyfish *Aequorea victoria* has widespread utilization for localization studies and identification of protein interactions and function. The GFP consists of 238 amino acids, with a total molecular weight of about 27 kDa. GFP does not influence the biological behaviour of its fusion partners (such as scFv), and that *vice versa*, GFP is not effected by other proteins fused to it (Hink et al., 2000; Lu et al., 2005). The GFP is a cytoplasmic protein with high stability, solubility, intrinsic fluorescence which can be expressed and is correctly folded within the reducing condition of cytosol (Casey et al., 2000). Insertion of antibody binding loops to a particular stable form of GFP created an intrinsically fluorescent affinity reagent, fluorobody, combining the advantages of antibodies

(high affinity and specificity) and with those of GFP (stability and solubility) (Zeytun et al., 2003). The scFv-GFP fusion can be produced as a functionally active protein inside the cytoplasm of prokaryote (Casey et al., 2000; Schwalbach et al., 2000) and eukaryote (Peipp et al., 2004) cells. The pTRAkt-ScRep1-GFP and pTRAkt-ScRep2-GFP constructs were made for expression of scFv-ScRep1 and scFv-ScRep2 as N-terminal fusion with GFP protein. This fusion ensured that all visualized green fluorescence resulted from the scFv-GFP fusion and not form native GFP.

To target scFv into the nucleus, ´´SV40 T antigen`` nuclear localization signal (PKKKRKV) (Kalderon et al., 1984) was introduced to the amino terminal of scFv-ScRep1, scFv-RWAV and scFv-ScRep1-GFP. The nucleus provides a reducing environment that may interfere correct folding of antibody fragments (De Jaeger et al., 2000). It has been proven that anti-Tat scFv fragment could be effectively localized and acted against corresponding antigen within the nucleus of eukaryotic cells (Mhashilkar et al., 1995). The nucleus targeted scFv could be applied for immunomodulation of abnormal proteins within the nucleus (Hink et al., 2000). Nuclear targeting of scFv specifying *plum pox virus* (PPV) replicase has been successfully used for reduction of viral infection in transgenic *N. benthamiana* (Esteban et al., 2003).

The pTRAkt-ScRep1, pTRAkt-ScRep2, pTRAkt-RWAV, pTRAkt-HScCP2, pTRAkt-HScCP3, pTRAkt-HScCP4, pTRAkt-NLS-ScRep1, pTRAkt-NLS-RWAV, pTRAkt-ScRep1-GFP, pTRAkt-ScRep2-GFP and pTRAkt-NLS-ScRep1-GFP constructs were transformed into *Agrobacterium* cells by electroporation. Recombinant agrobacteria colonies harbouring corresponding plasmids were selected following PCR check using pSS primer and cultured in YEB medium supplemented with kanamycin, rifampicin and carbencilin antibiotics. Transient expression of recombinant proteins was carried out by vacuum infiltration and/or injection to tobacco leaves. The plant extracts were analyzed four days later.

Agroinfiltration of tobacco leaves with DRep-DsRed caused severe necrotic local lesions at the infiltrated area (Figure 29). It has been shown that expression of recombinant geminivirus Rep in *N. benthamiana* plants causes hypersensitive response (HR) associated with local necrosis and a systemic burst of hydrogen peroxide production (van Wezel et al., 2002; Selth et al., 2004). The middle portion of the ACMV Rep, amino acids 119–179, is essential for induction of the HR phenotype. Another two regions of Rep, amino acids 1–85 and 86–118, have various effects on the Rep-mediated phenotype (van Wezel et al., 2002).

The extremities areas immediately outside of the necrotic lesions were visualised by fluorescent microscopy. Most of the infiltrated cells showed DRep-DsRed fusion protein expression as a red fluorescence localized predominantly within the nucleus compartment

(Figure 30-B) although some fluorescence was occasionally observed in the cytoplasm of some cells. In contrast, DsRed fluorescence in its native form, was not particularly restricted to the nuclei of cells, but occurred throughout the cytoplasm (Figure 30-A). The molecular weight of DRep-DsRed is 55.3 kDa, above the size for efficient passive nuclear diffusion (Bonner, 1978; Ribbeck and Gorlich, 2002). It seems that nuclear targeting behaviour of Rep mediated driving of the DRep-DsRed fusion protein into the nucleus. Some geminivirus proteins such as Rep need to be targeted to the nucleus and its components to favour viral encapsidation and replication (Kim et al., 2004). A nuclear localization domain has been identified within geminivirus Rep (Hefferon et al., 2006). Our observations are compatible with other results that confirmed localization of Rep-GFP fusion protein took place mainly within the nucleus (Hong et al., 2003; Hefferon et al., 2006).

Transient expression of GFP fusion scFvs were accomplished by infiltration of tobacco leaves with recombinant agrobacteria harboring ScRep1-GFP1, NLS-scFv-ScRep1-GFP and scFv-ScRep2-GFP constructs. The expression and localization of scFv-ScRep1-GFP1, NLS-scFv-ScRep1-GFP and scFv-ScRep1-GFP2 fusion proteins in transiently transformed tobacco leaves were visualized by fluorescent microscopy. All fusions were successfully expressed and localized in the cytoplasm and nucleus (Figure 31 A-D). Localization patterns of scFv-ScRep1-GFP1 and NLS-scFv-ScRep1-GFP fusion proteins were similar, which may indicate the NLS tag has no effect on nuclear targeting and scFv fusions could passively cross the nuclear membrane. Previous studies have shown the successful expression and localization of some scFv-GFP fusions within the cytosol of transiently transformed tobacco cells (Zakri, unpublished data).

Tobacco plants transiently expressing scFv-ScRep1, NLS-scFv-ScRep1, scFv-RWAV, NLS-scFv-RWAV, scFv-ScRep2, scFv-HScCP2, scFv-HScCP3 and scFv-HScCP4 proteins were analyzed to detect scFv fragments in crude extract by blotting assays. The results showed that the scFv-ScRep1, NLS-scFv-ScRep1, scFv-ScRep2 accumulated at detectable levels within the tobacco leaves 4 days after infiltration (Figure 32). These scFv fragments had been selected from phage display libraries but other scFv fragments constructed from hybridoma clones were not produced in detectable level. These results confirm that the scFv accumulation capacity within the cell is highly dependent on the properties of the expressed scFv fragment. Intrinsic properties of scFv fragments determine scFv stability in the reducing conditions of cytosol and its ability to overcome the entropic destabilization of scFv protein folding caused by the absence of disulphide bridge formation (Frisch et al., 1996; De Jaeger et al., 1999; Ewert et al., 2003). While the scFv fragments engineered from hybridoma cell lines

have been selected based on the activity of the complete antibody, phage display selection provide high pressure needed for enrichment of functional scFv fragments with more stable scaffolds (De Jaeger et al., 2000; Worn and Pluckthun, 2001; Prins et al., 2005). The results confirm accumulation of scFv-ScRep1 and scScRep2 without disulfide bond formation in the cytosol. The highly conserved intra-chain disulphide bonds are one of the main structural features of immunoglobulins and are critical factors in the correct folding of the native antibody domain structures. Generally, the scFv stability and functionality is impossible without disulphide bond formation, but some antibody scaffolds can tolerate the deletion of the disulphide bridge and keep their stability and functionality (Glockshuber et al., 1992; Frisch et al., 1996). Several antibodies in the Kabat databases lack disulphide bridge in their structure but are still functional (Rudikoff and Pumphrey, 1986; Frisch et al., 1996; Proba et al., 1997). Sometimes addition of stabilizing amino acids improves the functionality of specific immunoglobulin lacking disulphide bonds (Frisch et al., 1996). While many scFv are accumulated at low levels, targeting them into the ER could improve their accumulation (Artsaenko et al., 1995; Rosso et al., 1996).

To determine functionality of tobacco expressed scFv, the crude extract of leaves transiently expressing scFv-ScRep1, NLS-scFv-ScRep1, scFv-ScRep2, scFv-ScRep1-GFP and scFv-ScRep2-GFP were applied for detection of recombinant Rep by ELISA and/or Western blot assays. The results showed that crude extracts containing scFv-ScRep1, NLS-scFv-ScRep1 and scFv-ScRep2 could specifically bind to recombinant fusion Rep proteins but scFv-ScRep1-GFP and scFv-ScRep2-GFP failed to do so (Figure 35). These results provide further evidence to verify correct folding of scFv-ScRep1 and scFv-ScRep2 in reducing conditions of cell with antigen binding activities. These scFvs introduce antibody fragments with a framework well adapted for cytosolic expression. It is shown that scFv800E6 was efficiently produced in the cytosol and remained functional for binding to its corresponding antigens (Lombardi et al., 2005).

Further investigation to determine *in vivo* binding activities of scFv-Screp1 and scFv-ScRep2 were performed. scFv-Screp1-GFP and scFv-ScRep2-GFP fusions were individually co-expressed transiently with DRep-DsRed in the infiltrated tobacco leaves. The fluorescence microscopy was again performed 4 days after infiltration. As shown Figure 36 and 37, the expression and localization of GFP and DsRed fusions happened inside the cells. Some identical cells presented co-localization of both fusions. It is, however, difficult to determine if interaction between both fusion proteins is occurring at these loci.

IV.8 Generation and characterization of stable transformed *N. benthamiana* plants

Transgenic tobacco (*N. benthamiana*) plants were generated by the leaf disc transformation procedure with agrobacteria harbouring pTRAkt-ScRep1, pTRAkt-NLS-ScRep1, pTRAkt-RWAV, pTRAkt-HScCP2, pTRAkt-HScCP3, pTRAkt-HScCP4 and pTRAkt-ScRep1-GFP constructs. The regenerated plants were selected on MS medium containing kanamycin, transplanted into soil and kept under high humidity for next 2 weeks. The corresponding regenerated tobacco plants expressing ScRep1, NLS-ScRep1, RWAV, HScCP2, HScCP3 and HScCP4 proteins were briefly named SR, NSR, RW, HSC2, HSC3 and HSC4, respectively. Transgenic T0 tobacco plants were grown for 4-6 weeks in greenhouse and self-pollinated for next generation (T1) establishment. Most of rooted SR, NSR, RW and HSC2 tobacco lines successfully grew in soil and in comparison to wild type plants and did not show unusual appearance relative to production of transgene. However, most of the rooted T0 HSC3 (23 out of 30 plants) and HSC4 (26 out of 30 plants) lines failed to grow normally in soil and died 1-2 weeks after transplantation into the soil. The fact that we were unable to regenerate plants expressing HScCP3 and HScCP4 proteins may be explained by deleterious effects that constitutive over-expression of active transgene probably has on the cell and lead to abolish normal growth of relative lines. In some cases, harmful side effects of transgene peptides which are significantly different from the identified natural versions have been identified (Altpeter et al., 2005). Insertion of T-DNA in transgenic plants may alter function or expression of other genes involved in all aspects of plant biology (Wilson et al., 2006). Usually, the unexpected phenotypes in transgenic plants can be affected either by pleiotropic effects of integrated DNA on the host genome or by side effect of various stresses related due to new researches (Filipecki and Malepszy, 2006).

To study accumulation of transgene proteins within the transgenic plants, crude leaf extracts were prepared (II.2.8.5) and analyzed by immunoblotting experiments. These results indicated that SR lines expressing scFv-ScRep1 protein accumulated detectable amount of protein within the cytosol (figure 38), whereas independent RW, HSC2, HSC3 and HSC4 transgenic lines failed to accumulate detectable amount of relative transgene in the cell. Further PCR analysis using purified total DNA confirmed presence of transgene sequence within non accumulative T0 plants. It seems that these scFv that had been already raised from hybridoma clones, could not efficiently fold within the cytosol. It has long been proved that accumulation of expressed scFv in the cytosol exclusively depends on its intrinsic properties (Conrad and Fiedler, 1998; De Jaeger et al., 1999). Usually, scFv fragments developed form phage display libraries accumulate in higher level in comparison to those made from hybridoma clones.

Although the cytosol is a suitable compartment in which to inhibit virus replication, targeting of virus specifc antibodies to the secretory path way may block or at least reduce viral infection (Firek et al., 1993; Voss et al., 1995; Schouten et al., 1996; Fecker et al., 1997). Together these results confirm those obtained from transient expression analysis (III.6.2.1).

The Western blot analysis results revealed varying accumulation of ScRep1 protein with the expected size of 30 kDa within the cytosol of SR lines (figure 38). These results indicated that SR15, SR 22 and SR27 lines produced the highest amount of transgene proteins which are comparable with those obtained in the transient agroinfiltration. Also these results showed that some SR lines (SR 17) accumulated low amount of transgene within the cell and others like SR6 failed to accumulate detectable amount of protein. It has long been identified that independent transgenic lines vary in transgene expression. These variations could be caused either by different position of T-DNA integration or copy number of insertion in the plant genome (Hobbs et al., 1990; Filipecki and Malepszy, 2006). High level production of transgene protein in the cell is preferable since sufficiently amounts of scFv are needed for blocking the function of desirable antigen (De Jaeger et al., 1999).

The immunoblotting experiments of SRG lines leaf extracts failed to detect expressed ScRep1-GFP fusion protein. Using of polyclonal α-GFP antibody in this assay yeilded many non-specific bands on the membrane making it impossible to discriminate specific bands from others. As an alternative way, presence of ScRep1-GFP in T0 SRG lines was measured by their fluorescence ability.

Altogether these results indicate that the transient state of transgene protein accumulation in infiltrated leaves is not always comparable to results after stable integration, but it verifies suitability of transient expression to show the highest levels that could be obtained by stable transformants.

VI.9 Challenging of T0 transgenic tobacco lines with TYLCV

The protective effect of expressed scFv fragments in T0 tobacco lines were directly assayed through inoculation of these plants with TYLCV agroinfectious clone. The great potential of antibody mediated resistance approach using recombinant antibodies, such as scFvs, to prevent viral infections has already been verified. While application of this approach had been limited for plant viral diseases (Tavladoraki et al., 1993; Voss et al., 1995; Schillberg et al., 2000), but its feasibility against human viruses has been proved (Marasco, 1995). In the most cases coat proteins are targeted to obtain resistance against viral disease in plants (Tavladoraki

et al., 1993; Voss et al., 1995; Fecker et al., 1996; Schillberg et al., 2000; Schillberg et al., 2001).

Due to a potential negative effect of viral infection on reproduction processes of tobacco plants, the putative independent T0 lines were first self pollinated and allowed to grow till mature seeds were obtained. Inoculation of wild type and transgenic T0 lines was carried out with agroinfectious clone harbouring pBIN19-2TYLCV-Ir construct. The TYLCV agroinoculation is a simple and stightforward way for infection of tobacco plants and subsequent assays to study its protective ability against the viral disease (Bendahmane and Gronenborn, 1997). For this aim, old branches were removed and with 5-6 leaves remaining and inoculated through injection of 10 µl of agrobacterium suspension (OD600 ~ 0.05) into the petiol and stem of young branch. It was shown that following the agroinoculation of geminiviruses, a T-DNA containing a dimmer of the viral genome is transfered into plant cell, where one copy of viral DNA is released and replicated as a circular double stranded form (Stenger et al., 1991). The inoculated plants were weekly inspected for symptom developments. Early symptoms including leaf curling and reducing of size of new emerged leaves were revealed on non-transgenic and sensitive transgenic plants 3-4 weeks after inoculation (Fig. 39). Scoring of disease symptoms were continued for 5 weeks and those with no clear or reduced symptoms in comparison to wild type plants were selected and used for subsequent evaluations.

The accumulation of viral DNA and presence of virus particles in the inoculated plants were assayed through PCR, Southern blotting and TAS-ELISA analysis.

The PCR results showed its worth for detection of viral DNA within the infected plants through either purified total DNA or crude extract of tobacco leaves. This method has been accepted as a rapid, specific, reliable, and sensitive method for detection of geminiviruses in infected plants (Gharsallah Chouchane et al., 2006; Maruthi et al., 2007). It was able to detect virus even in the symptomless samples that were assigned as a negative by conventional assays. Comparative PCR analysis using purified DNA and crude leaf extract proved feasibility of later way for detection of viral DNA. This could lead to a decrease of time needed for purification of total DNA. However, in some cases PCR analysis using leaf extract failed to detect viral DNA within the infected plants. Apparently, this detection system is limited by low DNA template concentration and presence of inhibiting components in the plant leaf extract.

To detect and quantify virion particles in the inoculated plants, the TAS-ELISA analyses were accomplished. The results showed that this application is only useful for detection of infected

plants with severe symptoms and high virus concentration, but it failed to discriminate healthy plants from those with mild symptom containing low viral titres. These results are contradictory to reports that used TAS-ELISA as an efficient way to distinguish and comparison of TYLCV concentration among individual plants (Duan et al., 1997; Fuentes et al., 2006). The difficulty for detection of TYLCV virions via this system may be caused by its low concentration within the crude leaf extract. It is shown that TYLCV is a phloem limited geminivirus and is not present in the mesophyl (Rojas et al., 2001). This phenomenon could lead to drastically decrease detectable virion particles within the leaf extract. To improve detection system, several attempts like using higher concentration of antibodies and different leaf extraction methods without clear effects were accomplished.

To determine viral DNA accumulation within the inoculated plants, Southern blotting hybridization analysis was performed. Many previous reports clearly showed feasibility of this assay as a simple method to determine viral DNA accumulation (Bendahmane and Gronenborn, 1997; Abhary et al., 2006; Shivaprasad et al., 2006). For this purpose, total DNA from inoculated plants were extracted and subjected to agarose gel electrophoresis. The separated DNA fragments were blotted onto positively charged membrane and hybridized with digoxygenin labelled probe. The results showed its great worth for detection of different viral DNA conformations e.g. open circular, linearized and supercoiled dsDNA as well as ssDNA structure within the infected plants (figure 42). Since S1 nuclease enzyme is able to degrade ssDNA molecules, it was applied to distinguish ssDNA from dsDNA molecules. Upon S1 nuclease treatment expected ssDNA fragments were no longer detectable (Fig. 44).

Assessment of resistance status within the inoculated plants was evaluated 5 weeks after inoculation. This evaluation was initially accomplished by disease symptom. The complementary Southern hybridization analysis confirmed reduction or complete suppression of viral DNA replication in the symptomless plants. The samples with absence or remarkable reduction of disease symptoms were assigned as resistant plants.

As shown in table III-6, among the transgenic tobacco plants, SRG lines expressing ScRep1-GFP fusion protein revealed the highest resistance (28%) against TYLCV challenge as 7 out of 25 lines remained symptomless 5 weeks after inoculation. In the same time SR and RW lines presented 8 percent resistance against TYLCV infection while NSR, HSC2, HSC3 and HSC4 lines as well as non-transgenic plants were totally susceptible and showed severe symptoms.

VI.10 Challenging of T1 transgenic plants against TYLCV

The seeds obtained from self pollinated T0 lines were individually grown *in vitro* with kanamycin selection. Transgenic seedlings were screened based on their tolerance to antibiotic and those with 3-4 small leaves transferred to soil and assigned as individual T1 progenies. Alternatively, the SRG T0 seeds were directly sown into soil and T1 transgenic plants expressing ScRep1-GFP fusion protein were selected via fluorescent microscopy.

Table III-7 shows the total number of T1 trangenic progenies per number of cultured seeds. The seeds obtained from T0 SR-22 line germinated poorly in MS medium and raised seedlings failed to establish in soil and died after 2 weeks. This may be caused by deleterious effect of expressed scFv on tobacco plants.

The *N. benthamiana* is a very susceptible host for TYLCV infection and virus is able to accumulate to high levels within the infected young plants. This could lead to overcome the ability of transgenic plants and virus would be able to break the expected resistance. Therefore, finding the minimum effective inoculum to infect young tobacco plants is a major challenge. Initial experiments by injection of serial dilution of agrobacterium suspension into the stem and petiole of wild type tobacco were performed. As an alternative, inoculation of tobacco plants was performed by rubbing of a bacterial colony on the wounded surface of decapitated stem. These results indicated that the latter method caused lower severity and one week delay in appearance of disease symptoms. It seems that using this approach lead to optimal inoculum for infection of tobacco plants and is a promising method for TYLCV resistance assay on tobacco plants (Gronenborn, unpublished data).

Out of each transgenic line, 15 individual transgenic plants were selected and subjected for agroinoculation. A fresh agrobacterium culture harbouring pBIN19-2TYLCV construct was made on petri dishes containing solified YEB medium. Individual plants in 5-8 leaf stage were inoculated by removing the stem apex and rubbing of bacteria colony on the wounded surface. The excised apices were stored for subsequent molecular analysis. The inoculated plants were kept in similar situation and weekly inspected for symptom developments. To study the effect of transgene on TYLCV infection and ability of transgenic plants to confer disease resistance, the initial evaluation was based on disease symptomology in inoculated plants. Early symptoms were revealed 2-3 weeks after inoculation. The sensitive transgenic lines as well as wild type plants showed clear symptoms including leaf curling on new emerged leaf. Symptom scoring was done during 4 weeks. To evaluate viral DNA accumulation within the tobacco plants, symptomless plants as well as symptomatic ones were analyzed by hybridization assay. These results indicated that symptomless plants

contained no detectable viral DNA. In contrast, plants with severe symptoms also showed accumulation of viral DNA in an amount comparable to that of untransformed wild type plants. Four weeks after inoculation, some plants with milder symptoms were also obtained. Generally, hybridization analysis indicated direct relation between symptom severity and accumulation of viral DNA. Therefore, the TYLCV disease symptom status could provide confident and direct information about the viral DNA level within the inoculated plants, these results are compatible with other report as found similar correlations (Bendahmane and Gronenborn, 1997).

Resistance response against viral infection was evaluated 4 weeks after inoculation. Plants with remarkable reducion of disease symptoms or without distinct symptoms were assigned as resistant. Hybridization assays, showed that they have substantial reduction or complete suppression of viral DNA replication. The table III-7 shows that all T1 progenies obtained from SR27, RW14 and RW22 revealed typical TYLCV symptoms. The SRG28 and SRG18 lines expressing ScRep1-GFP protein showed highest resistance by amount of 40% and 33.3%, respectively. These results indicate that resistance phenotype is inherited and improved through the T1 generation from 28% in T0 to 40% in T1 progenies of SRG28 line.

Other lines including SRG4, SRG27, SRG34, SRG36 and SRG 42 presented intermediate level of protection by amount of 13.3, 20, 13.3, 6.6 and 20 percent resistance against TYLCV challenging.

Hereafter, the protective ability of transgene in the symptomless plants was studied. The observation revealed that some lines like SRG28-12 remained symptomless even 7 wpi. However, some initially resistant plants like SRG28-4 were unable to prevent TYLCV invasion for a long term and virus could overcome resistance mediated by scFv expression. Similar results have been reported for transgenic resistant plants challenged with TYLCV (Noris et al., 1996; Zrachya et al., 2007). This phenomenon could be explained by high replication ability of TYLCV within the *N. benthamiana* and incomplete inhibition of viral replication by scFv which, in turn, could lead to systemic infection and breaking resistance in transgenic plants. Concerning to this issue, two other important factors should be considered. First, use of the 35S promoter has been shown to have some drawbacks. It is not completely constitutive as previously believed, and can produce a mosaic pattern of expression (Neuhuber et al., 1994). The fluorescent microscopy results showed that some cells in SRG transgenic lines produced no or reduced amounts of ScRep1-GFP fusion protein allowing virus replication to occur. Second, the type and dose of inoculum may substantially affect the

level of resistance (Anderson et al., 1992). Apparently, use of lower agrobacterium dose may be resulted to observe more resistant plants.

The most promising lines, SRG28 and SRG18, showing higher resistance phenotype were selected for subsequent analysis. Quantitative PCR analysis was performed to compare amount of transcripts among the selected transgenic lines. These results indicated that plants with an elevated ScRep1-GFP transcripts level, like SRG28-12, consistently exhibited a higher degree of virus resistance. However, a strict correlation between the amount of the transcripts and degree of resistance is difficult to assess. It has been proved that resistance to plant viruses in antibody producing transgenic plants depends on the level of antibody expression (Biocca et al., 1995). However ability of low levels of cytosolic scFv to give highly resistant transgenic plants has been demonstrated for TMV (Zimmermann et al., 1998) suggesting that even small amounts of functional antibodies are efficient for virus inactivation.

Alternatively, comparative analses for intensity of fluorescence emission among T1 lines expressing ScRep1-GFP fusion protein were carried out. These results indicated that lines with higher transcripts level such as G28-12 emitted fluorescent light with higher intensity in comparison to those with less amounts of transgene transcripts. Generally, elevated amount of ScRep1-GFP transcripts is directly correlated with higher fluorescent intensity emitted from transgenic SRG lines. The copy number of foreign DNA integrated to host genome and position of integration on chromosomal loci significantly influence expression level of transgene (Finnegan and McElroy, 1994). Hence the variation of fluorescence emission in the various progeny lines may also be caused by these effects.

In addition, fluorescence emission intensity in different leaves of independent SRG plants at 3-4 leaf stage, was studied. These results indicated increasing rate of fluorescence from upper leaves to lower one. It may be caused by post translation modification occurred on expressed fusion protein. Similarly, higher accumulation of transgene within the lower leaves of transgenic tobacco plants expressing TYLCV Rep protein has been reported (Noris et al., 1996; Zrachya et al., 2007).

The data reported here show that transgenic tobacco expressing ScRep1-GFP are capable to neutralize TYLCV infection. It is possible that the produced cytosolic scFv fusion protein directly interact with newly senthesized viral Rep and block its active site or render the structure of the Rep. However, the mode of interaction between this fusion protein and the viral Rep requires further investigation. The geminivirus Rep is the only single viral protein indispensable for their DNA replication (Orozco et al., 2000). It is a multifunctional protein

whose activities comprise, the specific recognition of its cognate replication origin (Jupin et al., 1995), the sequence specific DNA cleavage and linkage (Laufs et al., 1995) and an ATPase activity (Desbiez et al., 1995). All these essential functions of Rep make it an ideal target for obtaining virus resistance by antibody-mediated suppression of its function.

By contrast, SR transgenic lines expressing ScRep1 alone presented limited ability to inhibit viral replication. Comparison of the results obtained from virus resistance assay of SRG and SR lines indicate that GFP fusion partner is probably responsible for improvement of protective ability in SRG lines. The GFP is a cytoplasmic protein with high stability and solubility. It is proved that insertion of antibody binding loops to such a particular stable form of GFP create an intrinsically fluorescent affinity reagent, fluorobody, combining advantages of antibodies (high affinity and specificity) and with those of GFP (stability and solubility) (Zeytun et al., 2003). The loss of protective ability within the T1 SR plants may be caused by low expression of scFv within the cells. It is possible that scFv fragment have not been accumulated in the plant cells at levels sufficient to completely neutralize the viral infection. Due to importance of TYLCV-mediated damage, substantial efforts have been focused to obtain virus resistant plants. Some successfully attempted approaches to obtain resistance against geminivirus infections involved introducing of natural tolerance loci from wild type tomato species into *L. esculentum* cultivars (Zakay et al., 1990), production of transgenic plants expressing an Rep antisense RNA (Bendahmane and Gronenborn, 1997), production of siRNA targeted coat protein in transgenic plant (Zrachya et al., 2007), introduction of defective interfering DNAs (Stanley et al., 1990) or intron-hairpin RNA derived from viral Rep protein (Fuentes et al., 2006) and expression of mutated movement proteins (Von Arnim and Stanley, 1992; Hou et al., 2000).

This study provides the first successful attempt to obtain resistant plants against plant DNA viruses via recombinant antibody mediated resistance. This approach could potentially provide an effective means for protecting plants against TYLCV infection. The main result of this work is reducing of viral replication in tobacco plants expressing the specific fusion scFv proteins. So far, all applications of antibody mediated resistance in plants have been accomplished against RNA viruses. Of these attempts, the most ones used coat proteins as a target to inhibit or decrease viral infection (Tavladoraki et al., 1993; Voss et al., 1995; Fecker et al., 1996; Schillberg et al., 2000). Expression of recombinant antibody binding to TMV coat protein resulted in reduced susceptibility of tobacco plants against viral infection (Voss et al., 1995). Similarly, constitutive intracellular expression of scFv fragment against AMCV coat protein lead to resistant *N. benthamiana* lines with a lower virus accumulation, reduced

incidence of infections and a delay in the viral symptom development (Tavladoraki et al., 1993). Because of high variability of viral coat proteins, and higher accumulation of viral coat proteins within the cells, these plants might not result in perfect and broad range resistance. In contrast, low accumulation of viral replicase protein within the plant cells provides suitable target for obtaining higher resistance through recombinant antibodies against replicases. Feasibility of this approach to develop broad spectrum resistance against *Tomato bushy stunt virus* (TBSV) has been verified. Here, tobacco plants expressing scFv against RNA-dependent RNA polymerase protein lead to high level resistance against infection with related virus and showed varying degrees of resistance against four plant viruses from different genera (Boonrod et al., 2004).

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VI Appendices

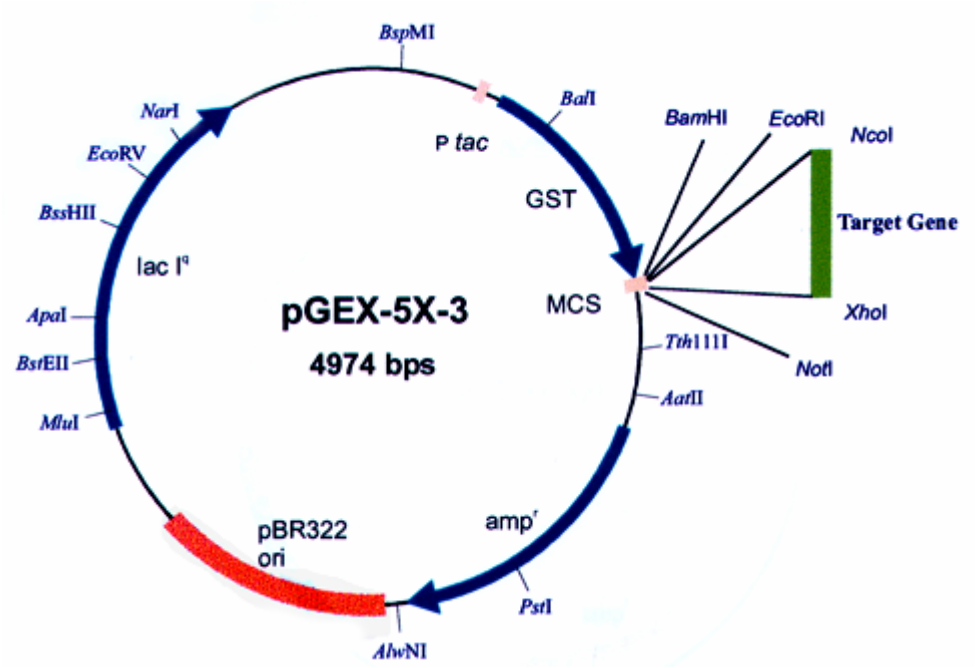
VI.1 List of abbreviations

°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
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	fragment crystallizable
G	glycine
GAM	goat-anti-mouse (antibodies)
gm	gram

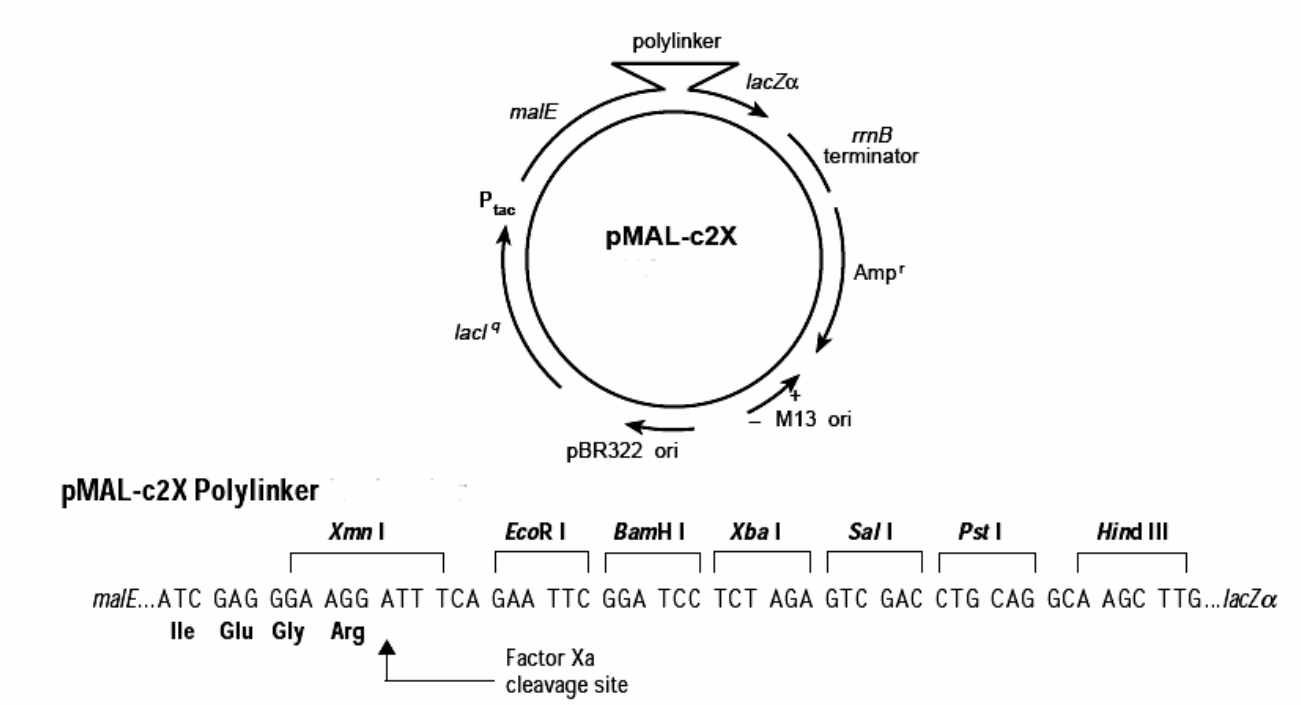
h	hour(s)
His	histidine
Ig	immunoglobulin
K_A	equilibrium association constant
kDa	kilodalton
kg	kilogram
Km	kanamycin
l	liter(s)
LBA	Luria broth with ampicillin
M	molarity (mol/L)
mAb	monoclonal antibody
min	minute(s)
mRNA	messenger ribonucleic acid
MW	molecular weight
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% (v/v) Tween20
PCR	polymerase chain reaction
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
v/v	volume per volume
w/v	weight per volume

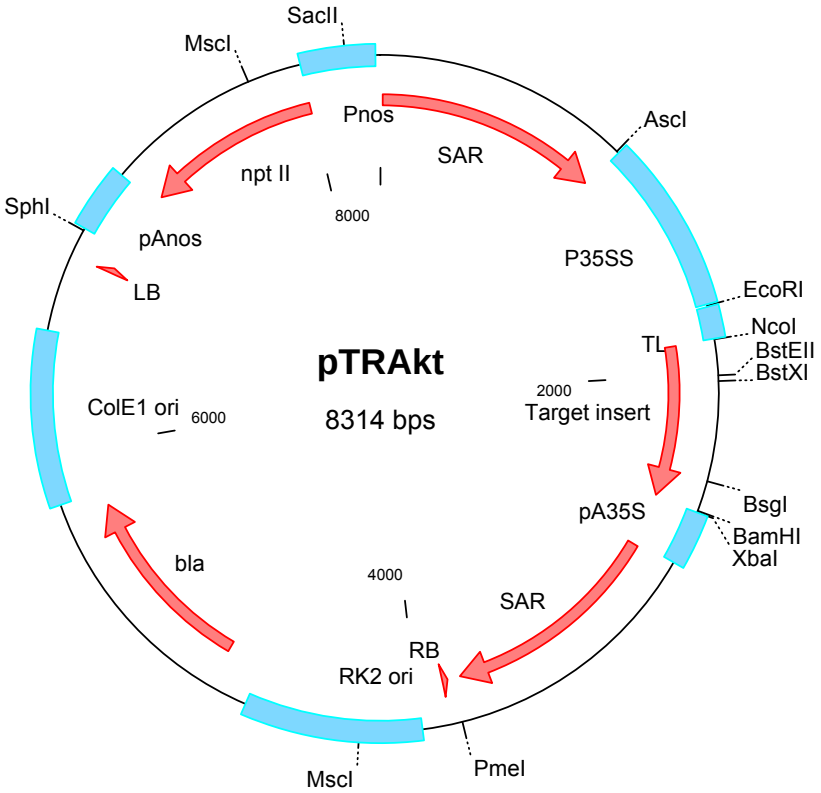
VI.2 Vector maps



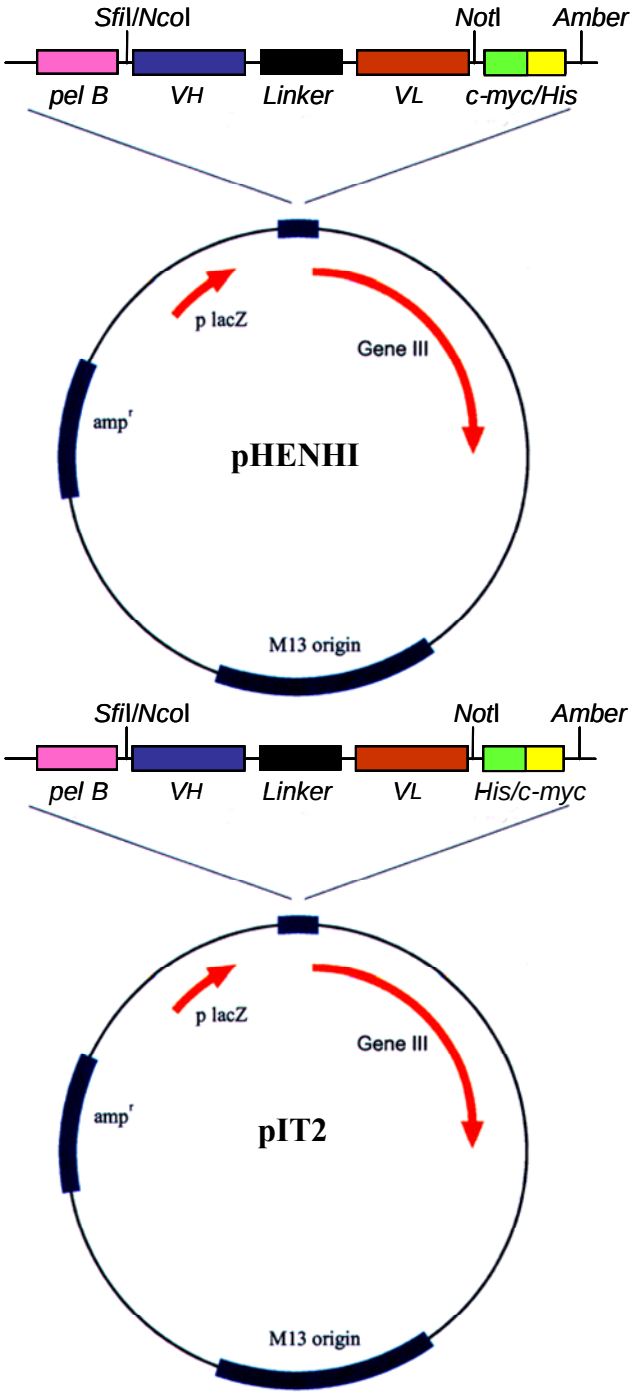
A) Map of the GST fusion vector (pGEX-5X-3) showing the reading frames and main features. MCS: multiple cloning sites, amp^r: ß-lactamase ampicillin resistant gene, p *tac:* promoter induced by IPTG, *lac* I^q: repressor protein coding region, pBR322ori: plasmid replication origin.



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-kt. p35S: 35S promoter from CaMV with duplicated 35S enhancer, CHS: 5' UTR of TEV, 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.

Figure 49: Schematic presentation of the vectors used in this study.

VI.3 Figures

VI.4 Tables

VII Acknowledgements

I would like to thank Professor Dr. Rainer Fischer (RWTH-Aachen) for giving me the opportunity to work in his Institute, providing excellent working facilities and supervision. My special thanks go to Dr. Ulrich Commandeur (RWTH-Aachen) for his supervision, unlimited dedication and commitment towards this work. His critical reading, discussion and correction of this thesis made it possible for me to present it in this form.

I would like to give some special thanks to Prof. Dr. Bruno Gronenborn (Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France) for his effective comments throughout the work. I also thank Karoline Richter, Markus Sack, Adel Zakri, Dr. Kerstin Uhde, Dr. Elsa Arcalis, Julian Rodriguez and Dr. Frank Müller. I thank Dr. Greta Nölke, Holger Spiegel, Yvonne Olbrich and other members of the Fraunhofer IME for their kind support and technical assistance during this work. Special thanks go to Dr. Flora Schuster for her skillful assistance in generation of transgenic tobacco plants. I wish to thank Dr. Thomas Rademacher for his great help during my work with fluorescent microscopy and for providing me with most of the plant expression vectors used through out this work. I would like to thank Ibrahim Al Amedi for his help in the green house and for taking great care of my transgenic plants.

I wish to express my special thanks to Sascha Offermann, Björn Dreesen, Bernhard Gilleßen, Rashad Kebeish, Frank Jansen, Dr. Christoph Peterhänsel and other members of the Institute for Biology 1 (Department of Molecular Genetics) for their kindness and cooperation. I would like to acknowledge Alexander Hynes (University of Calgary, Canada) for helpful comments and suggestions on the preparation of the manuscript.

I would like to thank Professor Dr. Fritz Kreuzaler and PD Dr. Christoph Peterhänsel for their acceptance to be co-examiners.

Special thanks go to the Iranian Organization of Agricultural Research and Education for the financial support of my Ph.D. studies in Germany.

I also thank my friends in Germany who stood with me at the time of need lightened the hardness of being away from home. I especially thank Ali, Javad, Morteza, Malek, Saeed, Naser, Shahram, Masoud and their families for all the wonderful moments we spent together.

Most of all, I would like to thank my family in Iran who stood with me all my life. I think now it is a good time point to remember and express my pure honor to the great spirit of my mother, as I always feel her spiritual support in all aspects of my life. Of course, I wish to express my endless thanks to my wife, Masoumeh, for her very kindly support and patience. My special thanks to the hope of my life, my son, Saleh, for his lovely smiles that always refresh my life.

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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, 2008