"Evaluation of a viral vector system, based on a defective interfering RNA of tomato bushy stunt virus, for protein expression and the induction of gene silencing"

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2 Abbreviations

Amp	ampicillin
AP	alkaline phosphatase
APS	ammonium persulfate
Asn	asparagine
bp	base pairs
DI	defective interfering particle
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpi	days post inoculation
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno sorbent assay
ER	endoplasmatic reticulum
EtOH	ethanol
GFP	green fluorescents protein
GUS	β-D-glucuronidase
His	histidine
IgG	immunoglobulin G
kb	kilo bases
kD	kilo dalton
Man	mannitol
MeOH	methanol
MES	2-Morpholinoethansulfonsäure
MS	Murashige and Skoog
nt	nucleotides
NTP	nucleotide triphosphate
ORF	open reading frames
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDS	phytoen desaturase
PEG	polyethylenglycol
PNK	polynucletide kinase
POD	peroxidase
PTGS	post transkriptional gene silencing
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	rounds per minute
SAP	shrimp alkaline phosphatase
scFv	single chain antibody variable fragment
SDS	sodiumdodecylsulfate
Ser	serine
TAE	tris acetate EDTA
TBE	tris borate EDTA
TBS	tris buffered saline
TBST	tris buffered saline tween
TBSV	tomato bushy stunt virus
TEMED	N,N,N,N,tetramethyl-ethyleneamine
Thr	threonine
VIGS	virus-induced gene silencing
X-GlcA	5-bromo-4-chloro-3-indolyl-β-G-glucoronic acid
YFP	yellow fluorescents protein

3 Abstract

In this thesis a viral vector system was developed based on a DI-RNA, a sub-viral particle derived from TBSV-BS3-statice. This newly designed vector system was tested for its applicability in protein expression and induction of gene silencing. Two strategies were pursued in this study. The first strategy being the replication of the DI-RNA by a transgenically expressed TBSV replicase and the second being the replication by a so called helper virus. It could be demonstrated by northern blot analysis that the viral replicase, expressed by the transgenic *N. benthamiana* plant line TR4 or supplied by the helper virus, is able to replicate DI-RNA introduced into the plant cells. Various genes were inserted into different DI constructs in order to study the vector system with regard to protein expression. However, independent of how the replicase was provided no detectable amounts of protein were produced in the plants. Possible reasons for this failure are identified: the lack of systemic movement of the DI-RNA in the transgenic TR4 plants and the occurrence of deletions in the inserted genes in both systems. As a consequence the two strategies were considered unsuitable for protein expression.

The DI-RNA vector system was able to induce silencing of transgenes as well as endogenous genes. Several different p19 deficient helper virus constructs were made to evaluate their silencing efficiency in combination with our DI-RNA constructs. In addition to the various helper virus constructs different inoculation methods were studied to adapt the vector system for a high efficiency, high throughput screening approach. However, it was found that our vector system can not compete with other existing VIGS (virus induced gene silencing) systems in this field.

Finally, the influence of DI sequences on mRNA stability on transient GFP and GUS expression experiments in GFP/GUS silenced plants was evaluated. However, *A. tumefaciens* infiltration with the binary vectors carrying the GFP gene did not result in GFP expression in wild type *N. benthamiana* plants. Therefore, further experiments with GFP silenced plants were not conducted. The alternative GUS reporter gene system was found to be unsuitable for distinguishing between expression levels of wild type plants and GUS silenced transgenic plants. Nevertheless, the results indicate a positive effect of the DI sequences on the level of protein expression and therefore further research into this area is recommended.

4 Introduction

4.1 Molecular farming

Commercial success of plant based production processes has demonstrated that plants expression systems possess an enormous potential for large scale production of recombinant proteins. Plants have several advantages in terms of practical, economical and safety issues compared to other expression systems. There are already several efficient plant based expression systems available and development still continues driven by those advantages. Many different proteins have been successfully produced in plants including antibodies, vaccines and hormones [Fischer and Emans, 2000; Giddings, 2001]. A key advantage of using transgenic plants as "bioreactors" is the comparatively low cost of large scale production. This is mostly due to the fact that no expensive fermenters and peripheral equipment are needed. Extensive research in this field is fueled by the possibility of high protein yields. For instance, yields of 0.1 to 1 % of total soluble protein are already competitive with other expression systems.

Conventional bioreactors have certain restrictions regarding the scale-up whereas plant systems can be scaled up rapidly in response to the market demand.

The largest part of the production costs are caused by downstream processing and purification of the recombinant protein. Regardless of the protein expression system the purification processes are similar and the costs do not vary greatly. However, several types of recombinant proteins produced in plants can be used in an unprocessed or only partially processed state. Since, plant material is usually unproblematic for human consumption, recombinant vaccines can be administered by the consumption of raw fruits or vegetables.

Accepting many advantages of plant based systems one has to acknowledge a major drawback which is the time needed to establish a stable transgenic plant line. Transient expression systems, as for example *Agrobacterium tumefaciens* infiltration and viral protein expression vectors try to address this problem.

Transient expression systems commonly use three different approaches (Fig. 1); biolistic delivery of DNA, infiltration with *Agrobacteria* and infection with modified viral vectors. However, the first system using particle bombardment is not suitable for the expression of large amounts of proteins in plants since only a small number of cells per leaf express the protein of interest (Fig. 1).

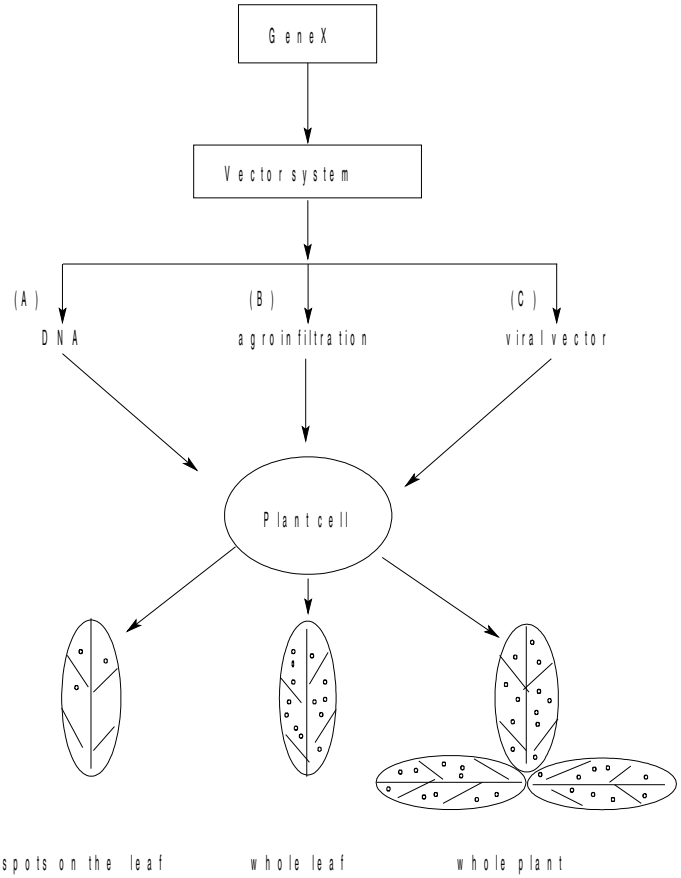


Figure 1: Transient expression systems most commonly used for the heterologous expression of proteins in plants. (A) Biolistic introduction of DNA directly into the plant cells. (B) Gene transfer using *Agrobacterium* infiltration. (C) Protein expression using a genetically modified viral vector. [Fischer et al., 1999]

Agroinfiltration utilizes the delivery of *Agrobacteria* into intact leaf tissue by vacuum infiltration or by infiltration using a needle-less syringe. This permits the use of conventional binary vectors. Several proteins have been successfully expressed in plants. Examples include scFvs, a protein containing only the variable regions of the heavy and light chain of an antibody linked by a glycine linker peptide, and full size antibodies [Vaquero et al., 1999] which demonstrates the capability of the system to express multi-component protein complexes. However, the *Agrobacterium* transient expression systems has its drawbacks concerning the large scale production of proteins, as it is not feasible to infiltrate large amounts of leaf material. The lack of systemic production of the heterologous protein and the susceptibility to gene silencing is also limiting the yield and the applicability of the system for

large scale productions. Systemic production is one of the major advantages of viral vector systems and therefore considerable research is done to develop novel viral vector systems.

4.1.1 Viral vectors in molecular farming

Viral vectors have several advantages in common with agroinfiltration. The rapidness in which milligram amounts of protein can be produced and also the ease of use are similar for both systems. So far the system has mostly been applied to test the usability of the plant expression system for a specific protein under small scale production conditions but field trials have also been conducted to produce recombinant protein [Fischer et al., 1999]. Additionally, viral vector systems are less susceptible to gene silencing due to the fact that most plant viruses have developed strategies to suppress this plant defense mechanism.

In virus-mediated expression systems, the gene of interest is cloned into a plant virus genome and plants are infected with the modified virus to produce the protein. Approximately 80 % of all plant viruses are RNA viruses and replicate to high titers which makes them ideal protein expression tools. Some plant viruses like TBSV additionally have a wide host range and are easily transmissible by mechanical inoculation making it possible to inoculate whole fields of crop plants. There are different strategies to insert the gene of interest into the viral vectors: Gene replacement, where viral genes, e. g. that encode the coat protein, dispensable for replication and infection are replaced by a foreign gene; Gene insertion, where the gene of interest is inserted into the viral genome under the control of a viral subgenomic promoter [Fischer et al., 1999]; Gene fusion, where the foreign gene is translationally fused to a viral gene and insertion of the heterologous gene into a satellite or any other subgenomic viral RNA. The most suitable strategy is dependent on the host/virus combination and the target gene. Most plant viruses have constraints on genome size and because of this, gene replacement is predominantly chosen.

The gene insertion technology is employed, if large coding sequences need to be expressed. Fusions with viral proteins were generally carried out with the coat protein and are very efficient for presenting foreign peptides on the surface of the viral particle [Joelson et al., 1997]. The insertion of genes into satellite or other subgenomic RNAs has not been widely applied up to now.

For inoculation of plants, the recombinant viral vectors are transcribed *in vitro* using

bacterial phage RNA polymerases like T7- and SP6- RNA polymerase. The synthesized RNA is inoculated by gently rubbing the leaves with an abrasive. For the infection of a large number of plants extracts of the initially infected plants can be used. Efficiency improvements of this technique may involve the use of *Agrobacteria* to introduce the viral vectors into the plant. However, there are several issues that still need to be approached with viral vectors. The stability of these recombinant vectors is often poor and the containment of the virus has to be addressed. One of the aims of this study was to approach the problem of containment so that replication of the viral sequence is only possible if the viral replicase is supplied *in trans*.

4.2 Post transcriptional gene silencing (PTGS)

As mentioned above gene silencing can be a problem in generating transgenic plants for effective protein expression in both stable and transient systems. Gene silencing is an evolutionary highly conserved defense mechanism against invasive nucleic acids, e. g. viruses and transposable elements. The phenomenon was first discovered in 1990 when plant biologists introduced an additional chalcone synthase gene into petunias to achieve a darker purple flower color [Napoli et al., 1990; Van Der Krol et al., 1990] . Surprisingly the flowers of the transformants were found to be white or patchy instead of darker. The introduced transgenes had silenced both themselves and the endogenous genes of the plants. This phenomenon was termed cosuppression. A similar effect was observed when plants were infected with a genetically engineered RNA virus harboring a plant gene fragment [Lindbo and Dougherty, 1992]. However, this process was not understood at that time. The results of Fire and coworkers [Fire et al., 1998] shed new light on the relationship between the two phenomena, since they could demonstrate that double stranded RNA (dsRNA) triggered gene silencing in *Caenorhabditis elegans*. Detailed examinations of gene silencing phenomena in plants revealed a similar mechanism. Simultaneous expression of sense and antisense transgene constructs [Waterhouse et al., 1998], induced silencing to a higher degree than expression of the sense construct alone. Similar to this RNA viruses replicating through dsRNA intermediates, multicopy transgenes, occasionally producing low levels of dsRNA and inverted repeat constructs likewise provide efficient silencing trigger molecules in plants. Considerable progress in this field has been achieved in the past years like the isolation of short interfering RNAs (siRNA), two 21 nt long RNA strands paired in a staggered duplex

that are derived from the silenced gene, but the understanding of the mechanism (Fig. 2) is not yet complete.

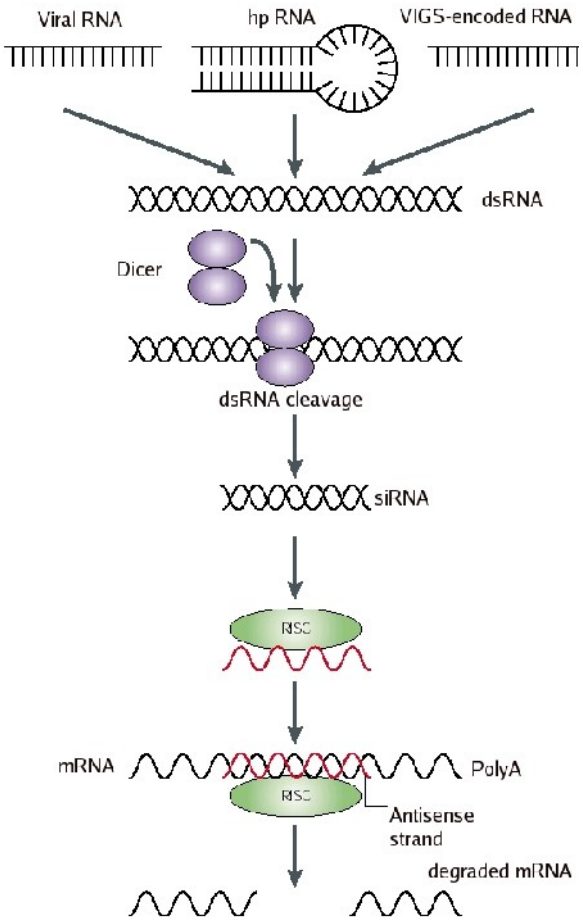


Figure 2: The current model of RNA-mediated gene silencing in plants. Double-stranded RNA (dsRNA) from replicating viral RNA, viral-vector-derived (VIGS, or virus-induced gene silencing) RNA or hairpin RNA (hpRNA) transcribed from a transgene, is processed by a Dicer-containing complex to generate siRNAs. An endonuclease-containing complex (called the RNA induced silencing complex, RISC), is guided by the antisense strand of the siRNA to cleave specific mRNAs, so promoting their degradation [Waterhouse and Helliwell, 2003].

The first step in the silencing process is the cleavage of the dsRNA into siRNAs by an enzyme called Dicer [Bernstein et al., 2001]. The next step is the removal of the sense strand of the siRNA, leaving the antisense strand which is complementary to the target gene. The antisense strand guides a protein complex named RISC (RNA induced silencing complex) to the mRNA of the target gene. The mRNA is then degraded by the protein complex. In plants,

gene silencing by siRNAs requires the activity of an RNA-dependent RNA polymerase (RdRP), which uses the antisense strand of a siRNA as primer in the synthesis of more dsRNA, thus amplifying the signal. This enables siRNA-mediated silencing to spread throughout the plant, by cell-to-cell movement of silencing associated RNA [Yoo et al., 2004], resulting in the resistance of the plant against further viral infection.

Gene silencing is becoming a valuable tool in genetic research since it can be applied to study the function of unknown genes. This and the fact that silencing is a major problem in the production of transgenic plant lines initiated intensive research in order to develop a better understanding of this phenomenon.

4.2.1 Virus-induced gene silencing (VIGS)

The term "virus-induced gene silencing" was first coined to describe the phenomenon of recovery from a virus infection as described in section 4.2. However, it has since been applied almost exclusively to suppression of gene expression by means of recombinant viruses [Ruiz et al., 1998; Baulcombe D.C., 1999]. VIGS is a valuable tool to study gene functions in reverse genetics since it allows the specific down-regulation of a particular gene or gene families [Baulcombe D.C., 1999]. Advantages of VIGS are the methodological simplicity, high speed and the circumvention of plant transformation. The latter makes VIGS especially interesting for species recalcitrant to transformation or for the evaluation of gene functions involved in early developmental stages. Gene knockout techniques traditionally used transformation as a delivery system. However, this is not possible with genes which are essential for the regeneration after transformation since knockout of these genes would result in the inability of the plants to regenerate. The VIGS system does not have this requirement for time-consuming transformation and tissue culture procedures. The resulting phenotypes can be observed within days after inoculation instead of months.

There are many different VIGS or VIGS-like systems already known. Examples include tobacco mosaic virus (TMV) [Kumagai et al. 1995], potato virus X (PVX) [Ruiz et al., 1998] and satellite tobacco mosaic virus (STMV) which uses TMV as helper virus [Gosselé et al., 2002]. There are also some DNA virus-based systems that have been used successfully for silencing [Peele et al., 2001]. The diverse systems utilize different inoculation procedures. As most plant viruses are RNA viruses the major inoculation method is based on direct cell

inoculation with RNA transcripts. A vector containing the viral genome under the control of a bacterial phage promoter, such as T7, SP6 or T3, allows *in vitro* transcription of large amounts of RNA. The RNA solution, together with an abrasive substance is mechanically inoculated onto plant leaves in order to deliver the RNA into parenchyma cells. DNA viruses have the advantage of eliminating the transcription step, allowing direct inoculation with plasmid DNA. Since *in vitro* transcription can be economically prohibitive on a large scale, plasmid DNA provides a cheaper alternative for application of a large number of vectors. Agroinfiltration is another method to introduce the viral genome into the plant. *Agrobacteria* infiltration is more suitable in the application of VIGS in high-throughput screening of cDNA libraries since it is cheaper and not as time consuming as RNA transcript rub-inoculation.

For the purpose of testing new VIGS systems there are a set of genes that have previously been reported to function in VIGS vectors, such as the phytoen desaturase (PDS) gene [Mann et al., 1994]. PDS participates in the carotenoid metabolic pathway, acting on the antenna complex of the thylakoid membranes and it protects the chlorophyll from photo oxidation. Deficiency in PDS activity results in white bleached spots on affected leaves. Further genes that have also been successfully adopted in VIGS systems include the *FtsH* protease, the GFP, and the cellulose synthase A gene. These approaches demonstrated that the entire coding sequence is not required to induce silencing. A partial sequence of 100 to 800 bp is sufficient.

Until now the VIGS system has only been efficiently applied to a few plant species, including *N. benthamiana,* tomato and barley. In order to expand this group of species that respond efficiently to VIGS, considerable effort is being put towards developing novel vectors. The tomato bushy stunt virus (TBSV)/defective interfering particle (DI) system was assumed to represent such a novel silencing vector because it has a broad host range, a small construct size and it can be introduced by *A. tumefaciens* infiltration.

4.3 Effect of DI sequences on gene silencing

DIs have been shown to effectively trigger gene silencing of transgenes [Hou and Qiu, 2003]. In addition, the DI-RNA is a poor target of the gene silencing mechanism [Szittya et al., 2002]. This phenomenon has not been analyzed to full extend and hence the mechanism is still unsolved. One possible explanation is based on the assumption that DI RNAs might be inaccessible to the RISC. Since DI-RNAs contain almost exclusively cis-acting sequence

elements required for replication and are tightly associated with the replication complex. Alternatively, the secondary structure of the RNA may prevent their degradation by PTGS as indicated by correlations of the size of DI RNAs and the degradation by PTGS [Szittya et al., 2002]. Further, DI sequences have been shown to enhance translation of uncapped RNA in protoplasts [Wu and White, 1999; Ray and White, 2003] since they carry a cap-independent translational enhancer (3' CITE). To investigate the suitability of DI sequences for protein expression in plants several projects have been conducted in our institute. One project showed that a binary vector construct containing a DI-GUS sequence led to a five times higher GUS activity than constructs without DI sequences when adopted in transient *Agrobacterium* infiltration experiments [Eilers, 2002]. In stable transformants harboring these constructs this difference was not observed. Since gene silencing is an even greater problem in transient expression than with stable transformants, it was thought that DI sequences may protect the mRNA from degradation by PTGS. To further investigate this possibility it was decided to construct additional DI binary vector constructs and infiltrate them in silenced GFP or GUS plants respectively. DI sequences could be a valuable tool to minimize the problem of gene silencing since it is a major problem in the generation of transgenic plants.

4.4 Tomato bushy stunt virus (TBSV)

Tomato bushy stunt virus (TBSV) is a member the *Tombusviridae* family [Brunt et al., 1997; Hull, 2002] and within this family TBSV belongs to the genus *Tombusvirus.* It forms isometric icosahedral particles which contain a positive-sense single stranded RNA (ssRNA) with a size of 4770 nt. The genomic RNA of TBSV contains 4 ORFs (Fig. 3). ORF 1 encodes a 33 kDa protein and read-through of its amber stop codon gives a 92 kDa protein. These two proteins constitute the viral RNA dependent RNA polymerase (RdRP) [Russo et al., 1994]. During replication two subgenomic RNAs are produced from which the remaining proteins are translated. Translation of ORF 2 from the subgenomic RNA 1 (sgRNA1) produces the 41 kDa viral coat protein which is dispensable for viral infection [Scholthof et al., 1993]. ORFs 3 and 4 are expressed from sgRNA2. The product of ORF 3 is a 22 kDa protein which facilitates cell-to-cell movement [Scholthof et al., 1995]. ORF 4 codes for a 19 kDa protein which is associated with necrosis formation, host-dependent induction of systemic invasion [Scholthof et al., 1995] and suppression of gene silencing [Qu and Morris, 2002]. Ribosome

scanning occurs, allowing translation of ORF 4 [Hull, 2002] since the initiation codon of ORF 3 is in suboptimal context. For plant mRNAs, the most crucial elements of AUG context are the purine at position +3, not present in the context of ORF 3, and guanine at position +4 [Kozak, 2002; Kozak, 1984].

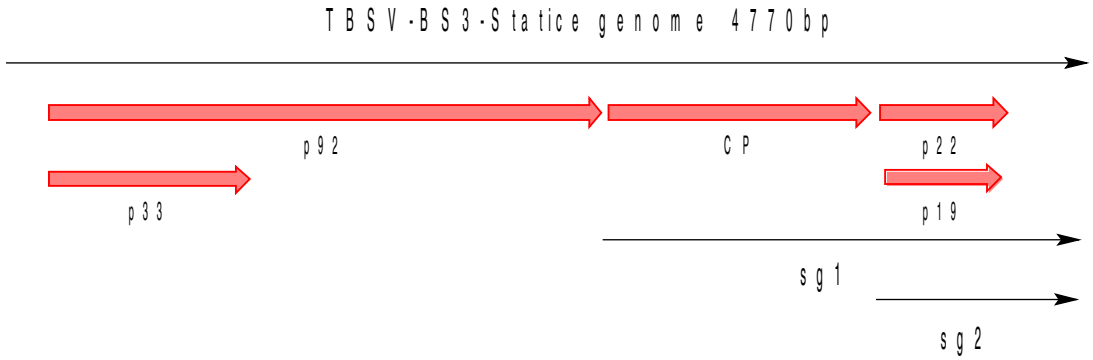


Figure 3: Schematic representation of the genomic RNA sequence of TBSV-BS3-Statice [Krczal et al., 1994; Galetzka et al., 2000]. The 4 ORFs of TBSV are depicted as red arrows. The p92 ORF is the product of read-through of the amber stop codon of p33. CP is translated from the sg1 RNA. p22 and p19 are translated from sg2 RNA.

4.4.1 Replication of TBSV

A RNA virus replicase specifically replicates the RNA of the parental virus or very closely related viral RNAs. This specificity is determined by recognition and attachment of RNA viral replicases to a certain structure in the viral RNA. The replication mechanism of (+) strand RNA viruses follows a basic scheme (Fig. 4).

The replicase synthesizes a (-) strand from the (+) template and then uses this (-) strand as template to produce new (+) strands. Synthesis of new RNA occurs from the 3' to the 5' end of the template. The replication proceeds in a protein/RNA complex called replication complex containing the template RNA, newly synthesized RNA, the viral replicase and host factors. The viral replicase of TBSV is a RNA dependent RNA polymerase (RdRP) and is associated with the peroxisomal membranes, the site of replication of TBSV [Russo et al., 1994].

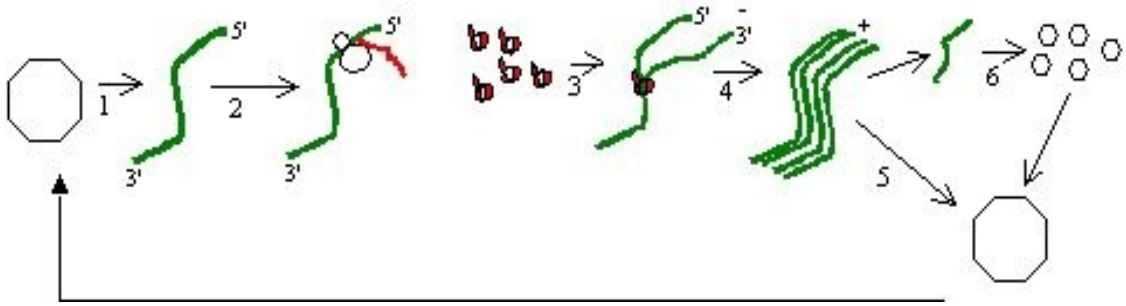


Figure 4: Infection cycle of a tombusvirus. A virus particle infects a plant and the RNA (+strand) is unpacked (1). At the ribosomes of the host the replicase is the first protein to be translated (2), which in turn synthesizes complementary RNA (-strand) (3). The complementary RNA serves as a template for the replicase to produce +RNA-strands (genomic as well as both subgenomic RNAs) (4) the genomic RNA will be packed into virus particles (5). The components of these particles are encoded by sg1 (subgenomic RNA 1) (6).

4.4.2 Defective interfering particles

Defective interfering particles (DI) are completely derived from the parental genome of TBSV and their size varies from approximately 400 to 800 nt. They are mostly found after experimental serial passages of the parental virus on *N. benthamiana* and are produced through stepwise deletions from the parental genome. They depend on the parental virus for replication and therefore interfere with its replication, thus their name defective interfering particles. They reduce the severity of the symptoms caused by their parental virus. The reduction of symptoms is a direct effect of the reduction in parent virus concentration which in turn is an effect of the more efficient recruitment of the replicase by the DI RNA. DIs can represent up to 60% of the viral RNA in infected plants but are not packaged into viral particles at the same percentage.

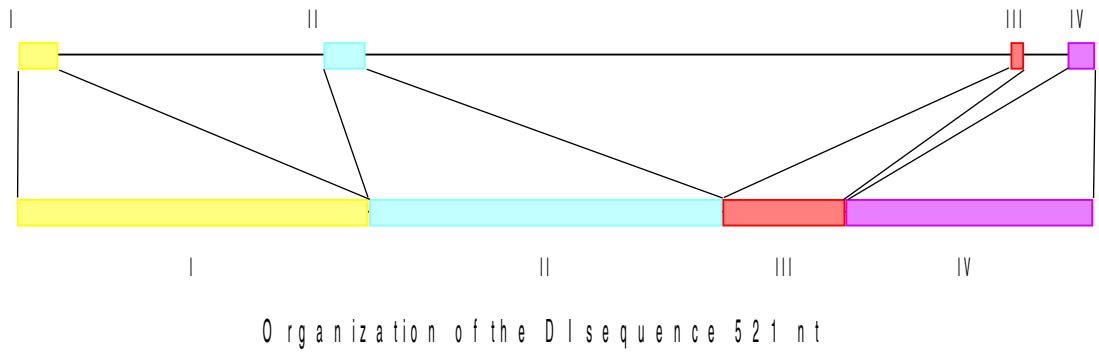


Figure 5: Schematic representation of the genomic TBSV RNA and the associated DI-RNA. The DI-RNA is generated through stepwise deletions from the viral genome. Roman numerals (I to IV) are used as names for the different regions.

DIs are composed of conserved non-contiguous regions of the viral genome (Fig. 5) that accumulate *de novo* after serial passage of the parent virus. The typical DI molecule contains four segments called region I to IV. Region I comprises the complete 5' untranslated region of the TBSV genome up to the start codon of ORF 1. Section II is a sequence just downstream of the amber stop-codon of p33. Regions III and IV are part of the 3' untranslated region of the viral genome. Infected plants contain two classes of TBSV DI RNA, the larger molecules still containing the sequence between regions III and IV. They are thought to be a precursor of the smaller second class of DI molecules. The DI shown in Figure 5 is one of the second class of molecules and was isolated by Galetzka and coworkers [Galetzka et al., 2000].

4.5 Aim

The main goal of this thesis was to develop a novel transient protein expression system which was capable of producing large amounts of recombinant protein. In order to avoid conflicts with already existing patents a new system was designed based on sub-viral RNAs so called defective interfering (DI) RNAs and tomato bushy stunt virus (TBSV). Two strategies were to be evaluated in the protein expression system:

(a) DI-RNA in combination with the wild type helper virus
(b) DI-RNA in combination with a transgenic *N. benthamiana* plant line (TR4) expressing the viral replicase.

The mode of operation for strategy (b) is outlined in Figure 6. A gene of interest is

inserted into a cDNA clone of the DI and plants are inoculated with RNA transcripts made from this clone. The viral replicase supplied by the helper virus or transgenically expressed in the plants multiplies the DI-RNA which is then translated by the plant ribosomes to the protein of interest.

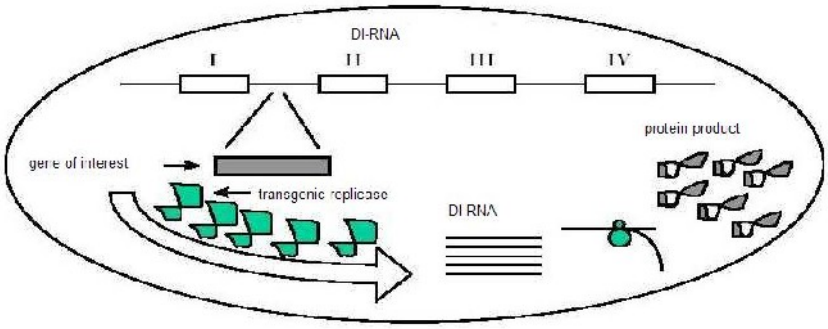


Figure 6: Schematic display of a plant cell of transgenic *N. benthamiana* TR4 plants expressing the TBSV replicase. Outline of the mode of operation of the transient expression system.

These two strategies were additionally tested for their use as a high through-put gene silencing induction system. The gene of interest being replaced by a gene fragment of the silencing target. It was thought that a silencing system based on TBSV would have advantages over existing systems since TBSV has a wide host range.

The investigation of positive effects of DI sequences on protein expression and potential protection from gene silencing was the final part of this study. In this part the expression level of several DI/reporter gene constructs was examined. Wild type plants were compared with transgenic plants in which the reporter gene was silenced.

5 Materials and Methods

5.1 Chemicals

All chemicals that were used in this work were p. a. quality.

5.2 Enzymes

• T4-polynucleotide kinase 10000 U/ml M0201S	New England Biolabs
• *Pfx* DNA polymerase 2000 U/ml M0254S	Invitrogen
• *Taq* DNA polymerase 5U/µl	Eppendorf
• T7 RNA polymerase 50000 U/ml M0251L	New England Biolabs
• All other enzymes	New England Biolabs Roche MBI Fermentas

5.3 Antibodies

• Anti-TBSV-IgG	Loewe
• Anti-TBSV-IgG-AP-conjugate	Loewe
• Anti-HexaHis	Novagen
• Anti-Mouse-IgG-IgG	Sigma
• Anti-Mouse-IgG-IgG-POD-conjugate	Boehringer

5.4 Plants

- *Nicotiana benthamiana*
- *Nicotiana tabacum*
- TR4 (*Nicotiana benthamiana* carrying the replicase gene from TBSV-BS3-Statice under the control of an enhanced 35S promoter)

5.5 Bacterial strains

Table 1: Bacterial strains used for cloning and plant infiltration.

Bacteria	***Function***	***Characteristics***	***Source***
DH5α	cloning	Φ d*lacZ*Δ M15	Invitrogen
XL1-Blue	cloning	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacl^{q}ZΔ M15 Tn10 (Tet^{r})]	Stratagene
Invα	cloning	F′ endA1 recA1 hsdR17 (r_k^-, m_k^+) supE44 thi-1 gyrA96 relA1Φ 80lacZ Δ M15 Δ lacZYA-argF) U169	Invitrogen
ATHV	plant infiltration		[Hood et al., 1986]

5.6 Plasmids

Table 2: Plasmids used in this thesis.

Name	***Antibiotic***	***Usage***
pUC19	Amp	cloning
pUC18	Amp	cloning
pBluescriptSK(+)	Amp	cloning
ppZP200	Strep/Spec	cloning of *Agrobacterium* infiltration constructs
pGJ357	Strep/Spec	cloning of *Agrobacterium* infiltration constructs
ppDI	Amp	cloning and production of RNA transcripts

Name	*Antibiotic*	*Usage*
pTBSV	Amp	cloning and production of RNA transcripts
pTBSV-M	Amp	production of RNA transcripts
pGJ-GUSInt	Strep/Spec	transient GUS expression experiments
pBAR-DI-GUSInt	Strep/Spec	transient GUS expression experiments

5.7 Cultivation media

LB liquid medium
10 g tryptone
5 g yeast extract
10 g NaCl
ddH_2O to 1 l
adjust the pH to 7.0 and then autoclave to sterilize

LB agar
10 g tryptone
5 g yeast extract
10 g NaCl
15 g agar
ddH_2O to 1 l

NZY+ medium
10 g NZ Amine (casein hydrolysate)
5 g yeast extract
5 g NaCl
adjust pH to 7.5 using NaOH
autoclave
Add the following supplement prior to use
12.5 ml 1M $MgCl_2$
12.5 ml 1M $MgSO_4$
10 ml of 2 M filter-sterilized glucose solution.

5.8 Cultivation conditions of greenhouse material

Plants were grown in the greenhouse at a temperature of 26 °C and day/night cycle of 16h/8h.

5.9 Molecular biology methods

5.9.1 Mini-preparation of plasmid DNA with Qiaprep spin miniprep kit

The overnight bacterial culture was submitted for plasmid DNA mini-preparation using the QIAprep spin miniprep kit. Isolation was performed according to the instructions of the manufacturer.

5.9.2 Mini-preparation of plasmid DNA from *Agrobacterium tumefaciens* with Wizard *Plus* DNA purification system

The bacterial culture was submitted for plasmid DNA mini-preparation using the Wizard *Plus* DNA purification system. Isolation was performed according to the instructions of the manufacturer. After isolation, *E. coli* cells were transformed with the purified DNA.

5.9.3 Cryopreservation of bacterial cells

Validated recombinant bacterial clones were maintained as glycerol stocks [300 µl glycerol (87%) + 700 L bacterial culture] at -80 °C.

5.9.4 Determination of DNA and RNA concentration by UV spectrometry

The concentration of DNA was measured by adding 5 µl of the DNA solution to 95 µl of water. The absorption at 260 and 280 nm was measured using a UV photometer (UV-160 1PC Shimadzu). $OD_{260} = 1$ is equal to a DNA concentration of 50 µg/ml [Sambrook et al., 1989].

The concentration of RNA was measured by adding 2 µl of the RNA solution to 98 µl of water. The absorption was measured at 260 and 280 nm using a UV photometer (UV-160 1PC Shimadzu). $OD_{260} = 1$ is equal to a RNA concentration of 42 µg/ml [Sambrook et al., 1989].

5.9.5 T-tailing of plasmid DNA

About 8 µg of *SmaI* digested and gel purified pUC19 plasmid was dissolved in 69 µl of

water. To the resuspended plasmid 10 µl of 10x *Taq* DNA polymerase reaction buffer, 20 µl of 10 mM dTTP solution and 1 µl of *Taq* DNA polymerase were added. The reaction was incubated for 3h at 70 °C. After incubation the DNA was purified by phenolization and subsequent precipitation. The DNA was dissolved in water and stored at -80 °C for analysis.

5.9.6 Restriction enzyme digests

For preparative digests 1 µg of DNA was digested with 10 U of restriction enzyme in the appropriate buffer in a total volume of 20 µl. A 1 µl aliquot was taken after 1 h for electrophoresis analysis.

5.9.7 Agarose gel electrophoresis

Analytical as well as preparative gel electrophoresis of plasmid DNA and PCR fragments was performed as described preciously [Sambrook et al., 1989]. The percentage of the agarose gels ranged from 0.8%-1.5% (w/v) made in 1x TAE, supplemented with the fluorescent intercalating dye ethidiumbromide (0.1 µg/ml). To determine the fragment size and estimate the concentration, DNA markers (Fig. 7) with known sizes of fragments were coelectrophoresed. Bands were visualized using an ultraviolet (UV) transilluminator (306 nm max) and photographs were taken using INTAS computer documentation system.

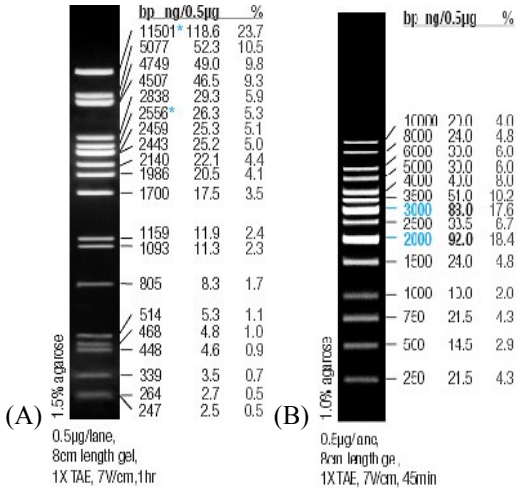


Figure 7: MBI Fermentas DNA markers used for agarose gel electrophoresis. (A) Lambda DNA PstI marker. (B) GeneRuler™ 1kb DNA ladder

5.9.8 Agarose gel extraction

Preparative gel electrophoresis was carried out for purification of DNA fragments. Gels were placed onto a UV transmittable plastic sheet and fragments of interest were excised with a sterile scalpel. DNA was extracted from the gel pieces using the QIAquick gel extraction Kit.

5.9.9 Klenow fragment “fill in” reaction

About 1µg of digested DNA was dissolved in 16.5 µl of water and 2 µl of the 10X reaction buffer provided by the manufacturer (MBI), 0.5 µl 2mM dNTP solution and 1µl of Klenow fragment enzyme were added to the reaction mixture. The reaction was incubated for 10 min at 37 °C, followed by the inactivation of the enzyme by incubation for 10 min at 70 °C.

5.9.10 Phenol/chloroform purification of nucleic acids

Phenolization was performed as follows. The nucleic acid solution was brought up to a volume of 50 µl with water and 50 µl TE buffer equilibrated phenol. The emulsion was vortexed, centrifuged (2 min at 13000 rpm) and the supernatant was transferred to new reaction tube. 50 µl of Tris buffered phenol/chloroform/isoamylalcohol (25:24:1) were added

and mixed. After centrifugation the supernatant was transferred to a new reaction tube and the nucleic acids were precipitated by adding 1/10 volume of 3M NaAc (pH 5.2) solution and 2.5 volumes of EtOH. The DNA was then pelleted by centrifugation (10 min at 18000 rpm) and subsequently washed with 70% EtOH. The pellet was dried and then dissolved in 10 µl of water.

5.9.11 Phosphorylation of nucleic acids using T4 polynucleotide kinase (T4 PNK)

Phosphorylation of DNA was done using T4 PNK from MBI Fermentas. Up to 1 µg of DNA was dissolved in 19 µl of water and 2.5 µl of 10X reaction buffer, 2.5 µl of 10mM ATP solution and 1 µl of T4 PNK were added to the reaction mixture. The reaction was incubated for 15 min at 37 °C, then the enzyme was inactivated by incubation at 70 °C for 10 min.

5.9.12 Dephosphorylation of digested plasmid DNA with shrimp alkaline phosphatase (SAP)

For the dephosphorylation of digested plasmid-DNA 20 µl of the digest reaction were mixed with 3 µl of 10X SAP (shrimp alkaline phosphatase) buffer, 6 µl of H_2O and 1µl of SAP. The reaction was incubated for one hour at 37 °C, and then the enzyme was deactivated by incubation for 20 min at 68 °C. The plasmid DNA was purified by agarose gel extraction (5.9.8).

5.9.13 Ligation with T4 ligase

A vector and DNA-fragment with compatible cohesive termini were ligated in a 10 µl reaction mixture containing DNA (molar ratio vector / DNA-fragment, 1:2), 1 µl 10x ligation Buffer and 1 µl T4 DNA ligase. The mixture was incubated on room temperature for 4 h or overnight.

5.9.14 Production of chemically competent *E. coli* cells

Bacteria were cultivated overnight at 37 °C, 180 rpm in liquid LB medium. 20 µl of the bacterial suspension were used to inoculate 10 ml of LB medium. After incubation at 37 °C, 180 rpm for 3.5 h, the bacteria were pelleted by centrifugation for 5 min at 6000 rpm. The supernatant was discarded and the pellet was gently resuspended with 4 ml of KZB buffer and kept on ice for 20 min. Centrifugation (6000 rpm, 5 min) the supernatant was discarded and the pellet resuspended with 600 µl of KZB buffer. 50-100 µl of suspended competent cells were used for transformation.

5.9.15 Production of electro competent *Agrobacterium tumefaciens* cells

Bacteria were cultivated overnight at 28 °C, 180 rpm in liquid LB medium. 5 ml of the bacterial suspension were used to inoculate 500 ml of LB medium. After incubation at 28 °C, 180 rpm for 3.5 h, the bacteria were incubated on ice for 30 min. The bacteria were then pelleted by centrifugation for 10 min at 4000 rpm. The supernatant was discarded and the pellet was gently resuspended with 5 ml of water. After resuspension the volume was adjusted to 500ml and the cells were centrifuged for 20 min at 4000 rpm. The supernatant was discarded and the cells were resuspended in 5 ml of water. The volume was adjusted to 500 ml and the bacteria were centrifuged for 20 min at 400 rpm. The supernatant was discarded and the pellet was resuspended in 80 ml of an ice cold 10% glycerol solution. After centrifugation (4000 rpm, 20 min) the supernatant was discarded and the pellet resuspended with 500 µl of ice cold 10% glycerol. 50 µl of resuspended competent cells were used for transformation.

5.9.16 Transformation of chemically competent *E. coli* cells

The cloned plasmids were transformed in competent *E. coli* (DH5-alpha, XLBlue, Inv-alpha). To 100 µl of competent *E. coli* 4 µl ligation mixture were added and incubated on ice for 30 min. A heat shock was performed by incubating the mixture at 42 °C for 45 s, then quickly put on ice. 175 µl of prewarmed SOC solution were added and incubated at 37 °C for 1 h. The mixture was plated on 50 µg/ml antibiotic supplemented LB solid media and incubated overnight at 37 °C

5.9.17 Transformation of electro competent *Agrobacterium tumefaciens* cells

The electro competent cells were thawed on ice and 1 µl of plasmid DNA was added. The cells were then transferred to a chilled electroproration cuvette, which was placed in the electroporator. The settings of the apparatus were as follows. Capacitance at 25 µF, charging voltage at 2.5 kV and resistance at 200-400 ohm. After the application of the electro pulse 450 µl of SOC medium were added. The suspension was transferred to a 2 ml reaction tube and incubated for 1 h at 28 °C. After incubation, 200 µl of the bacterial culture were plated on the appropriate antibiotics supplemented solid LB medium.

5.9.18 PCR and sequencing primers

Table 3: Primer pairs used for PCR reactions and sequencing

Name	*Sequence (5' - 3')*
GUS1	aat tgc cat ggt acg tcc tgt aga aac
GUS2	aat tgt acg tat cat tgt ttg cct ccc tgc
gfp5-NcoI	act ctt gac cat ggt aga tct gac tag ta
gfp3-KpnI	gct ggt acc aat tca cac gtg gtg gtg gt
p22-EcoRI-5	aga cga att cat gga tac tga ata cga aca
p22-KpnI-3	ttc cgg tac caa ctc aga ctg aag agc ctg tct
p19-ATG-Mut	cca tgg aat tca tgg ata ctg aat acg aac aag tca ata aac cct gga ac
5-CP-NcoI	aat tac cca tgg caa tga caa cga gaa ata ac
3-CP-KpnI	aat tta ggt acc tca tag taa gtt aac aac att ag
3-scFv.KpnI	gtc acg ggt acc att cag atc ctc ttc tg
5-scFv-NcoI	gcg gcc cag ccg gcc atg gcc gag
dpRT101_seq1	cga cgt tgt aaa acg acg gcc
dpRT101_seq2	ccc tta tct ggg aac tac tca
dpRT101_seq3	gtc ctc tcc aaa tga aat g
T7-TBSV-3	gag acg cgt aat acg act cac tat agg gct gca ttt ctg caa tg
DI-3-BamHI	gga ctc tag aag atc ccc ggg ct
TBSV-Blunt	aga aat tcc cca gga ttt ctc gac c
scFv-DIG	ggc ggt tca ggc gga ggt agc ggc gg
3-DI-III	aca tac gtt gtc aga ttc aca ctc g

Name	*Sequence (5' - 3')*
5-DI-III	gta aga cag gct ctt cag tct g
5-TBSV-T7-MunI	cat tat caa ttg taa tac gac tca cta tag gaa att ccc c
FtsH-5	caa atg gca gtt gca ctt gg
FtsH-3	cat cga tga aaa ggc tca tg
5-FtsH-KpnI	ttt tat ggt accc caa atg gca gtt gca ctt gg
3-FtsH-EcoRI	aaa aaa aga att ctc atc gat gaa aag gct cat
5-p19-Stop	cga ttt cga att aag ata atc ccc ttg gtt tct aga aaa gc
IR28	ggg ctg cat ttc tgc aat g
p19-ATG-M-TBSV-F	gga tac tga ata cga aca agt caa taa acc ctg gaa cga gct ata caa gg
p19-ATG-M-TBSV-R	cct tgt ata gct cgt tcc agg gtt tat tga ctt gtt cgt att cag tat cc
5-p19stop	cga ttt cga att aag ata atc ccc ttg gtt tct aga aaa gc
5-gfp-MunI-linker	caa ttg agt aaa gga gaa gaa ctt ttc act gga
3-gfpER-KpnI	ggt acc tta aag ctc atc atg ttt gta tag ttc atc cat gcc atg
5-gfp5-NcoI2	cca tgg gta tga aga cta atc ttt t
IR28 Marcello	gac tct aga gga tcc ccg ggc tgc att tct gca atg
5-TBSV-EcoRI	gaa ttc aga aat tcc cca gga ttt ctc g
3-TBSV-SalI	gtc gac ggg ctg cattt ctg caa tgt tcc gg
3-Rep-EcoRI	ggc aga att caa cct ctt cc
pUC-FW	cag cac tga ccc ttt tgg gac cgc
pUC-RV	agc gga taa caa ttt cac aca gga
35S-FW	cac tat cct tcg caa gac cc

5.9.19 PCR with *Taq* DNA polymerase

Taq DNA polymerase was only used for control PCR reactions and never for the amplification of DNA fragments for cloning.

For a total reaction volume of 50 µl, 1 µl of the appropriate 3'-primer, 1 µl of the appropriate 5'-primer, 1 µl of dNTP solution (10 mM), 2.5µl of DNA (~0.05 µg), 5 µl of 10X PCR-buffer and 0.5 µl of Taq DNA-polymerase (5U/µl) were added to 39.3 µl of water. The applied PCR cycler-program is shown in Figure 8.

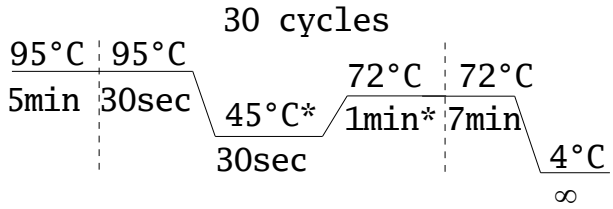


Figure 8: PCR cycler-program used for standard analytical PCR. *This temperature and the extension time depend on the used primer pair and the size of the desired PCR product.

5.9.20 PCR with *Pfx* DNA polymerase

Pfx DNA polymerase is a high-fidelity thermophilic DNA polymerase which was used for cloning PCR-reactions because of it's 3'-5' proofreading exonuclease activity and generates 95% of blunt ended PCR products [Takagi et al., 1997].

For a total reaction volume of 50 µl, 1 µl of the appropriate 3'-primer (10 pmol/µl), 1 µl of the appropriate 5'-primer (10 pmol/µl), 1.5 µl of dNTP solution (10 mM) 5 µl of DNA (~0.05 µg), 10 µl of 10X PCR-buffer (recommendation of the manufacturer) and 0.5 µl of *Pfx* DNA-polymerase (2U/µl) were added to 31 µl of water. The applied PCR cycler-program is shown in Figure 9.

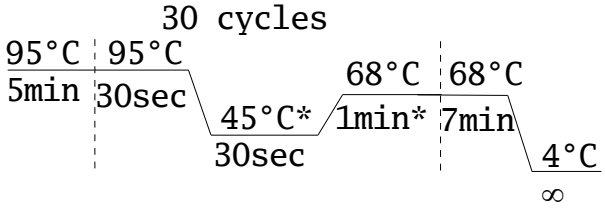


Figure 9: PCR cycler-program for standard preparative PCR reactions. *This temperature and the extension time depend on the used primer pair and the size of the dezired PCR product.

5.9.21 Sequencing PCR

Sequencing reactions were carried out using the following protocol. For a 10 µl reaction about 300-700 ng of DNA diluted in 5 µl of water were used, 1 µl of the appropriate primer

(10 pmol/µl), 2 µl of 5x sequencing-buffer and 2 µl of BigDye-Terminator enzyme mix (Genterprise, Mainz) were added. The reactions were run with the cycle program shown in Figure 10. The samples were then send to Genterprise GmbH in Mainz to be analyzed.

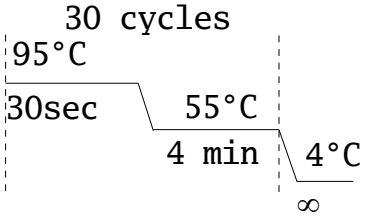


Figure 10: PCR cycler-program for standard sequencing PCR reactions.

5.9.22 QuickChange™ site-directed mutagenesis kit

The QuickChange site-directed mutagenesis kit from Stratagene was used according to the manufacturers instruction.

5.9.23 RNA extraction from plants

RNA extraction was done using phenol/chloroform extraction method with the following protocol. One small leaf was ground with liquid nitrogen using autoclaved mortar and pestles. Then 600 µl of EB-buffer were added and the suspension was transferred to a reaction tube. 600 µl of phenol were added to the suspension and vortexed vigorously for one minute. For isolation of protoplast RNA the frozen samples were just vortexed with buffer EB and phenol. The samples were then centrifuged for two min, the supernatant was transferred to a new cup and 200 µl of phenol and 200 µl of chloroform were added. After vortexing the emulsion was centrifuged for 10 min and the supernatant was again transferred to a new tube. 400 µl of chloroform were added to the supernatant and the emulsion was again vortexed and centrifuged for two min. After centrifugation the supernatant was again transferred to a new tube and 1 ml of ice-cold ethanol was added. The mixture was allowed to precipitate for 30 min at -20 °C and was then centrifuged for 15 min at 18000 rpm. The pellet was washed with 600 µl of ice-cold 70% ethanol and dried under vacuum. The pellet was dissolved in 35 µl H_2O and the quality of the RNA was checked by agarose gel electrophoresis of a 3 µl aliquot

of each extraction. Concentration of the RNA was determined by UV-spectrometry.

10X EB-buffer

7.7g Glycine

5.8g NaCl

20ml EDTA 0.5M (pH8)

2g SDS

1g N-Lauryl sarcosine

5.9.24 RT-PCR using SuperScript One-Step RT-PCR kit from Invitrogen

The reaction mixture for each sample consisted of 25 µl 2X reaction mix, 1 µl of sense primer (10 µM), 1 µl of antisense primer (10 µM), 1 µl of RT/Taq mix, 1 µl of template RNA solution and 21 µl of autoclaved distilled water. The reaction was performed in a PCR-cycler with the program shown in Figure 11.

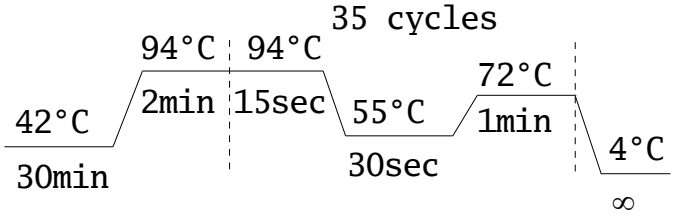


Figure 11: PCR cycler-program used for standard RT-PCR.

5.9.25 Northern blot

The RNA samples (5.9.23) were prepared for electrophoresis by adjusting the volume of the samples to 12.5 µl, so the concentration of the samples was three to 10 µg per 12.5 µl. 12.5 µl of denaturing puffer were added and the samples were then incubated at 65 °C for 10 min before they were kept on ice.

Subsequently, samples were mixed with RNA loading buffer and electrophoresed on a RNA-denaturating agarose gel [~1.2% (w/v); see below] for 4-5 hours at 75V.

The denatured agarose gel was proceeded for capillary transfer of RNA onto positively charged nylon membranes as described preciously [Sambrook et al., 1989]. The RNA was

immobilized on the membrane by incubation at 80 °C for 2 h. The membrane was proceeded for pre-hybridization with DIG Easy Hyp buffer (Roche Diagnostics) at 68 °C for 1 h and hybridized in a hybridization oven (Amersham) at 50 °C for 12-16 h. The RNA immobilized on the membrane was hybridized to a DIG labeled DNA probe made according to manufacturers instructions (DIG PCR labeling Kit, Roche Diagnostics) or to a DIG labeled DNA primer ordered from MWG Biotech. After hybridization the blot was washed twice for 5 min with 2 x SSC/0.1% SDS at 68 °C. Then again twice for 5 min with 0.1xSSC/0.1% SDS at 68 °C. Further development of the blot was done to the manufacturers instructions (Chemiluminescence DIG Detection Kit, Roche Diagnostics). The X-ray films were exposed for 1 to 10 min depending on signal intensity.

10x MOPS
400 mM MOPS (pH 7.0)
100 mM NaAc
10 mM EDTA

Denaturing puffer
500 µl deionized formamide
120 µl deionized formaldehyde
200 µl 10x MOPS
120 µl H_2O
1 µl EtBr

RNA-Loading buffer
30%Ficoll
10 mM EDTA (pH 8,0)
0.25% bromophenole blue
0.25% xylencyanol

RNA-Denaturating gel
100 ml H_2O
11.5ml MOPS(10x)
1.38g agarose
after boiling the solution was cooled to 60 °C and 3.45ml formaldehyde were added.

20x SSC
175.3 g NaCl
88.2 g Na-Citrate
ad. 1000ml
pH 7.2
10xSSC, 5xSSC, 2xSSC/0,1 % SDS and 0.1x SSC/0.1% SDS were made from 20xSSC

Maleat buffer
11.6 g maleic acid
8.76 g NaCl
pH 7.5

Developing buffer
12.11 g Tris-HCl
5.84 g NaCl
pH 9.5

5.9.26 *In vitro* transcription of RNA

About 2 µg of the appropriate plasmid were digested (2 h at 30 °C, 20 µl total reaction volume) with 20 U of the restriction enzyme *SmaI* to linearize the DNA. Gel electrophoresis was done to check if digestion was complete. Alternatively to obtain (-) strand RNA a PCR was done using the TBSV-Blunt/T7-TBSV-3 primer pair. This introduces a T7 promoter at the 3' end of the DI sequences. To purify the DNA, 20 µl phenol and 20 µl chloroform were added to the reaction mixture, vortexed and centrifuged for 2 min at 13000 rpm. The supernatant was transferred to a new reaction tube, 40 µl of chloroform were added, this mixture was vortexed and centrifuged for 2 min at 13000 rpm. The supernatant was transferred and the DNA was precipitated by adding 4 µl of a 2 M NaCl solution and 88 µl of ice-cold ethanol. The reaction was then centrifuged for 10 min at 18000 rpm, the obtained pellet was washed by adding 200 µl of 70% ethanol and another centrifugation step (2 min at 13000 rpm). The DNA was dissolved in 80 µl of H_2O, 10 µl of RNA NTP's (2.5 µM of each NTP), 10 µl of 10X T7 RNA polymerase buffer, 1 µl of RNase inhibitor (RNA Guard, Invitrogen) and 2 µl of T7 RNA polymerase were added to the solution. The reaction mixture was incubated for a minimum of 3 h at 37 °C. To check if RNA is present in the reaction mixture gel electrophoresis was performed with 2 µl of the mixture. For transfection of protoplasts the reaction was treated with DNase to remove the plasmid-DNA. To 100 µl of transcription reaction 5 µl of DNase were added and incubated at 37 °C for 1 h. After incubation the RNA was purified by phenol/chloroform as described in section 5.9.10.

5.10 Protein analysis

5.10.1 Histochemical GUS staining

For the histochemical detection of β-D-glucuronidase (GUS) expression in plants leaves were covered in an appropriate volume of GUS staining solution and incubated for at least 3 h at 37 °C or overnight. After incubation the leaves were destained (removal of chlorophyll) in 70% EtOH. The staining patterns were documented using a standard computer scanner.

X-GlcA stock solution
50mg/ml X-GlcA in DMF

$K_3[Fe(CN)_6]$ stock solution
100mM $K_3[Fe(CN)_6]$

$K_4[Fe(CN)_6]$ stock solution
100mM $K_4[Fe(CN)_6]$

GUS staining solution
50mM Sodium phosphate buffer pH 7
0.025% Triton X 100
0.5 mM $K_4[Fe(CN)_6]$
0.6mM $K_3[Fe(CN)_6]$
500 µg/ml X-GlcA

5.10.2 Sample preparation for SDS-PAGE

For the use in SDS-PAGE (5.10.3) plant leaves together with 200 µl of SDS-PAGE sample buffer were ground with mortar and pestles. The samples were then incubated at 100 °C for 10 min after which they were centrifuged for 10 min. The supernatant was transferred and used for SDS-PAGE.

5.10.3 SDS-PAGE

For the experiments the Biorad mini-protein III dual slab gel was used. For a total volume of 12.5 ml (sufficient for 2 gels) of 12.5% running gel, 5.2 ml of 30%/0.8% acrylamide/bisacrylamide (Roth), 3.1 ml distilled water, 0.125 ml (10%) SDS solution, 4.2 ml 1.5 M Tris-HCl (pH 8.8), 16 µl TEMED (N,N,N,N,tetramethylethylenediamine) and 125 µl of 10% APS as reaction starter were mixed. The mixture was then poured between the glass plates of the gels. Then it was overlaid with 1-2 ml water saturated butanol. After polymerization of the gel, the overlay was removed and the surface of the gel was cleaned with running buffer. The 5% collecting gel, which was composed of 0.67 ml 30%/0.8% acrylamide/bisacrylamide, 3.63 ml water, 0.1 ml SDS (10%), 0.67 ml 1M Tris-HCl (pH 6.8), 200 µl 10% APS and 12 µl TEMED, was poured and then the combs were placed. Electrophoresis was carried out in Laemmli [Laemmli, 1970] buffer system. After filling the upper and lower reservoir with running buffer, the combs were removed and protein samples (25 µl per well) were applied to the slots using a Hamilton syringe. For protein stacking the gel was run at 90 volt and for the protein separation the gel was run at 175 Volt.

10X Protein electrophoresis buffer

144g Glycine
30g Tris-Base
100 ml 10% SDS-solution
ad. 1000 ml ddH_2O
pH 8.8

4X Tris-HCl / SDS buffer

6.5 g Tris-Base
ad. 100 ml ddH_2O
adjust pH to 6.8
add 400 mg of SDS

Protein sample buffer

7 ml 4X Tris-HCl / SDS buffer
3 ml Glycerol
1 g SDS
0.93 g DTT
1.2 mg Bromophenol blue
ad. 10 ml ddH_2O

10% Ammonium persulfate (APS)

0.2 g APS
ddH_2O to 2 ml
store at -20 °C

10% SDS (sodium dodecyl sulfate)

10 g SDS
ddH_2O to 100 ml

5.10.4 Coomassie staining

The gels were stained for 30 min by shaking in the staining solution and were destined for 2 h with destaining solution. Staining solution was composed of 2 g Coomassie-Brilliant-Blue R250 , 450 ml methanol, 50 ml acetic acid and 500 ml of H_2O. Any undissolved particles were filtered off before use. Destaining solution was prepared using 300 ml of methanol, 100 ml of acetic acid and 600 ml of H_2O.

5.10.5 Western blot

All fiber pads, filter papers, and the transfer membrane were soaked in transfer buffer previous to the assembly of the transfer cassette. It was made sure that no bubbles were trapped in the filters or fiber pads. (PVDF membranes were prewetted in methanol before they were soaked in transfer buffer.). The transfer cassette was assembled according to the manufacturer's instructions.

The transfer was run at 175 mA for 45 min [Kyhse-Andersen, 1984] in a semi-dry blot

apparatus from Biorad or at 30 V, or over night in an tankblotting apparatus from the same company.

After the transfer, the membrane was placed in blocking solution on a rocker platform for at least 60 min at RT or over night at 4 °C. The membrane was incubated with primary antibody diluted (1:1000) in blocking solution on a rocker plate 1 h at RT. The membrane was washed twice with TBST and twice with blocking solution each for 10 min. The secondary antibody POD-conjugate (1:10000 in blocking solution) was incubated with the membrane for 30 min at RT on a rocker plate. The membrane was washed 4 times with TBST for 10 min each. Substrate solution was added and allowed to react for 1 min. Membrane was removed from the substrate and laid between to transparency films. Air bubbles were removed using a paper towel. Film exposure was done for 1-30 min and development was done according to manufacturer's instructions.

Transfer buffer for westernblotting
3.03 g Tris-Base
14.4 g Glycine
100 ml Methanol
ad. 1000 ml ddH_2O
pH 8.4

TBS buffer pH 7.4
50 mM Tris-HCl
200 mM NaCl

TBST buffer pH 7.4
50 mM Tris-HCl
200 mM NaCl
0.1% Tween 20

5.10.6 ELISA (enzyme linked immuno sorbent assay)

ELISA was performed using 96 well Nunc Maxisorb ELISA microtiter plates. The plates were coated with the first antibody by incubating each well with 200 µl of first antibody solution (1:200 in coating buffer) for 4 h at 37 °C. After 4 washing steps the samples were applied to each well (200 µl) and incubated over night at 4 °C.

After another 5 washing steps and the addition of 200 µl per well of secondary antibody (coat protein-antibody-AP-conjugate) solution (1:200 in sample buffer) the plates were incubated at 37 °C for 4 h. Enzymatic assay was performed after 5 washing steps and addition

of 200 µl of substrate solution per well. Detection of the results was done after 1-2 h incubation with an ELISA reader (Anthos Reader, Anthos Microsystems).

ELISA Coating buffer
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
ddH_2O to 1000ml pH 9.6

ELISA Sample buffer
8 g NaCl
2.9 g Na_2HPO_4 x $12H_2O$
0.2 g KH_2PO_4
0.2g KCl
0.5 ml Tween 20
add ddH_2O to 1 L pH 7.4

ELISA Conjugate buffer
20 g polyvinyl pyrrolidone
2 g BSA
0.1 g NaN_3
add ddH_2O to 1 L pH 7.4

ELISA Substrate buffer
97 ml diethanolamine
0.2 g $MgCl_2$ x 6 H_2O
add ddH_2O to 1 l, pH 9.8

ELISA Substrate solution
1mg/ml 4-nitrophenyl phosphate di-Na-salt in substrate buffer
Prepare immediately prior to use.

5.11 Inoculation of *Nicotiana benthamiana* with RNA transcripts

For the inoculation of 10 *N. benthamiana* plants with RNA transcripts about 40 µl of each transcription reaction were used. Inoculation buffer was added up to a volume of 600 µl. 20 µl of this solution were applied to each leaf (three leaves per plant) and were spread with autoclaved glass spatulas.

Inoculation buffer
522 mg K_2HPO_4
375 mg Glycerol
1 g Bentonit
1 g Cenit
adjust pH to 9.2 with KOH

5.12 *Agrobacterium* infiltration

Agrobacterium infiltration was essentially done as described by Kapila and coworkers [Kapila et al., 1997]. The *Agrobacteria* strain transformed with the desired plant transformation vector was grown in 20 ml of YEB media (pH 5.6) containing the appropriate antibiotics (300 µg/ml spectinomycin, 300 µg/ml streptinomycin an 15 µg/ml rifampicine) and 10 µM acetosyringone at 28 °C over night to an OD600 of maximum 1.0. The bacteria were then pelleted and resuspended in induction media at OD 600 of 0.8. After incubation for 2 hours at room temperature the bacterial suspension was applied to the plants using a needle-less syringe.

YEB media pH 5.6
5 g Beef extract
1 g Yeast extract
5 g Sucrose
25 ml 0.4 M MES pH 5.6
add ddH_2O to 1 L pH 5.6

Induction media
1 ml 1M MgCl
2.5 ml 0.4 M MES pH 5.6
20 µl 0.5 M Acetosyringone
add ddH_2O to 100 ml pH 5.6

5.13 Fluorescence microscopy and photography of GFP

GFP expression was monitored with a hand-held UV light or under a Leica MZ12 microscope with GFP Plus fluorescence unit. Photos were taken with Nikon Coolpix digital camera and a yellow optical filter.

5.14 ClustalX alignments

For multiple alignments the ClustalX program [Thompson et al., 1997] was used with the following alignment parameters. A gap opening penalty of 10, a gap extension penalty of 0.2 and a DNA transition weight of 0.5. The DNA weight matrix was IUB.

5.15 Protoplast isolation and transfection

The transfection was done as described preciously [Sheen, 2001] with some modifications. The leaves were cut from the plants and put in a petri dish. They were washed with MgMan solution and cut into 0.5-1 mm leaf strips with a fresh razor blade without excessive wounding. For about 10 g of leaves 50 ml of enzyme solution were used. The leaf strips were transferred to a petri dish together with the enzyme solution and covered with a filter pad to ensure that the strips are submerged in the enzyme solution during vacuum infiltration. A vacuum was applied for 10 min and then released rapidly. The digestion was continued for another three hours in the dark, without shaking. After incubation the enzyme solution turned green indicating the release of protoplasts. Protoplasts were released by shaking at 80 rpm for 1 min. To float the protoplasts, they were centrifuged at 80 g in a round-bottomed tube for 10 min. The floating protoplasts were collected with a Pasteur pipette, then washed and resuspended in W5 solution (2x10 ml). About 5-7x10^5 protoplasts were pelleted for each transfection.

For PEG transfection all steps were carried out at room temperature. To the protoplast pellet 40 µl of purified and DNA free RNA transcripts were added (as positive control 20 µg of pCat-gfp [Reichel et al., 1996] was adopted). Then 200 µl of a 10% mannitol solution were added, gently mixed for 15 s 300µl 40% PEG solution were added to the reaction and gently mixed for 15 s. Next, two times 600 µl of 10% mannitol were added and mixed gently. The mixture was incubated for 15 min on ice. After incubation the protoplasts were pelleted by centrifugation at 70 x g for 6 min. The supernatant was removed and the protoplasts were resuspended in 2 ml MS 0.4. After incubation at 25° C for 24 h (16 h light/8h dark) the protoplasts were harvested by centrifugation at 100 x g, frozen in liquid nitrogen and stored at -80 °C for analysis.

Enzyme solution

1-1.5 % cellulase R10

0.2-0.4 % macerozyme R10 (Yakult Honsha, Tokyo, Japan)

14 % sucrose

10 mM $CaCl_2$

20 mM KCl

20 mM MES, pH 5.7

0.1 % BSA (Sigma A-6793)

The enzyme solution is light brown but clear (passed through a 0.22 µm filter).

PEG solution (40 %, v/v)

40 g PEG4000 (Fluka, #81240)

3 mM $CaCl_2$

4 mM MES

ad 100ml

W5 solution

154 mM NaCl

125 mM $CaCl_2$

5 mM KCl

4 mM MES (pH 5.7)

MgMan solution

10 % mannitol

15 mM $MgCl_2$

4 mM MES (pH 5.7)

MS 0.4

2.2 g MS salts incl. vitamins

0,4 M sucrose

(pH 5.7)

6 Results

6.1 Functionality of the TBSV replicase expressed in transgenic plants

The transgenic *N. benthamiana* plants (TR4) used in this study carried the viral replicase gene under the control of an enhanced 35S promoter. These plants have been previously tested for the presence of replicase mRNA by northern Blot analysis (Galetzka, unpublished results). To determine whether the replicase was functional, plants were inoculated with a TBSV mutant [Boonrod et al., 2005] which carries a nonfunctional replicase gene. The mutation rendered the RNA binding domain of the replicase non functional but replication of the virus is still possible if a functional replicase is provided *in trans*. If the transgenic viral replicase is functional it should be able to complement the viral replication *in trans* which would result in virus infection.

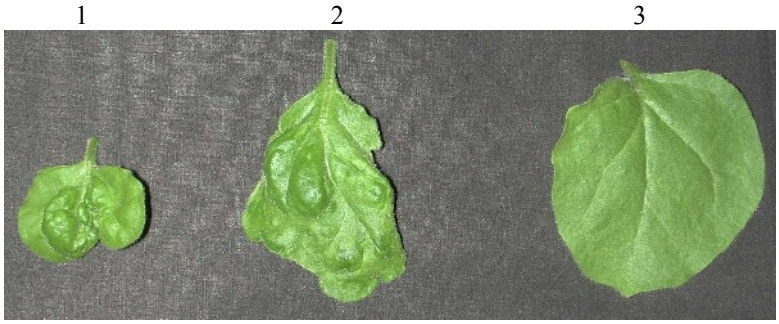


Figure 12: Leaves of TR4 and *N. benthamiana* plants inoculated with RNA transcripts of a mutated TBSV clone. 1 and 2 are systemically infected TR4 leaves (11 dpi) showing virus infection symptoms, 3 is the nontransgenic control.

TR4 plants as well as non transgenic *N. benthamiana* were inoculated with RNA transcripts of a TBSV mutant carrying a nonfunctional replicase gene (TBSV-M). Viral infection symptoms were observed after 11 days only in the TR4 plants (Fig. 12). ELISA tests confirmed these results by detection of the viral coat protein (data not shown). Infectivity of the TBSV-M was about 80-90% in the T1 generation of TR4 plant lines, demonstrating that this “complementation system” can be utilized to test defective interfering RNA (DI) vector-mediated protein expression (see below).

6.2 Protein expression using the DI vector system

In order to use DI vectors for protein expression different constructs were generated containing reporter genes and viral genes. The constructs described here were all based on a plasmid previously designed in our institute [Galetzka et al., 2000]. The plasmid (ppDI) carries a DI sequence isolated from TBSV-BS3-Statice bearing a multiple cloning site (MCS) between region I and II. This position proofed to be the most stable insertion site for foreign genes in the related cymbidium ringspot tombusvirus [Burgyán et al., 1994]. It also contains the T7 promoter sequence (5'-taatacgactcactatag-3' [Jorgensen, 1991]) for generation of *in vitro* RNA transcripts. Several experiments were performed to evaluate the use of the DI-RNA system for the expression of proteins in plants. The experiments were carried out on transgenic, replicase expressing, TR4 plant lines as well as on wild type *N. benthamiana* as control. The expression experiments were additionally carried out with TBSV as helper virus, to exclude the possibility that the activity of the heterologous expressed replicase is a limiting factor.

6.2.1 Cloning strategy of the ppDI-GUS construct

By using the GUS1 and GUS2 primers (5.9.18) in a PCR reaction a *NcoI* site was introduced at the 5' end of the GUS gene (Fig. 13). The 35SGUS plasmid (John Innes Center) was used as template.

```
GUS1
aatgccATGGTACGTCCTGTAGAAACC
------TACCATGCAGGACATCTTTGG---*GUS 2kb*----GCAGGGAGGCAAACAATGATAC--------
                                            CGTCCCTCCGTTTGTTACTATGcatgttaa
                                            GUS2
```

Figure 13: Schematic presentation of the PCR primers and their template binding sites. Primers are depicted with a grey background. Capital letters represent complementary sequences of primer and template. The *NcoI* site is underlined.

The PCR product was digested with *NcoI*, run on a 1% (w/v) agarose gel and the expected ~2 kb fragment was purified (5.9.8). The purified fragment was ligated into the ppDI vector which had been cut with *NcoI* and *SnaBI* (blunt end). The recombinant plasmids (ppDI-GUS) were identified by *SnaBI* digestion. Plasmids releasing a ~1.4 kb fragment were regarded as positive.

6.2.2 Inoculation experiments with DI-GUS RNA transcripts

TR4 plant lines as well as nontransgenic *N. benthamiana* were inoculated (5.11) with RNA transcripts obtained from the ppDI-GUS plasmid. After one and ten days the inoculated leaves were harvested and GUS-staining (5.10.1) was performed. None of the analyzed leaves showed any GUS activity (data not shown). To determine whether the failure of GUS expression was based on an affected viral replicase, inoculation experiments were repeated using TBSV-BS3-Statice transcripts as "helper virus". Upon co-inoculation with helper virus the DI vector was assumed to be replicated by the TBSV-BS3-Statice expressed RdRP. However, these experiments also did not result in GUS expression. Since it has been shown that large inserts tend to be unstable in viral vectors [Scholthof, 1999] and GUS being a large insert with 2 kb, it was decided to use GFP (0.8 kb), a single chain antibody fragment with a C-terminal Myc-tag (scFv, 0.7 kb) against the FUS3 plant transcription factor [Meinke et al., 1994], the coat protein of TBSV (1 kb), and the movement protein of TBSV (0.5 kb) as alternative reporter genes. Especially the viral coat protein was a promising candidate for the expression using this system as it has been previously reported for a different t*ombusvirus* [Burgyán et al., 1994].

6.2.3 Introduction of GFP, scFv, and viral coat protein genes into the vector

The GFP gene was cloned according to the following strategy. The gene was amplified by PCR to generate a *KpnI* site at the 3' end. The *KpnI* and the already existing *NcoI* site at the 5' end were used for cloning. The plasmid pCambia1302 (Accession AF234298) was utilized as PCR template. The GFP gene in this plasmid carries a C-terminal His-tag.

```
gfp5-NcoI
actCTTGACCATGGTAGATCTGACTAGTA
   GAACTGGTACCATCTAGACTGATCAT-mGFP 0.8kb--ACCACCACCACGTGTGAATTGGTgaccag
                                          TGGTGGTGGTGCACACTTAACCATGGTCG
                                           gfp3-KpnI
```

Figure 14: Schematic presentation of the PCR primers and their template binding sites. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. The *NcoI* and *KpnI* sites are underlined.

The PCR product was digested with *NcoI* and *KpnI*, run on an 1% (w/v) agarose gel and

the expected ~0.8 kb fragment was purified (5.9.8). The fragment was ligated into the ppDI vector which had been digested with the same restriction enzymes. The recombinant plasmids (ppDI-mGFP, Appendix, Fig. 52) were identified by *NcoI/KpnI* digestion. Recombinant plasmids releasing a ~0.8 kb fragment were regarded as positive.

The viral coat protein gene was amplified by PCR using the 5-CP-NcoI/3-CP-KpnI primer pair to introduce a 5' *NcoI* site and a 3' *KpnI*. pTBSV [Galetzka et al., 2000] a full-length TBSV-BS3-Statice clone was used as template.

```
5-CP-NcoI
aattaccccATGGCAATGACAACGAGAAATAAC
gtgttctcTACCGTTACTGTTGCTCTTTATTG--CP 1.2kb-  GATTACAACAATTGAATGATACTagagaacactcc
                                              CTAATGTTGTTAACTTACTATGAggtacctaaatt
                                              3-CP-KpnI
```

Figure 15: Schematic presentation of the PCR primers and their template binding sites. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. The *NcoI* and *KpnI* sites are underlined.

The PCR product was digested with *NcoI* and *KpnI*, run on an 1% (w/v) agarose gel and the expected ~1.2 kb fragment was gel-purified (5.9.8). The purified fragment was then ligated into the ppDI vector which had been likewise digested. The recombinant plasmids (ppDI-CP, Appendix Fig. 53) were analyzed by *NcoI/KpnI* digestion. Recombinant plasmids releasing a ~1.2 kb fragment were regarded as positive.

The scFv gene was cloned into ppDI according to the above strategy using the 5-scFv-NcoI/3-scFv-KpnI primer pair and FC8-scFv plasmid (Boonrod, unpublished results) as template. The resulting plasmid map is presented in the Appendix (Fig. 51).

6.2.4 Insertion of the viral movement protein into the vector

It was assumed that one problem of the DI vector system was based on the inability of the DI-RNA to move systemically through the plant. Thus, the viral movement protein was cloned into the DI vector to enable DI-RNA movement. As the viral movement protein ORF contains an internal *NcoI* site the cloning strategy described above could not be applied here. Thus, the gene was amplified by PCR using the primers 5-p22-EcoRI and 3-p22-KpnI. Amplification of the pTBSV plasmid DNA with this primer pair resulted in the generation of

an *EcoRI* site at the 5' end and a *KpnI* site at the 3' end. The PCR product was digested with *EcoRI* and *KpnI*, run on an 1% (w/v) agarose gel and the expected ~0.5 kb fragment was gel-purified (5.9.8). The purified fragment was ligated into the likewise digested ppDI vector. The recombinant plasmids (ppDI-p22, Appendix, Fig. 54) were analyzed by *EcoRV* digestion. Recombinant plasmids releasing a ~0.5 kb fragment were regarded as positive.

Apart from the viral movement protein ORF this construct also contained the p19 ORF. A mutation of the p19 ORF was required because a functional p19 would interfere with our experiments. A primer was designed (p19-ATG-Mut, Fig. 16) that allowed to mutate the start codon of the p19 gene without altering the amino acid composition of p22. The start codon of p19 was changed from ATG to CTG. This substitution resulted in a codon change in the p22 gene from a CCA to CCC (Fig. 17). Both the CCA and the CCC code for proline. Therefore, the amino acid sequence of p22 was not altered. The mutation was introduced via PCR amplification with the p19-ATG-Mut primer together with the 3-p22-KpnI primer.

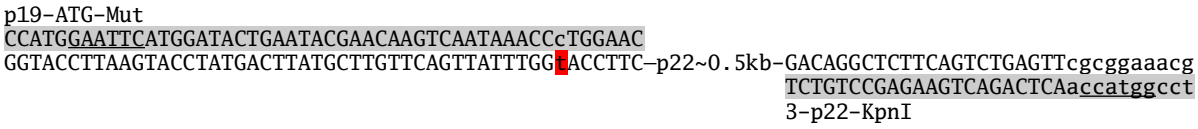


Figure 16: Schematic presentation of the PCR primers and their template binding sites. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. The *EcoRI* and *KpnI* sites are underlined. The ATG mutation is shown with a red background.

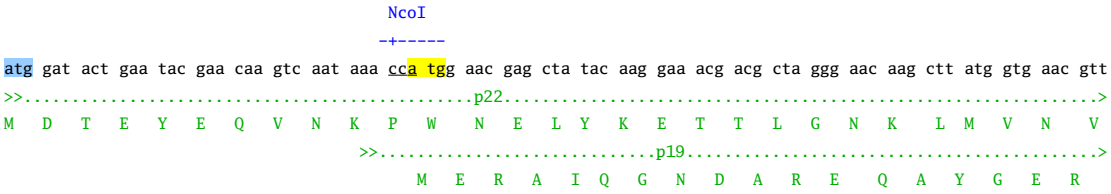


Figure 17: Schematic display of the partial coding sequence of the p22 and the p19 from TBSV-BS3-Statice. The start condons of p22 and p19 are depicted with a light blue and yellow background, respectively. The change of the atg of p19 to a ctg results in removal of the *NcoI* site and in blocking of p19 translation.

Digestion of the PCR product and ligation were done according to the same protocol as for the ppDI-p22. Recombinant plasmids were screened by digestion with *NcoI/KpnI* and by

comparison of the corresponding restriction pattern with the ppDI-p22 pattern. The resulting plasmid was named ppDI-p22M (Appendix, Fig. 55).

6.2.5 Inoculation experiments with DI reporter gene RNA transcripts

TR4 plant lines were inoculated with RNA transcripts of different reporter gene constructs. The reporter genes were additionally co-inoculated with DI-p22M, to evaluate if co-inoculation with DI-p22M transcripts and thus expression of p22 leads to systemic spread of the DI-RNA. On to non transgenic *N. benthamiana* they were inoculated together with the TBSV transcripts. As a control the transcripts were inoculated without TBSV transcripts. DI-CP transcripts were co-inoculated with FC8 transcripts, which is a coat protein deletion mutant of TBSV. A coat protein deletion mutant had to be used since we wanted to express CP from the DI-RNA. The detection of proteins was done with various methods depending on the reporter gene.

Plants inoculated with scFv transcripts were analyzed by western blot (5.10.5), three days after inoculation. Detection of the antibody fragment was done using the C-terminally fused Myc-tag. In none of the inoculation experiments scFv protein could be detected (data not shown).

The green fluorescent protein (GFP) detection was done by fluorescence microscopy and by western blot analysis using a His-tag antibody. Western blot detection of the protein was unsuccessful for all tested plant transcript combinations. The fluorescence of the GFP could not be easily distinguished from the auto fluorescence of damaged plant cells. The control experiments without GFP constructs showed fluorescence to nearly the same extent as the plants inoculated with GFP constructs (data not shown). However, single cells could be identified as GFP expressing cells. This result could not be regarded as positive, as single cell expression was not considerably higher than in non transgenic, uninfected control plants.

The “coat protein inoculated” plants were analyzed by an ELISA test (5.10.6) with an antibody against the viral particle. Again none of the tested plants showed a positive signal (Data not shown).

Table 4: Summary of the different transcript combinations used in the inoculation experiments. *Only single cells could be detected and no systemic expression of GFP.

DI Reporter gene transcripts	***N. benthamiana***	***N. benthamiana with TBSV transcripts***	***TR4 plant lines***	***TR4 plant lines with DI-p22M transcripts***	***N. benthamiana with FC8 transcripts***
GUS	neg.	neg.	neg.	neg.	not tested
scFv	neg.	neg.	neg.	neg.	not tested
GFP	neg.*	neg.*	neg.	neg.	not tested
coat protein	neg.	not tested	neg.	neg.	neg.

Table 4 shows a summary of the inoculation experiment results with different plant-transcript combinations and the corresponding results.

To determine whether the lack of gene expression was due to a failure of the RNA to replicate, northern blot analysis of TBSV/DI-scFv-infected plants was performed. It was decided not to analyze TR4 plants by northern blot because a preliminary RT-PCR experiment did not show any systemic spread of the DI-RNA (data not shown). Therefore, it was expected that northern analysis would produce no detectable signal even with RNA extracted from primary infected leaves.

6.2.6 Northern blot analysis of DI-scFv/TBSV-infected plants

Non transgenic *N. benthamiana* plants were inoculated with a mixture of genomic TBSV and (-) strand DI-scFv RNA transcripts. The (-) strand DI-scFv RNA transcripts were used, because the (-) strand is preferably utilized by the viral replicase as template to synthesize the (+) strand. It serves as template for a 10 to 100 times higher number of the (+) strand in viral infections [Hull, 2002]. The (+) strand represents the mRNA encoding all viral proteins [Russo et al., 1994].Therefore, the (-) strand is more effective as inoculum, because the first replication step (synthesis of (-) strand from the (+) strand) is not necessary.

Leaves were harvested at 1 dpi, 2 dpi, and 5 dpi. Total RNA was extracted and subjected to northern blot analysis. Detection was done using two different probes (DIG labeled primers), one complementary to the 3' end of the DI (+) strand and the other complementary

to the (-) of the scFv insert. Unfortunately, the primer that binds to the 3' end of DI (+) strand can also bind to the 3' end of TBSV. Therefore, the genomic and subgenomic viral RNAs were detected with this probe as well.

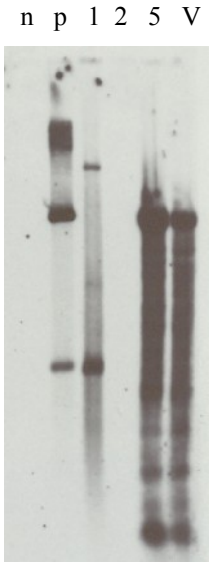


Figure 18: Northern blot with (+)strand 3' specific probe. Lanes: n total RNA from *N. benthamiana* as negative control; p RNA transcripts of (+) strand Di-scFv as positive control; 1, 2 and 5 total RNA extracted from *N. benthamiana* plants 1, 2 and 5 dpi, inoculated with (-) strand RNA transcripts of Di-scFv and together with (+) strand TBSV transcripts; V total RNA from *N. benthamiana* infected with TBSV

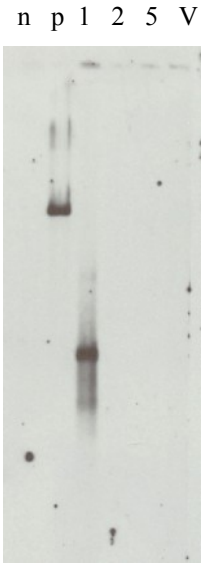


Figure 19: Northern blot with (-)strand scFv specific probe. Lanes: n total RNA from *N. benthamiana* as negative control; p RNA transcripts of (+) strand Di-scFv as positive control (because a (-) strand specific probe was used only the template DNA is visible and not the RNA transcript); 1, 2 and 5 total RNA extracted from *N. benthamiana* plants 1, 2 and 5 dpi, inoculated with (-) strand RNA transcripts of Di-scFv and togehter with (+) strand TBSV transcripts;V total RNA from *N. benthamiana* infected with TBSV

The Di-scFv RNA could only be detected 1 dpi (Fig. 18 and 19). Samples taken 2 and 5 dpi did not show a clear band of the corresponding fragment. This finding indicated that the DI was either not replicated or that it was not visible due to the strong signal originating from the replicating virus. The fact that the scFv-specific probe also failed to give a hybridization signal supports the first theory. However, it could not be excluded that scFv sequence was unstable and deleted from the DI. The signal obtained at 1 dpi with the (+) strand specific probe does not necessarily result from DI replication because a PCR product was used as template for the production of the RNA transcripts and could still present in the sample. If the

PCR template is still present in the sample it would hybridize with the DIG probe and produce the observed signal.

Further experiments using protoplasts isolated from the transgenic tobacco plants were done to determine whether the DI RNA could not be replicated or if the scFv insert was removed from the viral sequence. The protoplast system was adopted, because it eliminates the necessity to use wild type virus as helper for the replication of the DI-RNA. The viral replicase expressed by the transgenic plants replacing the replicase supplied by the helper virus. Further it is not effected by the inability of the DI-RNA to spread systemically through plants because in this system the uptake efficiency is higher than with rub-inoculation of the transcripts.

6.2.7 Protoplast transfection with DI RNA transcripts

To determine whether the DI sequence derivatives are replicated by the recombinant viral replicase that was introduced into the TR4 plant lines, protoplast transfection experiments were performed. Protoplasts were isolated from *N. benthamiana* and TR4 plant lines. They were transfected with RNA transcripts from the DI, DI-gfp and the DI-scFv, respectively. As a control, the plasmid pCAT-gfp [Reichel et al., 1996] was adopted. This Plasmid carries a GFP gene under the control of an enhanced 35S promoter. All transfections were carried out with TR4 as well as *N. benthamiana* protoplasts. The DI-gfp and pCat-gfp transfected protoplasts were examined after 24h using fluorescence microscopy (Fig. 20 and 21). Only in the control experiment with pCat-gfp DNA fluorescence was detectable. In the DI-gfp transfection experiments green fluorescence was not observed.

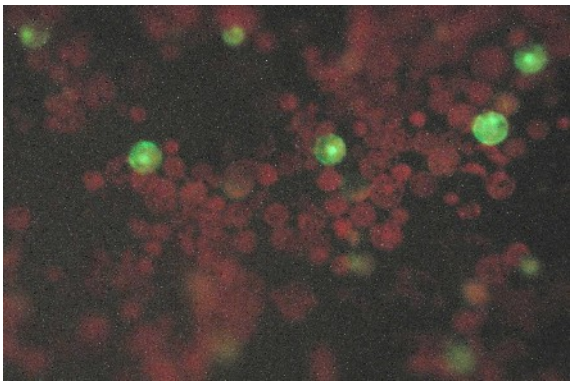


Figure 20: Fluorescence microscopy photograph of the TR4 plant line protoplasts transfected with 20 µg of pCat-gfp plasmid DNA. Pictures were taken 24h after transfection. Transfected protoplasts show the green fluorescence of the GFP, not transfected protoplasts show red autofluorescence of the plant chlorophyll.

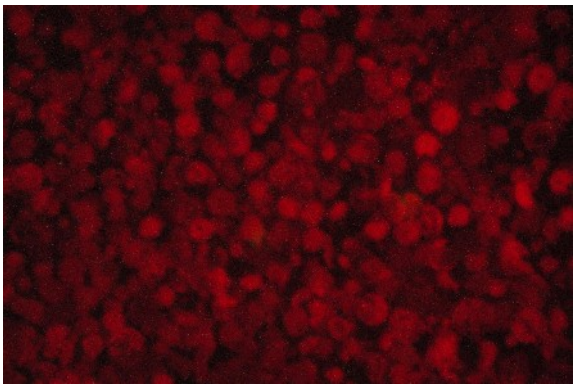


Figure 21: Fluorescence microscopy photograph of the TR4 plant line protoplasts transfected with DI-gfp RNA transcripts. Pictures were taken 24h after transfection. Not transfected protoplasts show red autofluorescence of the plant chlorophyll. No green fluorescence was detectable in this experiment.

After visual inspection all protoplasts were harvested, frozen in liquid nitrogen and stored at -80 °C for RNA extraction to be further analyzed by northern blot (6.2.8).

6.2.8 Northern blot analysis of protoplast RNA

Total RNA of TR4 and *N. benthamiana* protoplasts transfected with DI, DI-scFv, and DI-gfp was extracted as described in section 5.9.23. Control reactions were conducted with untransfected TR4 protoplasts as well as transfected *N. benthamiana* protoplasts. The RNA was analyzed by Northern hybridization (5.9.25) using a DIG labeled primer complementary to the 3' end of the DI sequences. The samples were analyzed 24h after transfection.

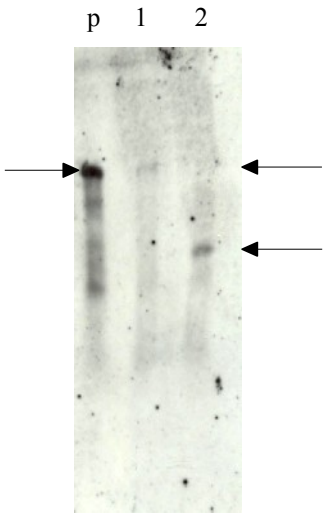


Figure 22: Northern blot analysis with (+) strand 3'- specific probe. Lanes: (p) positive control (RNA transcripts of the DI-scFv); (1) total RNA extracted from TR4 protoplasts transfected with DI-scFv transcripts harvested 24h after transfection; (2) total RNA extracted from TR4 protoplasts transfected with DI transcripts harvested 24h after transfection

Both the DI as well as the DI-scFv RNA could be detected in the Northern blot (Fig. 22). It can be concluded that the observed signal is due to replication of the DI-RNA by the transgenically expressed viral replicase, as the control transformations of *N. benthamiana* protoplasts did not show a signal. Protoplasts transfected with DI-gfp did not show a band of the corresponding size.

6.2.9 Construction of the ppmDI plasmid

It was assumed that translation of the DI-RNA could be affected since no protein expression could be achieved using the described DI constructs. Recent publications [Wu and White, 1999; Fabian and White, 2004] indicated that a part of the 3' end of TBSV is essential for translation of the viral genome. This sequence corresponding to a part of the viral 3' UTR is missing in our DI constructs and was therefore introduced into a new construct.

The 3' UTR of TBSV was PCR amplified using the 5-DI-III/IR28 primer pair and pTBSV as template. The amplified fragment contained region III, region IV of the DI molecule and

the so called region 3.5 (Fig. 23) of the parental virus. The size of the PCR product (~370 bp) was analyzed by agarose gel electrophoresis. The PCR product was the primer/template in an extension PCR in the next step.

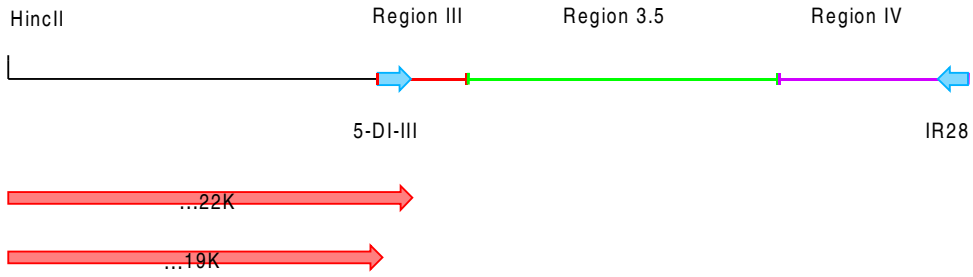


Figure 23: Schematic representation of 3' end of TBSV-BS3-Statice. The primer pair 5-DI-III/IR28 is depicted as light blue arrows and their orientation is indicated. Region III, 3.5 and IV are shown in red, green, and purple respectively. The 3' end of the p22 and p19 open reading frames are displayed as red arrows.

The 5' end of the DI molecule was amplified with the 5-TBSV-T7-MunI/3-DI-III primer pair. By using the 5-TBSV-T7-MunI forward primer a *MunI* site was introduced at the 5' end. The plasmid ppDI was utilized as template. The resulting ~430 bp fragment (Fig. 24) was analyzed by agarose gel electrophoresis and used as the second primer/template in the following extension PCR.

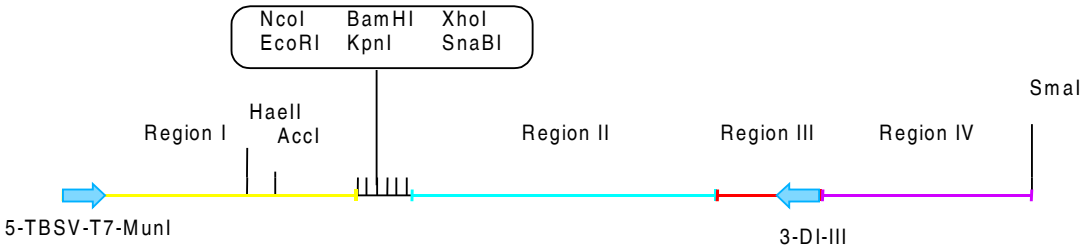


Figure 24: Schematic representation of the complete DI molecule. The primer pair 5-TBSV-T7-MunI/3-DI-III is depicted as light blue arrows and their orientation is indicated. Region I, II, III and IV are shown in yellow, cyan, red, and purple respectively. The MCS is shown in black together with unique restriction sites.

The extension PCR was done according to the standard PCR protocol with the exception that instead of template DNA and primers only the two PCR products were added (1 µl of each). The two PCR products possess an overlapping section (region III, Fig. 25) which enables them to act as primers and initiate the synthesis of the complementary strand (Fig.

25). This initiating reaction step was repeated for 10 cycles resulting in a linear amplification of the final PCR product. After 10 cycles the primers 5-TBSV-T7-MunI and IR28 were added to the reaction and PCR was continued for another 20 cycles to exponentially amplify the entire product.

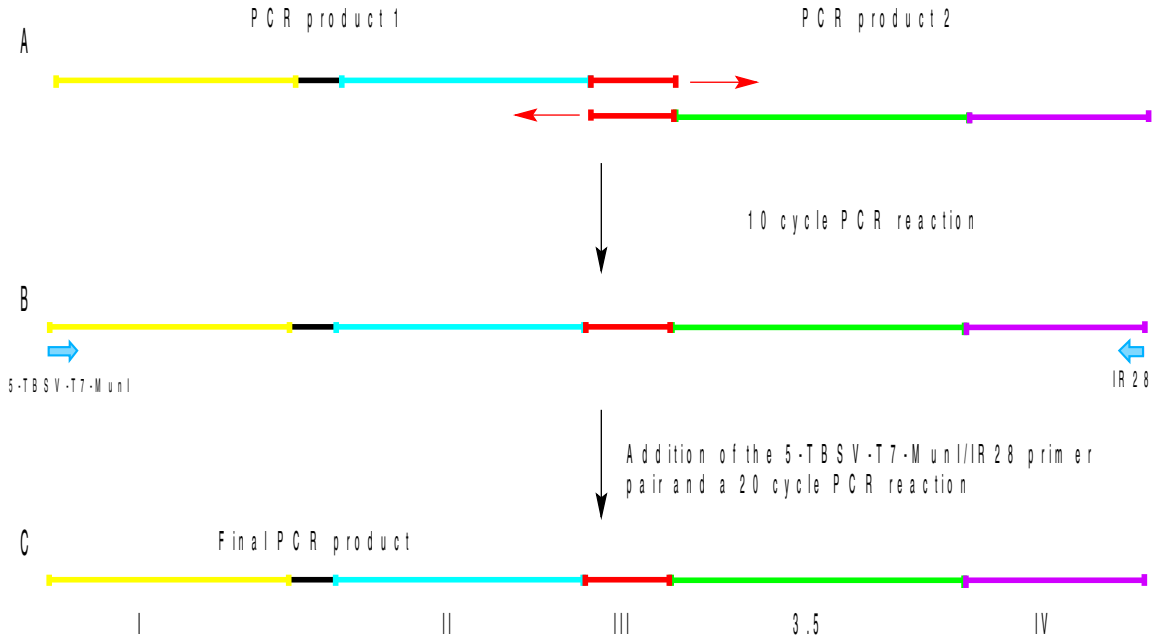


Figure 25: Schematic representation of the extension PCR. (A) Is showing PCR product 1 and 2 and their complementary sequences, the red arrows indicate the polymerase synthesis direction. (B). Addition of the 5-TBSV-T7-MunI/IR28 primer pair (light blue). Region I, II, III, 3.5, and IV are shown in yellow, cyan, red, green, and purple, respectively. The MCS is shown in black.

The final PCR product was analyzed using agarose gel electrophoresis and digested with *MunI*. *MunI* produces an *EcoRI* compatible overhang and the 3' end of the PCR product had a blunt end. The digested fragment was purified by agarose gel extraction (5.9.8) and ligated into an *EcoRI/SmaI* - digested pUC18. Recombinant plasmids (ppmDI, Appendix, Fig. 56)were digested with *BamHI*. Plasmids releasing a 570 bp fragment were regarded as positive.

6.2.10 Insertion of GUS, GFP, SCFV, YFP, CP and p22 into the ppmDI construct

The reporter genes from the ppDI constructs were also cloned into the ppmDI construct. The ppDI constructs containing the scFv, coat and movement protein genes were digested

with *NcoI* and *KpnI*, releasing the corresponding genes. Released fragments were gel purified and ligated into the likewise digested ppmDI plasmid. Recombinants were screened for the presence of *NcoI* and *KpnI* sites.

For the GUS and GFP constructs other plant optimized genes than in the DI constructs were inserted. The source of the GUS gene was pCambia-1381 (accession number AF234302) which was digested with *NcoI* and *BstEII* (blunt). The released fragment was gel purified and ligated into the *NcoI/ SnaBI* - digested ppmDI plasmid. Recombinant plasmids were digested with *HincII* and regarded as positive if a 1.2 and a 0.5 kb fragment was released.

The GFP gene was taken from the plasmid pCat-gfp [Reichel et al., 1996]. It was amplified via PCR using the primer pair 5-NcoI-gfp/3-KpnI-gfp that enabled the introduction of a 5' *NcoI* site and a 5' *KpnI* site. The PCR product was digested with *NcoI/KpnI* and gel purified. It was then ligated into the *NcoI/KpnI* digested ppmDI plasmid. Resulting recombinants were screened by *HincII* digestion and plasmids releasing a ~0.9 kb fragment were regarded as positive.

It was thought that yellow fluorescent protein (YFP), a derivative of GFP, represents a suitable alternative, because GFP fluorescence was not easily detectable if it is expressed in single cells. The YFP gene was obtained from the pEYFPC1 vector (Clonetech) by a *NcoI/KpnI* digest. Released fragments were gel purified and ligated into the likewise digested ppmDI plasmid. Recombinant plasmids were digested with *PstI* and plasmids releasing a 590 and 550 bp fragment were regarded as positives. Plasmid maps of these constructs are displayed in Appendix 9.5.

6.2.11 Inoculation experiments with mDI RNA transcripts

RNA transcripts of the different mDI reporter gene constructs were inoculated on TR4 plant lines, with and without mDI-p22 transcripts. On non transgenic *N. benthamiana* they were inoculated together with TBSV transcripts and as a control without TBSV transcripts. The mDI-CP transcripts were co-inoculated with FC8 transcripts that were derived from a coat protein deletion mutant of TBSV. Depending on the reporter gene the procedure for protein detection varied.

Plants inoculated with scFv transcripts were analyzed by western blot (5.10.5), three days

after inoculation. Detection of the antibody fragment was done using the C-terminally fused Myc-tag. In none of the inoculated plants the scFv protein could be detected (data not shown).

The presence of coat protein was analyzed by an ELISA (5.10.6), with an antibody against the viral particle. Again none of the tested plants that were inoculated with coat protein-containing transcripts gave a positive signal (data not shown).

For GFP and YFP detection fluorescence microscopy was applied. Similar problems occurred as in section 6.2.5. The fluorescence of GFP and YFP could not be distinguished from the autofluorescence of dead plant cells.

From the mDI-GUS inoculated plants, leaves were harvested after one and ten days and GUS-staining (5.10.1) was performed. None of the analyzed leaves showed any GUS activity (data not shown).

6.3 Induction of gene silencing using the DI vector system

The second part of this project was to adapt and optimize the DI vector system for the induction of gene silencing. In order to do this several plasmid constructs were created. The first goal was to create two p19 deficient mutants, as p19 is a strong suppressor of gene silencing, it was expected that this would increase the silencing signal. Different DI silencing constructs were created carrying sequences homologous to different endogenous genes and transgenes. Inoculation experiments with these constructs were performed to evaluate the potential of the DI system as a silencing inducer.

6.3.1 Cloning of pTBSVΔp19

It was necessary to create a deletion mutant of TBSV which does not express p19, because p19 of TBSV is a strong gene silencing suppressor [Qiu et al., 2002]. The site-directed mutagenesis kit from Stratagene was applied (5.9.22) to create this mutant. The primer pair was p19-ATG-M-TBSV-F and p19-ATG-M-TBSV-R (Fig. 26). The primers are complementary to each other and the A of the ATG start codon is changed to a C. This does not change the amino acid sequence of p22 (Fig. 17). This change also removes the *NcoI* site which cuts the ccatgg sequence .

```
p19-ATG-M-TBSV-F
5'-GGATACTGAATACGAACAAGTCAATAAACCcTGGAACGAGCTATACAAGG-3'
5'-GGATACTGAATACGAACAAGTCAATAAACCaTGGAACGAGCTATACAAGG-3' pTBSV
3'-CCTATGACTTATGCTTGTTCAGTTATTTGGgACCTTGCTCGATATGTTCC-5'
                                        p19-ATG-M-TBSV-R
```

Figure 26: Schematic representation of the PCR primers and their binding sites on the template. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. Sequence changes are shown with a red background. The ATG of p19 is underlined.

The PCR reaction mixture was made up of 5 µl 10x reaction buffer, 1 µl template (50 ng of pTBSV), 0.9 µl of the forward primer, 0.9 µl of the reverse primer 1 µl dNTP mix, 1 µl of *Pfu* Turbo Polymerase and 40.2 µl of water. The PCR program applied in this mutagenesis is shown in Figure 27.

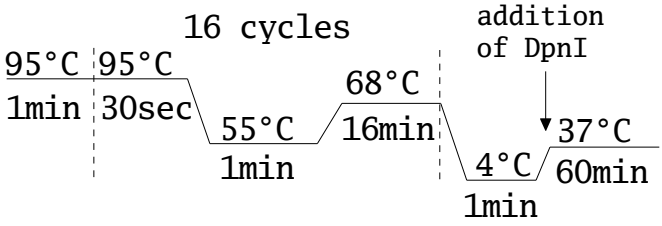


Figure 27: PCR program used for the site directed mutagenesis kit. After the PCR the reaction mixture was incubated with DpnI for 1 hour at 37 °C to remove the template DNA.

1 µl of the PCR reaction mixture was used to transform *E. coli* XL1-Blue competent cells. The transformants were analyzed by digestion with *NcoI*. Positive clones were linearized, opposed to the original plasmid which released a 2 kb fragment. One positive transformant (pTBSVΔp19, Appendix, Fig. 63) was selected and sequenced.

6.3.2 Construction of pTBSVΔΔp19

Several other mutations were done further downstream of the start codon, to ensure that the p19 deletion mutant does not revert to the original sequence. Three base pairs in the sequence were changed to introduce two stop codons, one amino acid change as well as a XbaI site (Fig. 28). The changes in the sequence did not change the amino acid sequence of p22.

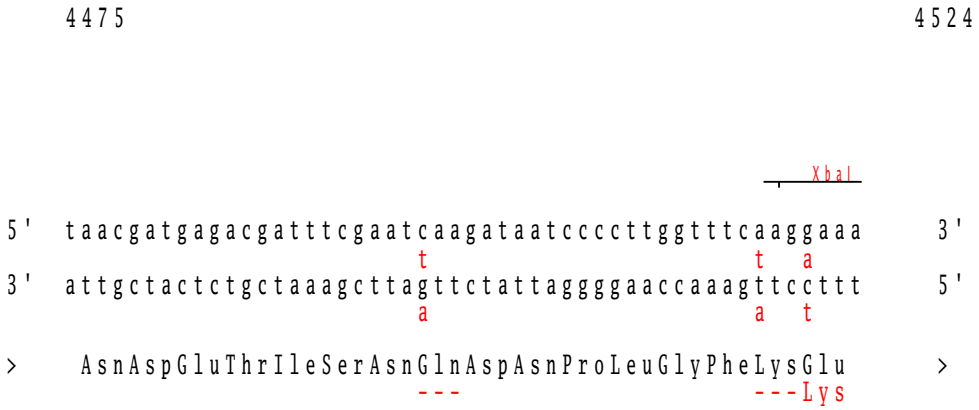


Figure 28: Schematic representation of the sequence changes introduced in pTBSVΔp19. The original DNA and amino acid sequences (p19 reading frame) are shown in black. Base changes as well as amino acid changes are depicted in red. The newly introduced *XbaI* site is also shown in red. The stop codons are represented by three red dashes. Numbers in the upper corners are the base pair positions on pTBSV.

The changes were introduced by amplifying a fragment of the pTBSV plasmid by PCR using the 5-p19-stop and DI-3-BamHI primers (Fig. 29).

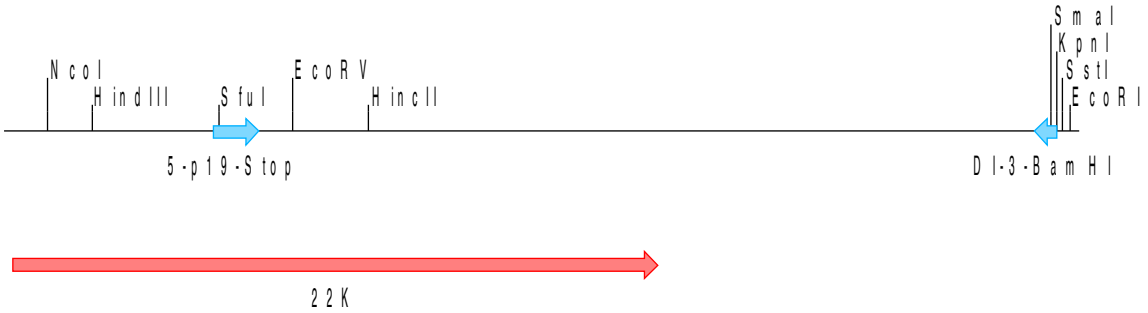


Figure 29: Schematic view of the 3' end the pTBSV plasmid and the binding sites of the 5-p19-Stop/DI-3-BamHI primer pair.

The PCR product was digested with *SfuI* and *Cfr9I* which is an isoschizomer of *SmaI* that produces sticky ends. The PCR product was then analyzed on an agarose gel and purified by gel extraction (5.9.8). The purified fragment was ligated into the pTBSVΔp19 which was digested *SfuI/Cfr9I* and the resulting ~7 kb fragment was gel purified before ligation. Transformants were screened by digestion with *XbaI* and *EcoRI*. Plasmids showing a 2.7 kb, a 2.2 kb, a 1.9 kb, and a 0.7 kb band were regarded as positive. Positive recombinant plasmids

(pTBSVΔΔp19, Appendix, Fig. 64) were selected for sequencing to confirm the desired mutations.

6.3.3 Isolation of a *FtsH* gene fragment from *N. benthamiana*

Total RNA was extracted from *N. benthamiana* leaves and reverse transcribed and amplified as described in section 5.9.24. The primers were FtsH-5 and FtsH-3 which were designed according to the cDNA sequence corresponding to the C-terminus of the FtsH protein of *N. tabacum* (accession number AB017480 [Seo et al., 2000], Fig. 30). The PCR product was ligated directly in *SmaI* linearized and T-tailed pUC18. Recombinant plasmids were digested with *KpnI* and *SphI*, positive transformants released a ~0.4 kb fragment. A positive recombinant plasmid was selected for sequencing. The results of the sequencing and the alignment with the *N. tabacum* FtsH sequence (Fig. 30) indicated that the plasmid carried the desired fragment.

```
FtsH N. benthamiana ------------------------CAAATGGCAGTTGCACTTGGTGGAA
FtsH N. tabacum     GTACAGCAGGAGCTACCTAGAGAATCAAATGGCAGTTGCACTTGGTGAAA

FtsH N. benthamiana GAGTTGCTGAGGAGGTCATTTTTGGACAAGACAATGTAACAACTGGGGCA
FtsH N. tabacum     GGGTTGCTGAGGAGGTTATTTTTGGACAAGATAACGTAACAACTGGGGCA

FtsH N. benthamiana TCTAATGATTTCATGCAAGTTTCACGAGTGGCGAGACAGATGGTTGAGAG
FtsH N. tabacum     TCTAACGATTTCATGCAAGTTTCACGAGTGGCAAGGCAGATGGTTGAGAG

FtsH N. benthamiana GCTTGGGTTCAGCAAAAAGATTGGCCAAGTTGCCATTGGCGGAGGTGGAG
FtsH N. tabacum     ATTAGGGTTCAGCAAAAAGATTGGACAAGTTGCCATTGGAGGAGGTGGAG

FtsH N. benthamiana GGAATCCTTTCTTGGGCCAGCAGATGTCAACCCAGAAAGACTATTCCATG
FtsH N. tabacum     GAAATCCTTTCCTAGGTCAACAGATGTCAACCCAGAAAGACTACTCCATG

FtsH N. benthamiana GCTACAGCCGACATTGTTGATGCTGAGGTAAGGGAATTGGTGGATAAAGC
FtsH N. tabacum     GCTACAGCCGATGTGGTTGATGCTGAAGTAAGGGAATTGGTTGAAAGAGC

FtsH N. benthamiana ATACTCGAGGGCAACGCAAATAATCACAACTCACATTGACATCCTACACA
FtsH N. tabacum     ATATGAAAGGGCAACAGAGATTATCACAACACACATTGACATCCTACACA

FtsH N. benthamiana AACTTGCTCAGCTGCTGATAGAGAAAGAAACTGTTGACGGCGGAGAGTTC
FtsH N. tabacum     AGCTTGCTCAGCTGTTGATAGAGAAAGAAACTGTTGATGGTGAAGAGTTC

FtsH N. benthamiana ATGAGCCTTTTCATCGATGG----------------------------
FtsH N. tabacum     ATGAGCCTTTTCATCGATGGCAAGGCCGAGCTATACATTTCTTGGGTCTC
```

Figure 30: Clustalw alignment of the *FtsH* sequence of *N. tabacum* and the isolated *FtsH* fragment. Sequence differences are depicted in a light grey background. The primer sequences are depicted in a dark grey background.

6.3.4 Insertion of the *FtsH* fragment into the ppDI vector

To use the *FtsH* gene fragment as a gene silencing inducer it was cloned into the ppDI vector. The fragment was amplified by PCR using the 5-KpnI-FtsH/3-EcoRI-FtsH primer pair to introduce a *KpnI* site at the 5' end as well as a *EcoRI* site at the 3' end (Fig. 31).

```
ttttatggtacccCAAATGGCAGTTGCACTTGG
tcgagccatgggaGTTTACCGTCAACGTGAACC-FtsH- TACTCGGAAAAGTAGCTACTCccctaggagatc
                                        ATGAGCCTTTTCATCGATGAGaattctttttt
```

Figure 31: Schematic representation of the PCR primers and their binding sites on the template. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. The *EcoRI* and *KpnI* sites are underlined.

The PCR product was digested with *EcoRI* and *KpnI*, run on a 1% (w/v) agarose gel and the expected ~0.4 kb fragment was purified by gel extraction (5.9.8). The purified fragment was then ligated into the ppDI vector which had been digested with the same restriction enzymes. The recombinant plasmids (ppDI-FtsH, Appendix, Fig. 65) were analyzed by *EcoRI/KpnI* digestion. Recombinant plasmids releasing a ~0.4 kb fragment were regarded as positive. Using these two restriction enzymes causes the fragment to be inserted into the vector in a (-) sense orientation.

6.3.5 Insertion of a phytoene desaturase (PDS) gene fragment into the ppDI vector

As second endogenous gene a partial PDS sequence from *N. tabacum* was inserted into the ppDI vector. The vector pT3T7SM-PDS(+)-GpG (Wassenegger, unpublished) was digested with *BamHI* restriction enzyme releasing a 359 bp fragment. The fragment was analyzed on an agarose gel and purified by gel extraction (5.9.8). It was then ligated into the likewise digested and dephosphorylated ppDI vector. Recombinant plasmids were screened by *BamHI* digestion and plasmids releasing a 359 bp fragment were regarded as positive. To determine the orientation of the PDS insert positive plasmids were then digested with *HindIII*. The (+) orientation resulting in a 2634, 839, and 773 bp restriction pattern, the (-) orientation

resulting in a 2634, 882, and 730 bp pattern. The (-) as well as the (+) orientation were adopted in gene silencing studies. The resulting plasmids were called ppDI-PDS(-) and ppDI-PDS(+) respectively (Appendix, 9.8).

6.3.6 Construction of a GFP silencing vector

A fragment of the GFP gene was cloned in a (-) orientation into the ppDI vector (Fig. 32), to evaluate the DI vector as a silencing inducer for GFP as described in [Hou and Qiu, 2003]. The plasmid ppmDI-GFP was digested with HincII and KpnI, releasing a 362 bp fragment. The fragment was purified by agarose gel extraction (5.9.8). The ppDI vector was cut with *KpnI* and *SnaBI*, purified by phenol/chloroform extraction and ligated with the 3' GFP fragment. Both *HincII* and *SnaBI* are blunt end cutters and can be ligated to each other.

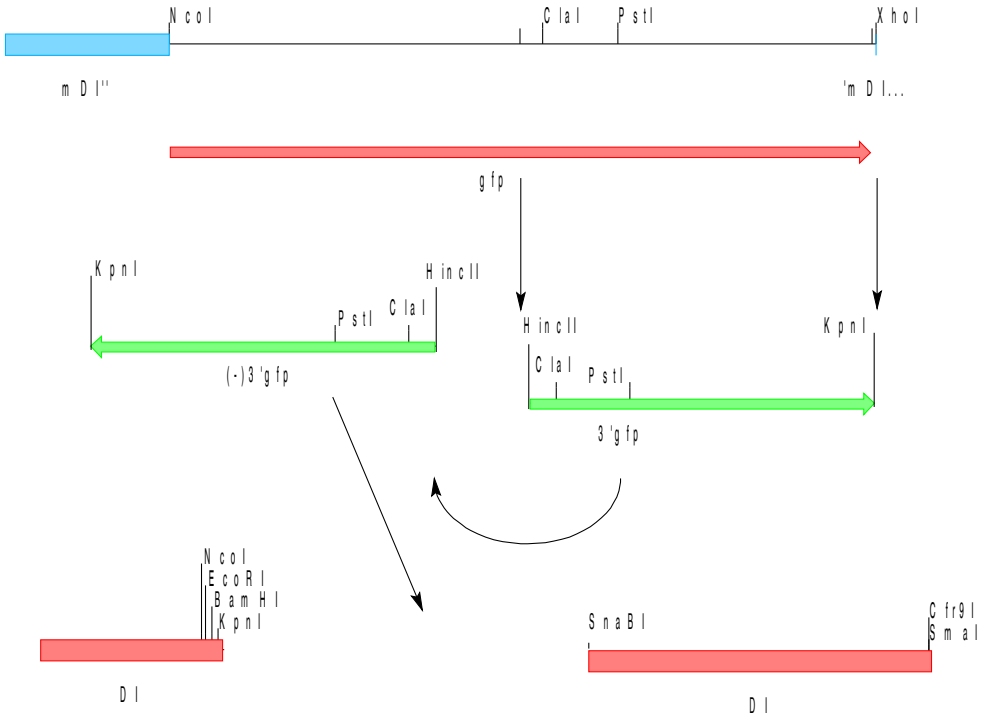


Figure 32:Schematic representation of the cloning strategy for ppDI-(-)3'gfp. Digestion of ppmDI-GFP with *KpnI/HincII*. Head to tail insertion of the 3' GFP fragment into the *KpnI/SnaBI* digested vector.

Recombinant plasmid (ppDI(-)3'gfp, Appendix, 66) were screened by digestion with *PstI*. Clones with a 2.6 kb, 0.9kb, and 0.7 kb restriction pattern were regarded as positive.

6.3.7 Inoculation experiments with TR4 plants

RNA transcripts of the silencing constructs ppDI-*FtsH* and ppDI-PDS were rub-inoculated on TR4 plants. In none of the plants could the appropriate silencing phenotype be observed (data not shown). Summary of the data can be seen in Table 5.

6.3.8 Inoculation experiments with the GFP silencing construct

Transgenic *N. benthamiana* expressing GFP (16C, [Ruiz et al., 1998]) were inoculated with RNA transcripts of ppDI (control) and ppDI-(-)3'gfp together with RNA transcripts of wild type TBSV as well as different p19 mutants of the virus (see section 6.3.1 and 6.3.2).

After inoculation the plants were visually examined under UV light every two days and photos were taken as described in section 5.13. The silencing was detected by the appearance of the red chlorophyll autofluorescence. Onset of GFP silencing was observed about 6-10 dpi depending on the viral helper construct.

Co-inoculation of DI-(-)3'gfp with wild type virus and pTBSVΔp19 (data not shown) construct resulted in severe viral symptoms typical for TBSV infections (Fig. 33) but the infection was not lethal for the majority of the plants. This is due to the symptom attenuation caused by co-infection with DI sequences [Havelda et al., 1998]. Control experiments with DI RNA without GFP insert exhibited no silencing symptoms. The inoculated leaves showed little or no silencing. After 10 days systemic infection symptoms were observed with wild type virus. Infection with pTBSVΔp19 exhibited symptoms two to three days later than wild type virus. At this time onset silencing was observed. The silencing signal moving along the veins as seen in Figure 33 (A). After 20 days the newly formed leaves were all GFP silenced (Fig. 33 (B)).

(A) (B)

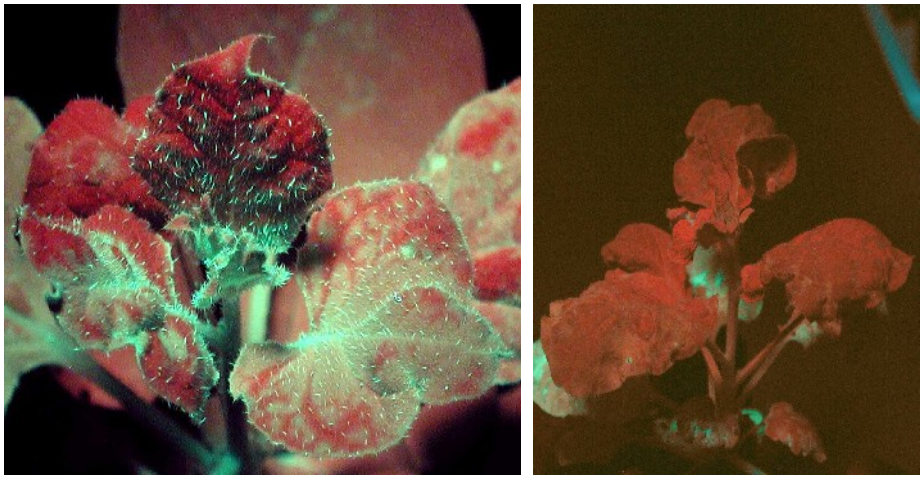


Figure 33: Fluorescence photography of 16C plants infected with TBSV and DI-(-)3'gfp RNA transcripts, (A) shows the plant 10 dpi. The spread of the silencing signal and severe symptoms are seen. (B) shows the newly formed leaves and stems with red autofluorescence of the plant chlorophyll.

Co-inoculation with pTBSVΔΔp19 RNA transcripts resulted in localized silencing in the inoculated leaves (Fig. 34 (A)). The inoculated plants showed no viral symptoms and a systemic infection was not detectable by visual examination. However, after 20 days the spread of the silencing signal was observed on the apical leaves of approximately 10% the plants (Fig. 34 (B)). Summary of the data can be seen in Table 5.

(A) (B)

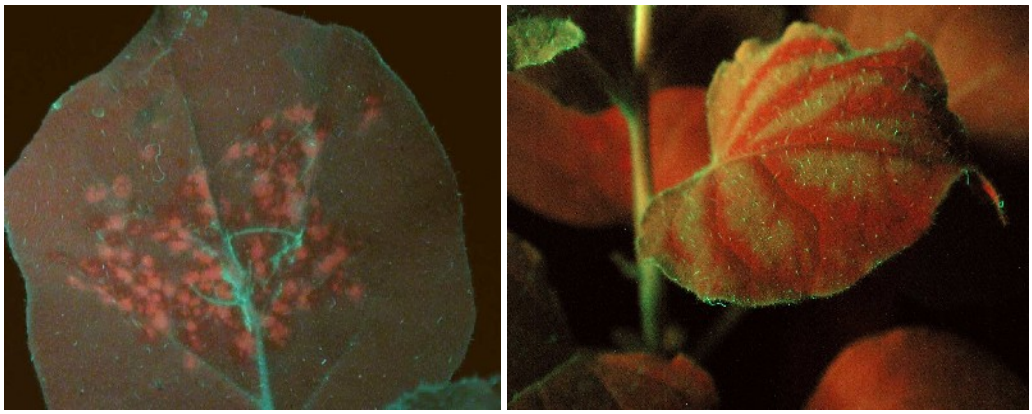


Figure 34: Fluorescence photography of (A) TBSVΔΔp19 co-inoculated 16C leaf 6 dpi. Local silencing of GFP is visible in the center. (B) Apical leaf of a TBSVΔΔp19 co-inoculated 16C plant 20 dpi. Spread of the silencing along the plant veins is visible.

6.3.9 Inoculation experiments with the *FtsH* and PDS silencing construct

N. benthamiana plants were inoculated with RNA transcripts of ppDI, ppDI-*FtsH*, or ppDI-PDS together with RNA transcripts of wild type TBSV as well as different p19 mutants of the virus (see section 6.3.1 and 6.3.2). After inoculation the plants were visually examined every two days and photos were taken as described in section 5.13. The silencing was detected by the appearance of leaf variegation and of a bleached leaf phenotype typical for *FtsH* and PDS silencing [Saitoh and Terauchi, 2002; Gosselé et al., 2002].

Plants infected with wild type TBSV showed typical viral symptoms 9 dpi. However, no silencing symptoms were visible for the DI, DI-*FtsH*, and DI-PDS co-inoculated plants (data not shown).

Plants co-inoculated with RNA transcripts derived from pTBSVΔp19 together with DI-*FtsH* RNA developed viral symptoms 3 to 5 days later than plants infected with a wild type virus-DI RNA combination. Leaf variegation and bleached leaf spots were visible in systemically infected apical leaves 14 dpi (Fig. 35). DI RNA inoculated control exhibited no silencing symptoms.

Figure 35: Photography (A) and (B) of TBSVΔp19/DI-FtsH co-inoculated *N. benthamiana* 14 dpi. Leaf variegation and bleached spots are visible in (A) and (B).

Plants co-inoculated with RNA transcripts derived from pTBSVΔp19 together DI-PDS RNA developed viral symptoms 3 to 5 days later than plants infected with a wild type virus-DI RNA combination. Leaf variegation and bleached leaf spots were visible in systemically infected apical leaves 20 dpi. The bleached spots were larger and more extensive compared to

the *FtsH* silenced plants at 30 dpi (Fig. 35). DI RNA inoculated control exhibited no silencing symptoms.

(A) (B)

Figure 36: Photography (A) and (B) of TBSVΔp19/DI-PDS co-inoculated *N. benthamiana* 30 dpi. Leaf variegation and bleached spots are visible in (A) and (B).

Co-infection with pTBSVΔΔp19 derived transcripts resulted in no visually observable viral symptoms as described before (6.3.8). However, in contrast to the GFP silencing observed with TBSVΔΔp19/DI-(-)3'gfp inoculated 16C plants, no *FtsH* or PDS silencing was observed (data not shown). Summary of the data can be seen in Table 5.

Table 5: Summary of the different combinations of viruses, plants and DI constructs used in silencing experiments and the corresponding results.

Virus construct	***Plants***	***DI construct***	***Silencing symptoms***	***Silenced gene***
n.a.	TR4	DI-PDS	no silencing	PDS
		DI-*FtsH*	no silencing	*FtsH*
TBSV	16C	DI-(-)3'gfp	complete systemic silencing	GFP
	N. benthamiana	DI-PDS	no silencing	PDS
	N. benthamiana	DI-*FtsH*	no silencing	*FtsH*

Virus construct	*Plants*	*DI construct*	*Silencing symptoms*	*Silenced gene*
TBSVΔp19	16C	DI-(-)3'gfp	complete systemic silencing	GFP
	N. benthamiana	DI-PDS	white spots on systemic leaves indicated silencing	PDS
	N. benthamiana	DI-*FtsH*	white spots on systemic leaves indicated silencing, not as strong as with PDS	*FtsH*
TBSVΔΔp19	16C	DI-(-)3'gfp	first symptoms of systemic silencing, not complete	GFP
	N. benthamiana	DI-PDS	no silencing	PDS
	N. benthamiana	DI-*FtsH*	no silencing	*FtsH*

6.3.10 Construction of infectious TBSV clones for *A. tumefaciens* infiltration experiments

To make the infection process of plants more suitable for high throughput screening, we decided to construct a binary vector carrying the complete TBSV genome under the control of the 35S promoter. This would allow to infect plants by simple *A. tumefaciens* infiltration instead of rub inoculation with RNA transcripts.

As it is important to maintain the wild type 5' end, the virus had to be inserted directly after the transcription initiation site of the 35S promoter. To achieve this a previously constructed plasmid (ppS) was utilized for the construction of the 35S promoter-virus construct. The plasmid harbors a *StuI* site directly downstream of the transcription initiation site [Töpfer et al., 1987] as seen in Figure 37. Furthermore, it was desirable to use an enhanced 35S promoter with a duplicated transcriptional enhancer as described by Kay, Töpfer and coworkers [Kay et al., 1987] and [Töpfer et al., 1987].

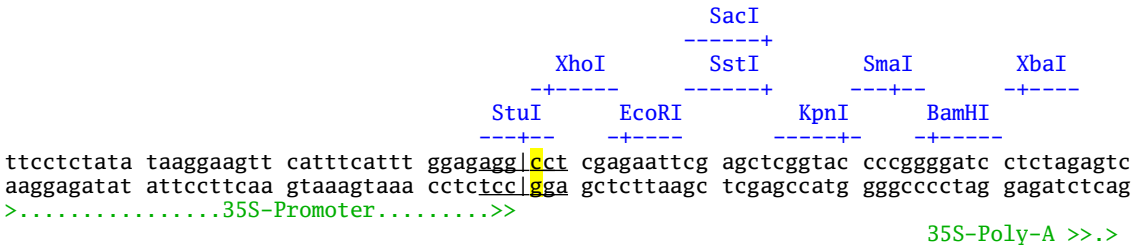


Figure 37: Schematic representation of the MCS and the transcription initiation site of the ppS plasmid. The StuI site is underlined and the first transcribed based is depicted with a yellow background. 35S promoter and polyadenylation signal sequences are indicated were appropriate.

The duplication was done essentially as previously described [Kay et al., 1987]. The resulting plasmid, ppdS (Appendix, Fig. 69) was used in the construction of the 35S promoter-virus plasmid. The cloning strategy is outlined in Figure 37.

The 5' end of the the virus was amplified by PCR using the TBSV-Blunt/3-Rep-EcoRI primer pair. The resulting 2.2 kb fragment was digested with *EcoRI* and purified. Subsequently the purified fragment was ligated into the *StuI/EcoRI* digested ppdS plasmid. Recombinants were screened by *EcoRV* digestion and plasmids showing a 4.1 kb – 1.2 kb – 0.4 kb restriction pattern were called ppdS-5'TBSV and utilized for further cloning steps. With this strategy transcripts made of the 35S promoter have the native 5' end of the virus. In the next step the 3' half of the viral genome is released from the plasmid pTBSV by digestion with *EcoRI/CfRI9* and purified. The 3' end of the viral genome was ligated into the *EcoRI/CfrI9* digested ppdS-5'TBSV vector. Transformants were digested with *EcoRV* to screen for positive clones. Plasmids showing a 4.1 kb – 2.5 kb – 1.2 kb – 0.4 kb restriction pattern were selected as positive clones called dTBSV (Appendix, Fig. 70).

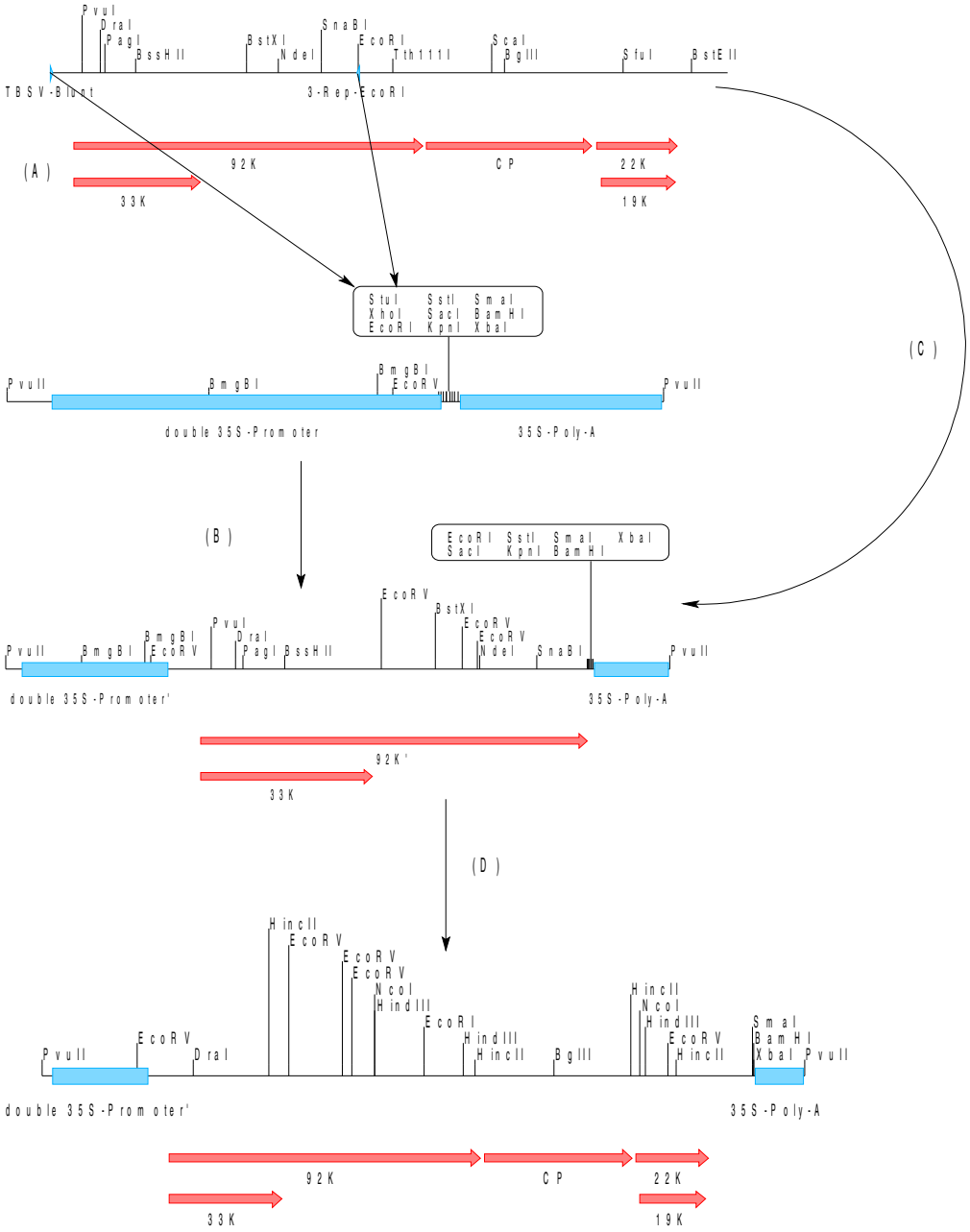


Figure 38: Cloning strategy for the construction of dTBSV. (A) PCR with TBSV-Blunt/3-Rep-EcoRI primer pair and (B) ligation of the digested PCR product into ppdS. (C) Release of the 3' end of the Virus from pTBSV and (D) ligation of the purified fragment into ppdS-5'TBSV.

To transfer the 35S promoter-virus cassette into the binary vector, dTBSV was digested with PvuII releasing the complete cassette from the pUC backbone. After purification the cassette was ligated into the *SmaI* linearized ppzp200 plasmid. Recombinant plasmids (ppzp-TBSV, Appendix, Fig. 71) were analyzed by *HindIII* digestion and plasmids showing a 8 kb –

2.6 kb – 1.4 kb – 0.7 kb restriction pattern were selected as positive.

With plasmid DNA purified from positive *E. coli* clones, *Agrobacterium tumefaciens,* strain ATHV, was transformed by electroporation (5.9.17). Verified transformants were utilized in *Agrobacterium tumefaciens* infiltration experiments.

6.3.11 Construction of an infectious TBSVΔp19 clone for *A. tumefaciens* infiltration experiments

As the with the infectious clone for RNA transcript production it was necessary to produce a p19 deficient mutant of the *A. tumefaciens* infiltration vector constructed in section 6.3.10. In order to introduce this mutation the plasmid pTBSVΔp19 was digested with *BglII* and *SmaI* to release a 1.5 kb fragment carrying this mutation. The binary ppzp-TBSV vector was likewise digested with *BglII/SmaI.* The 11.2 kb vector backbone was purified and ligated with the *BglII/SmaI* fragment from pTBSVΔp19. Selection of the positive transformants was done by screening the plasmids with a *EcoRI/NcoI* digestion. Plasmids with a 9.3 kb – 3 kb – 0.4 kb restriction pattern (ppzp-TBSVΔp19, Appendix, Fig. 72) were selected as positives and transformed into *A. tumefaciens* (5.9.17). Verified transformants were used in *A. tumefaciens* infiltration experiments.

6.3.12 Construction of a binary vector for the inoculation of plants with DI-PDS

An alternative method for the inoculation of plants was devised, because the production of RNA *in vitro* transcripts is expensive and time consuming. Further, Gossele and coworkers [Gosselé et al., 2002] had a stronger induction of silencing when they utilized a binary vector for the introduction of the silencing construct. The cloning strategy for the binary vector construct is depicted in Figure 39.

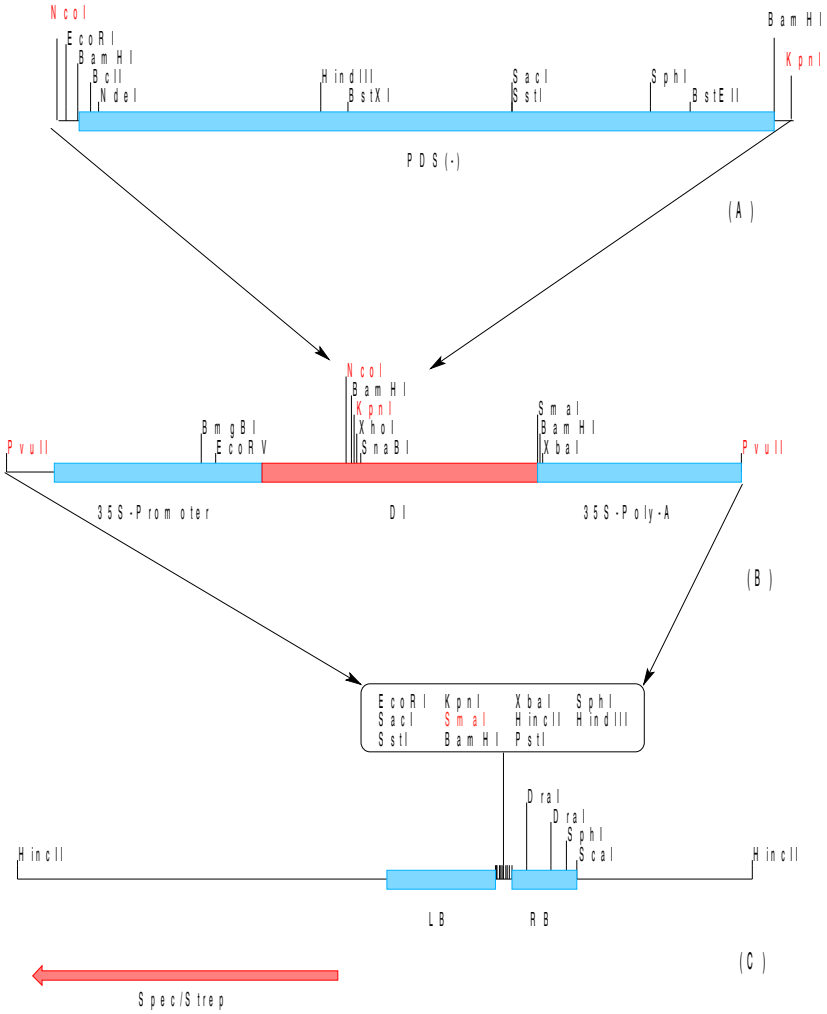


Figure 39: Cloning strategy for the construction of ppzp-SDI-PDS(-). (A) Digestion of ppDI-PDS(-) with *NcoI/KpnI* (highlighted in red), isolation of the desired fragment and ligation into the likewise digested ppSDI. (B) Digestion of ppSDI-PDS(-) with *PvuII* (highlighted in red), isolation of the desired fragment and ligation into the *SmaI* (highlighted in red) digested and dephosphorylized ppzp200 (accession number U10460) plasmid.

The first step was to insert the PDS fragment into a plasmid which ensures that the DI transcription is controlled by the 35S promoter. This plasmid, ppSDI, was previously constructed in our group and carries the DI sequence directly after the transcription initiation signal of the 35S promoter. Both ppDI-PDS(+) and ppDI-PDS(-) were digested with *NcoI* and *KpnI* releasing the PDS fragment. The fragments were purified by agarose gel extraction (5.9.8) and ligated into the likewise digested vector ppSDI. Transformants were analyzed by digestion with *PvuII*. Plasmids releasing a 1.8 kb fragment were regarded as positive.

The constructs ppSDI-PDS(+) and (-) were both digested with *PvuII* and the released 1.8 kb fragments purified by agarose gel extraction. After *SmaI* digestion the ppzp200 binary vector (accession number U10460, [Hajdukiewicz et al., 1994]) was treated with SAP (5.9.12) and purified by phenolization, subsequent precipitation with EtOH and resuspension in 10 µl of water. Both purified PDS fragments were ligated into the ppzp200 vector and transformed in chemically competent XL Blue cells. Transformants were screened by *HindIII* digestion and plasmids (ppzp-SDI-PDS(+) and ppzp-SDI-PDS(-), Appendix 9.11) releasing a 839 bp fragment were regarded as positive.

With plasmid DNA purified from positive *E. coli* clones, *Agrobacterium tumefaciens,* strain ATHV, was transformed by electroporation (5.9.17). Verified transformants were used in *Agrobacterium tumefaciens* infiltration experiments.

6.3.13 *A. tumefaciens* infiltration experiments with DI-PDS binary vectors on TR4 plants

Transgenic replicase expressing TR4 plants were infiltrated with *A. tumefaciens* bacterial suspension as described in section 5.12 to evaluate if this inoculation method was sufficient to induce a systemic silencing. *A. tumefaciens* cultures were utilized which carried the ppzp-SDI-PDS(+) and (-) plasmids. However, none of the infiltrated plants showed silencing of PDS (data not shown).

6.3.14 *A. tumefaciens* infiltration experiments with DI-PDS binary vectors

Wild type *N. benthamiana* plants were inoculated with TBSV, TBSVΔp19, and TBSVΔΔp19 RNA transcripts. Two h after rub-inoculation the plants where infiltrated with ppzp-SDI-PDS(+ and -). The infection behavior of the different virus mutants was the same as in previous experiments. Wild type TBSV infected plants showed the first systemic symptoms after 6-7 dpi, TBSVΔp19 infected plants after 10 dpi and TBSVΔΔp19 infected plants showed no symptoms. However, none of the ppzp-SDI-PDS(+ and -) infiltrated plants showed any silencing symptoms (data not shown) which is in contrast to the previously described experiments using RNA transcripts of DI-PDS.

6.3.15 *A. tumefaciens* infiltration experiments with DI-PDS and virus binary vectors

To evaluate the effectiveness of *Agrobacterium tumefaciens* mediated infection and induction of gene silencing with TBSV in comparison to the RNA transcript rub inoculation, *N. benthamiana* plants were infiltrated with *A. tumefaciens* bacterial suspension as described in section 5.12. The bacterial suspensions were a mixture of *A. tumefaciens* carrying the ppzp-TBSV or ppzp-TBSVΔp19 plasmid together with *A. tumefaciens* carrying the ppzp-SDI-PDS(-) or ppzp-SDI-PDS(+) plasmids.

Figure 40: Systemically infected *N. benthamiana* leaves showing PDS silencing symptoms. Viral symptoms like severe stunting of leaves can also be seen. Plants were infiltrated with ppzp-TBSV and ppzp-SDI-PDS(+) 10 days before.

Plants infiltrated with with a combination ppzp-TBSV and ppzp-SDI-PDS(+ or -) developed the first systemic after 5-7 dpi which is one to two days earlier then on RNA transcript inoculated plants. Additionally, in contrast to the inoculation with RNA transcripts plants infiltrated with a mixture of ppzp-TBSV and ppzp-SDI-PDS(+ or -) exhibited PDS silencing symptoms (Fig. 40). There was no significant difference between plants infiltrated with the (+) or (-) sense PDS constructs. Therefore, further experiments were done with the (+) sense construct alone.

N. benthamiana plants infiltrated with a combination ppzp-TBSVΔp19 and ppzp-SDI-PDS(+) exhibited comparatively mild viral infection symptoms after approximately 25 dpi. Minor silencing symptoms appeared after 30 dpi (Fig. 41). An interesting difference between the wild type virus and the TBSVΔp19 mutant was that silencing symptoms mostly appeared

along the veins of the plants with the wild type virus and with the TBSVΔp19 mutant there were mostly scattered spots on some leaves.

(A) (B)

Figure 41: (A and B) Systemically infected *N. benthamiana* leaves showing PDS silencing symptoms. Plants were infiltrated with ppzp-TBSVΔp19 and ppzp-SDI-PDS(+) 30 days before.

Additionally the infection success rate was approximately 50% not 100% as with wild type virus. Plants inoculated only with ppzp-TBSVΔp19 exhibited the same behavior which indicates that the limited infection success rate is not due to co infection with DI-RNA.

6.3.16 Northern blot analysis of TBSVΔp19/DI-FtsH infected plants

Total RNA was extracted (5.9.23) from 3 pTBSVΔp19/DI-*FtsH* infected *N. benthamiana* plants to determine the stability of the FtsH insert in the DI sequence. systemically infected apical leaves were extracted in order to eliminate detection of RNA inoculum. The RNA was analyzed by Northern blot (5.9.25), using a DIG labeled DNA probe complementary to the FtsH sequence in the DI construct.

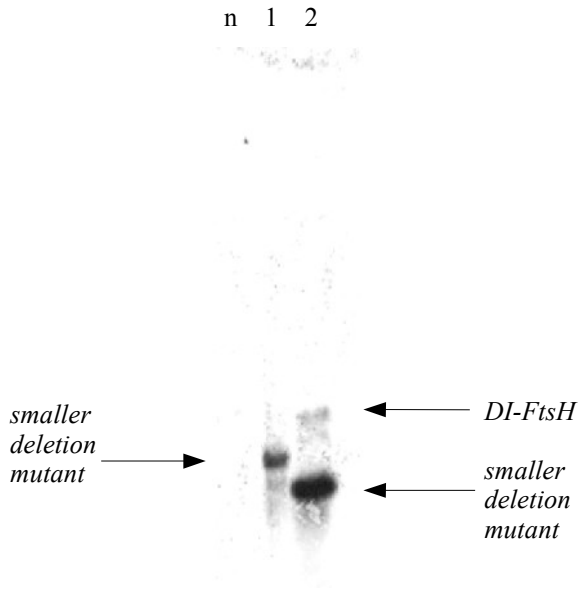


Figure 42: Northern blot analysis with DIG labeled FtsH - specific probe. Lanes: (n) neg. control RNA extracted from uninfected *N. benthamiana*; (1) total RNA extracted from TBSVΔp19/DI-FtsH infected *N. benthamiana* plants ; (2) same as lane 1.

The prominent band in Figure 42 is smaller than the expected DI-FtsH band. In different samples there are different populations of RNA, in lane 2 a large band can be seen corresponding to the original DI-FtsH RNA. However, the stronger signal comes from the smaller band in that sample. The smaller RNA is probably a deletion mutant of the original DI-FtsH inoculum. The RNA still retained a part of the FtsH sequence because it is readily detected by the DIG labeled FtsH probe. To assess the possibility that a part of the DI molecules still retained the complete FtsH sequence RT-PCR experiments and subsequent sequencing were performed.

6.3.17 RT-PCR and cloning of the DI-*FtsH* fragment

The identical RNA as analyzed by Northern blot was reverse transcribed and amplified by PCR using the FtsH-3-EcoRI/3-DI-III and TBSV-Blunt/FtsH-5-KpnI primer pairs (Fig. 43). In each primer pair one *FtsH* specific primer was used because two virus specific primers would have caused interference by the viral genome. The resulting PCR products were of the

expected size of 580 bp and 640 bp, respectively. Both fragments were ligated into pTPCR, a PCR cloning vector based on pUC19 [Wassenegger et al., 1994], without prior purification of the reaction mixture. Recombinant plasmids were screened by EcoRI/XbaI digestion and sequenced to determine whether deletions occurred during replication of the DI-RNA.

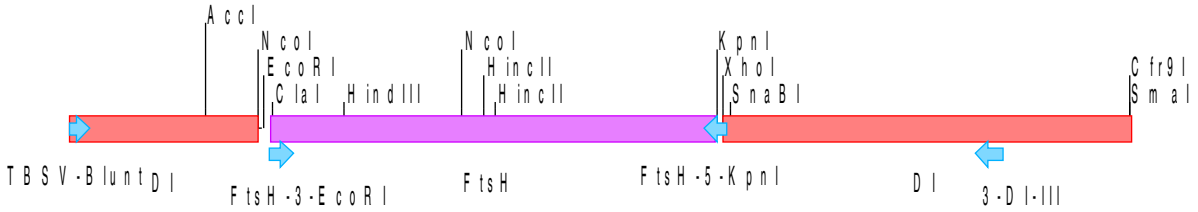


Figure 43: Schematic representation of the DI-FtsH sequence and the binding sites of the FtsH-3-EcoRI/3-DI-III and the TBSV-Blunt/FtsH-5-KpnI primer pairs. The FtsH insert is depicted in light purple, primers are depicted in light blue and the DI sequence is red. Common restriction sites in the construct are displayed were appropriate.

A ClustalX alignment was done (see Appendix 9.1), to compare the isolated sequences. Interestingly, no deletions were found in the sequence which indicates that the primers adopted for this experiment, selected specifically molecules carrying the complete *FtsH* insert. One isolated sequence carried two single base pair substitutions in the *FtsH* fragment. Since RNA dependent RNA polymerases have a high error frequency this was expected because there is no selection pressure to maintain the original sequence.

6.3.18 Differences between TBSVΔp19 and TBSVΔΔp19

The common consensus in the literature is that p19 is not necessary for efficient infection with TBSV [Scholthof et al., 1995; Scholthof et al. 2, 1995]. However, we experienced differences in the infection behavior of our p19 mutants. The severity of the symptoms varied significantly from wild type to TBSVΔp19 to TBSVΔΔp19. TBSVΔΔp19 for example did not show any systemic symptoms, in 9 out of 10 infection experiments, when co-infected with DI-RNA. However, if inoculated only with genomic viral RNA mild symptoms could be observed after 10 days. RNA viruses have a high mutation rate thus it is possible that the mutations that were introduced, reverted back to the original sequence. The 3' end of the viral genome from TBSV, TBSVΔp19, and TBSVΔΔp19 was isolated from infected *N.*

benthamiana plants using RT-PCR and sequenced, to investigate this possibility.

Total RNA was extracted from TBSVΔp19, TBSVΔΔp19, TBSV/mDI, TBSVΔp19/mDI and TBSVΔΔp19/mDI infected *N. benthamiana* plants as described in section (5.9.23). One of the plants inoculated with the TBSVΔΔp19/mDI combination showing systemic symptoms and one showing no systemic symptoms. From these plants 2 samples were taken, one inoculated leaf of each and one systemic leaf of each (see Table 6).

Table 6: Listing of the RNA samples extracted from *N. benthamiana* and analyzed by RT-PCR and subsequent sequencing of the cloned inserts.

Isolated from	***Number of samples taken***	***RT-PCR positive samples***	***Number of samples sequenced***
TBSV/mDI infected *N. benthamiana*	2 systemic leaves	2	1
TBSVΔp19/mDI infected *N. benthamiana*	2 systemic leaves	2	2
TBSVΔΔp19/mDI infected *N. benthamiana*	2 inoculated and 2 systemic leaves	2 positive from the inoculated leaves 1 positive from the systemic leaves	3
TBSVΔp19 infected *N. benthamiana*	1 systemic leaf	1	1
TBSVΔΔp19infected *N. benthamiana*	1 systemic leaf	1	1

RNA quality was evaluated by agarose gel electrophoresis. The 3' end of TBSV was amplified using the SuperScript One-step RT-PCR kit described in section 5.9.24. The primer pair was 5-EcoRI-p22/3-DI-III. The resulting PCR product was 615 bp long and was analyzed using agarose gel electrophoresis (Fig. 44). The differences in the intensities of the PCR bands correlate to the quality of the total RNA extracted. Control reactions showed no band of the corresponding size.

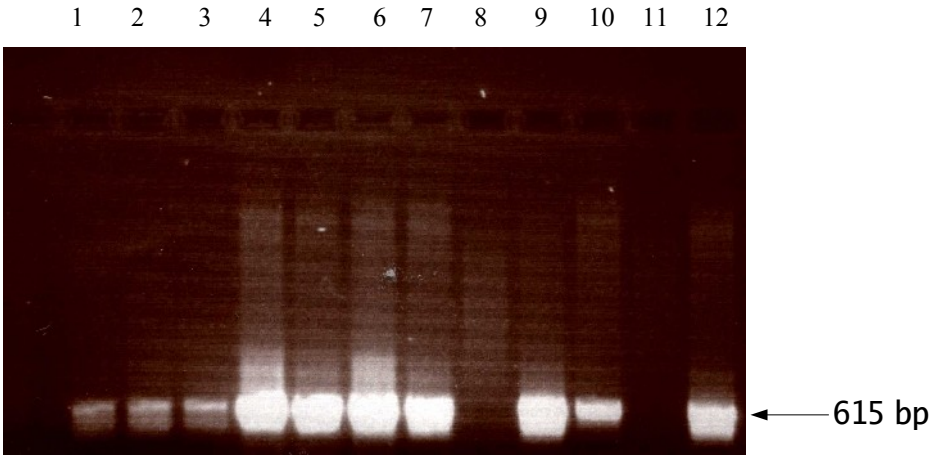


Figure 44: 1% agarose electrophoresis gel with RT-PCR reaction products from total RNA extracted from: (1, 3) TBSVΔΔp19/mDI infected *N. benthamiana* (inoculated leaf); (2) TBSVΔp19 infected *N. benthamiana*; (4, 6) TBSV/mDI infected *N. benthamiana*; (5, 9) TBSVΔp19/mDI infected *N. benthamiana*; (7) TBSVΔΔp19 infected *N. benthamiana*; (8, 10*, 12*) TBSVΔΔp19/mDI infected *N. benthamiana* (apical leaf); (11) uninfected *N. benthamiana*.

*** The plant the RNA was extracted from showed systemic symptoms see text for details.**

Ligation into pTPCR followed the electrophoresis. Transformants were screened by *HincII* digestion and plasmids releasing a 310 or 348 bp (+ and – orientation respectively) fragment were regarded as positive. One positive plasmid of each transformation reaction was selected for sequencing with the pUC universal forward primer. The inserts of the sequenced plasmids all showed the expected sequence except one derived from a TBSVΔΔp19/mDI infected plant which reverted to the original sequence of TBSV. This plant was also the only one showing systemic symptoms and a positive RT-PCR signal (Fig. 44). As the other plants infected with this batch of RNA transcripts showed no symptoms or a RT-PCR signal a contamination with wild type virus was ruled out. These results are in consensus with the literature regarding p19 which state that p19 is not necessary for an efficient systemic infection of *N. benthamiana* with pronounced symptoms [Scholthof et al., 1995; Scholthof et al. 2, 1995]. However, they do not explain the differences in the symptom severity between TBSVΔp19/mDI and TBSVΔΔp19/mDI inoculated *N. benthamiana.*

6.4 Protection from gene silencing

Different binary vector constructs were created, to evaluate the use of the DI sequences as a means to protect transgenes from silencing. A construct harboring a GFP gene in combination with 2 different DI sequences was created and transient expression experiments were carried out. Further an already existing DI-GUS [Eilers, 2002] binary vector construct was applied in infiltration studies of GUS silenced *N. tabacum* plants.

6.4.1 Construction of a binary vector harboring DI-gfpER and mDI-gfpER

A binary vector harboring the DI-GFP and mDI-GFP sequences under the control of the 35S promoter was constructed, to evaluate the use of the DI sequences as a means to protect transgenes from the gene silencing mechanism. Since this vector was to be utilized for the stable transformation of plants, an ER leader peptide - and an ER retention signal - sequence were added to the coding sequence as described in [Haseloff et al., 1997]. To do preliminary tests GFP silenced plants (Wassenegger unpublished results) were infiltrated with the DI-GFP and mDI-GFP constructs respectively and the resulting GFP expression was evaluated.

6.4.1.1 Cloning of pBSK-mgfp5

Construction of this vector was done in multiple steps starting from the plasmid pT3T7-mgfp5 (Wassenegger personal communication) which carries the mentioned sequences for the localization of the protein to the ER. The plasmid was digested with *HindIII* and *SacI* releasing the 860 bp GFP fragment. The fragment was purified by gel extraction and ligated into the likewise digested vector pBluescript-SK(+) (pBSK, Stratagene). Recombinant plasmids were screened by *KpnI* restriction and plasmids releasing a 886 bp fragment were regarded as positive.

6.4.1.2 Cloning of pUC-gfpER

Since our fluorescence microscopy equipment was more suitable for the detection of a "red shifted" [Heim and Tsien, 1996] GFP the fluorophor coding sequence of pCAT-gfp [Reichel et al., 1996] was introduced into the sequence of the new construct. To additionally

introduce a 5' *MunI* as well as a 3' *KpnI* site the DNA was amplified with the primer pair 5-gfp-MunI-linker/3-gfpER-KpnI. The *MunI* site was chosen because it produces *EcoRI* compatible overhangs. The sequence of the primers was constructed so that later ligation of the *MunI* overhang to the *EcoRI* overhang of the pBSK-mgfp5 plasmid results in translation of the leader peptide fused to GFP (Fig. 46).

```
5-gfp-MunI-linker
caattgaGTAAAGGAGAAGAACTTTTCACTGGA
cggtaccCATTTCCTCTTCTTGAAAAGTGACCT-pCat-gfp -GTACCGTACCTACTTGATATGTTTattccta
                                            CATGGCATGGATGAACTATACAAAcatgatgagcttTAAggtacc
                                            5-gfp-MunI-linker
```

Figure 45: Schematic representation of the 5-gfp-MunI-linker/3-gfpER-KpnI PCR primers and their binding sites on the pCat-gfp template. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. *MunI* and *KpnI* sites are underlined. The *NcoI* site and the stop codon found in pCat-gfp are depicted with a light yellow background. Shown in red letters is the sequence coding for the ER retention signal peptide HDEL. The newly introduced stop codon is shown in bold letters.

After the PCR reaction the PCR product was purified by phenolization and subsequent precipitation by ethanol (5.9.10). The fragment was phosphorylated as described in section 5.9.11 and ligated into a *HincII* digested pUC19. Recombinant plasmids were screened by *MunI* digestion and plasmids releasing a 562 bp fragment were regarded as positive.

6.4.1.3 Cloning of pBSK-gfpER

To exchange the fluorophor coding sequence of mgfp5 with that from pCAT-gfp, pUC-gfpER was digested with *MunI*, releasing a 562 bp fragment. The fragment was purified by gel extraction (5.9.8). The vector pBSK-mgfp5 was digested with *EcoRI* and *MunI* (*EcoRI* and *MunI* have compatible overhangs). This resulted in a 3189 bp and a 562 bp fragment. The 3189 bp fragment was purified by gel extraction and the *MunI* fragment from the pUC-gfpER digest was ligated into this vector backbone (Fig. 46). Ligation of a *MunI* overhang with an *EcoRI* overhang results in a sequence that can not be cut by both the restriction enzymes, which was done to allow the usage of *EcoRI* in one of the later steps in the construction of the binary vector. The exchanged fragment also lacks the *NcoI* site the original sequence of mgfp5 carries.

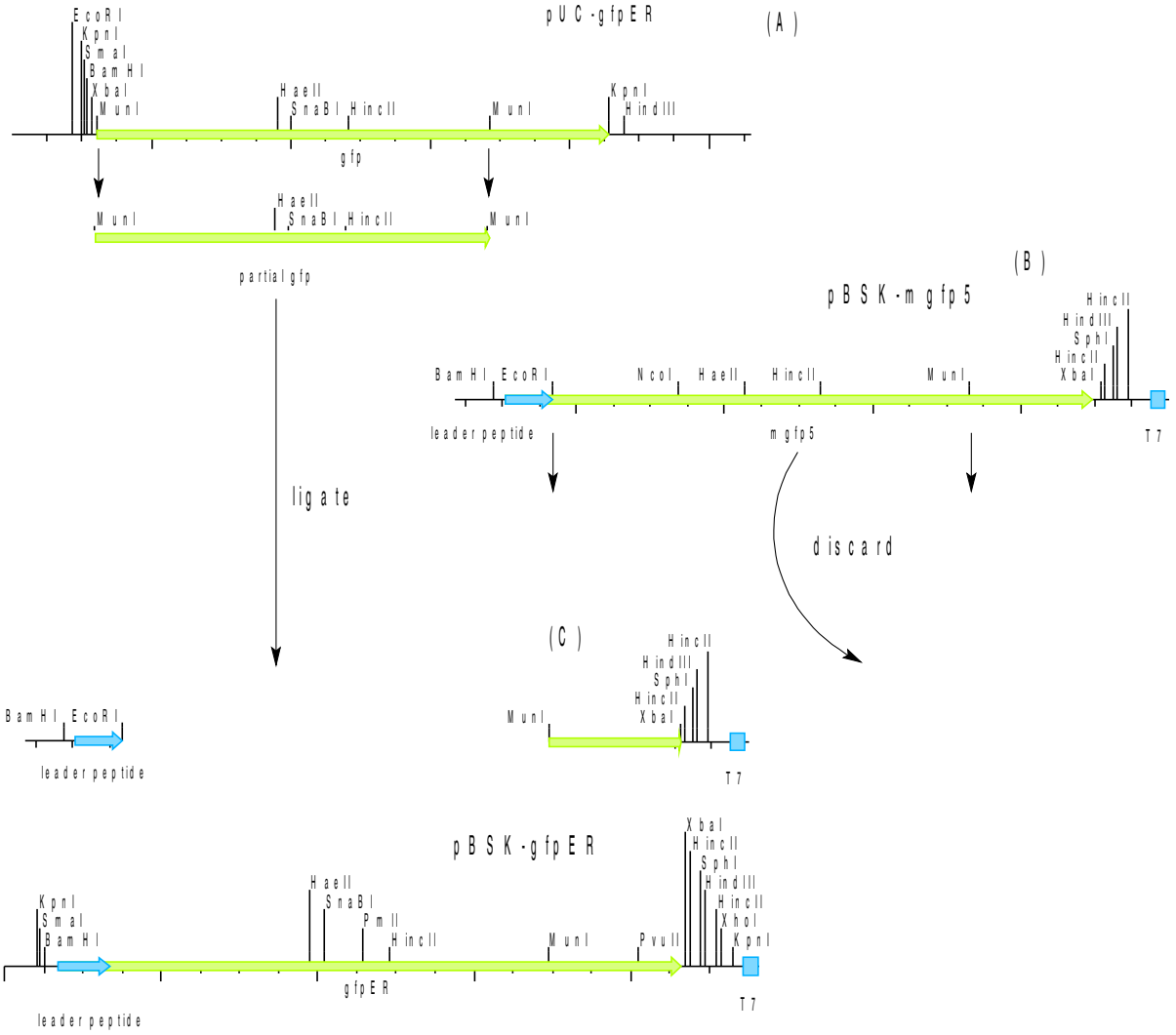


Figure 46: Schematic representation of the cloning strategy applied to obtain the pBSK-gfpER plasmid. (A) Digestion of pUC-gfpER with MunI yields the desired partial GFP fragment. (B) Digestion of pBSK-mgfp5 with *EcoRI/MunI* yields the desired plasmid backbone. (C) Ligation of the two nucleic acids yields the desired pBSK-gfpER construct. The GFP insert is depicted in light green, the leader peptide for the localization to the ER and the T7 promoter of pBSK are depicted in light blue. Common restriction sites in the construct are displayed were appropriate.

Recombinant plasmids were screened by *PstI* digestion and plasmids releasing a 289 bp fragment were regarded as positive clones carrying the insert in the right orientation.

6.4.1.4 Cloning of pTPCR-gfpER

For the cloning of the GFP gene that was inserted into pBSK-gfpER the introduction of an *NcoI* site at the 5' end was necessary. To do this GFP was amplified by PCR using the 5-gfp5-NcoI2/3-gfpER-KpnI primer pair (Fig. 47).

```
5-gfp5-NcoI
ccatgggtATGAAGACTAATCTTTT
tatattgtTACTTCTGATTAGAAAA-----pBSK-gfpER-----GTACCGTACCTACTTGATATGTTTGTACTACTCGAAATTCCcaga
                                               CATGGCATGGATGAACTATACAAACATGATGAGCTTTAAGGtacc
                                               3-gfpER-KpnI
```

Figure 47: Schematic representation of the 5-gfp5-NcoI/3-gfpER-KpnI PCR primers and their binding sites on the pBSK-gfpER template. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. *NcoI* and *KpnI* sites are underlined.

The PCR product was ligated into pTPCR a T-tailed PCR cloning vector [Wassenegger et al., 1994]. Recombinants were screened by *NcoI/KpnI* digestion and plasmids releasing a 803 bp fragment were regarded as positive and selected for sequencing.

6.4.1.5 Cloning of ppDI-gfpER and ppmDI-gfpER

For the construction of ppDI-gfpER and ppmDI-gfpER pTPCR-gfpER was digested with *NcoI* and *KpnI*. The resulting 803 bp fragment was electrophoresed, purified by gel extraction and then ligated into the likewise digested vectors ppDI and ppmDI. Recombinants were screened by *PstI* digestion and plasmids showing a 2658 bp – 1153 bp – 858 – bp or a 3365 bp – 853 bp restriction pattern for ppDI-gfpER and ppmDI-gfpER respectively were regarded as positive.

6.4.1.6 Cloning of pTPCR-DI-gfpER and pTPCR-mDI-gfpER

An *EcoRI* site at the 5' end and a *BamHI* site at the 3' end had to be introduced, to transfer the DI sequences into a 35S promoter cassette. The sites were introduced by PCR amplification using the primer pair 5-TBSV-EcoRI/Ir28 Marcello.

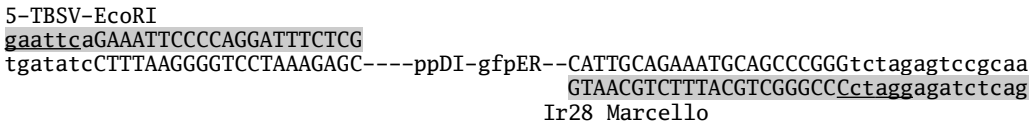


Figure 48: Schematic representation of the 5-TBSV-EcoRI/Ir28 Marcello PCR primers and their binding sites on the ppDI-gfpER template. Primers are depicted with a grey background. Capital letters show complementary sequences of primer and template. *EcoRI* and *BamHI* sites are underlined.

PCR amplification was done using ppDI-gfpER and ppmDI-gfpER as template. Both PCR products were ligated into the plasmid pTPCR a T-tailed PCR cloning vector. The transformants were screened by *EcoRI* digestion, plasmids releasing a 1376 bp and 1569 bp fragment for DI-gfpER and mDIgfpER respectively were regarded as positive. Positive clones were sequenced to verify the coding sequence of the GFP. The results showed a one base deletion in the 3' primer 3-gfpER-KpnI which resulted in the loss of the stop codon in that primer. Reintroduction of the stop codon is described in section 6.4.1.7.

6.4.1.7 Cloning of pTPCR-DI-gfpERK and pTPCR-mDI-gfpERK

Both plasmids were linearized with *XhoI* and the resulting 4 base 5' overhangs were filled in by a Klenow fragment reaction (5.9.9), to reintroduce the deleted stop-codon into the pTPCR-DI-gfpER and pTPCR-mDI-gfpER constructs. The plasmids were religated and screened for the absence of the *XhoI* restriction site. Positive clones were selected and sequenced because only a complete fill in of 4 bases or an addition of 1 base resulted in the reintroduction of the stop codon 6 or 5 codons downstream of the original site (Fig. 49).

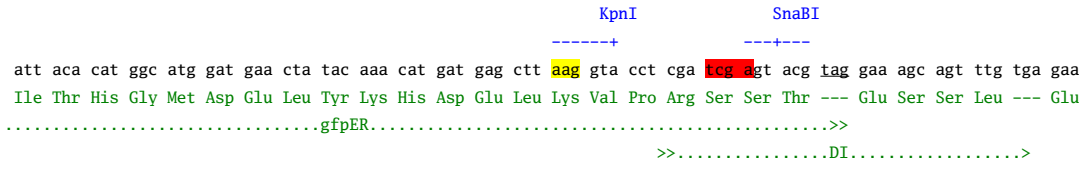


Figure 49: Schematic representation of the reintroduction of the deleted stop codon in pTPCR-DIgfpERK. Amino acid coding sequence is displayed as green text. Nucleotides introduced by the fill in reaction are depicted with a red background. The position of the original stop codon is displayed with a yellow background.

For both constructs positive clones were found, the plasmids were named pTPCR-DIgfpERK and pTPCR-mDIgfpERK respectively to differentiate between constructs without stop codon. The next cloning steps were carried out with the "K" constructs.

6.4.1.8 Transfer of DI-gfpERK and mDI-gfpERK constructs into 35S promoter cassette

The pTPCR-DI-gfpERK and pTPCR-mDI-gfpERK plasmids were digested with *EcoRI* and *BamHI* and the desired fragment purified by agarose gel extraction (5.9.8), to clone the DI-gfpERK and mDI-gfpERK constructs into 35S promoter cassette. Ligation of the purified fragment into the likewise digested pRT101 [Töpfer et al., 1987] yielded the pRT-DI-gfpERK and pRT-mDI-gfpERK constructs. Transformants were screened by PstI digestion and plasmids having a 2.6 – 1.1 – 1.0 – kb (mDI construct) and a 2.6 – 1.1 – 0.8 kb(DI construct) band pattern were regraded as positive.

6.4.1.9 Cloning of pGJ-DI-gfpER and pGJ-mDI-gfpER

The final step in the construction of the binary vector was the insertion of the 35S-DIgfpERK construct into the pGJ357 plasmid, a ppzp200 [Hajdukiewicz et al., 1994] based binary vector harboring a kanamycin resistance gene. The promoter cassette containing the DI and mDI constructs was released from the pRT101 plasmid by digestion with HindIII. The fragment was purified and ligated into, the likewise digested and dephosphorylated, pGJ357. Recombinant plasmids, pGJ-mDIgfpERK and pGJ-DIgfpERK, were screened by digestion with EcoRI and plasmids releasing a 2.3 kb fragment were selected as positive clones with the "right" (head to head) orientation.

With plasmid DNA purified from positive *E. coli* clones, *Agrobacterium tumefaciens,* strain ATHV, was transformed by electroporation (5.9.17). Verified transformants were used in *Agrobacterium tumefaciens* infiltration experiments.

6.4.2 Agroinfiltration for transient expression of GFP constructs on *N. benthamiana*

N. benthamiana plants were agroinfiltrated with ATHV bacteria carrying the pGJ-mDIgfpERK and pGJ-DIgfpERK constructs to evaluate the GFP expression of the 2 plasmids. As control plants were infiltrated with pGJ-gfp, a binary vector construct based on pGJ357 harboring a GFP gene under the control of an enhanced 35S promoter, as described preciously [Reichel et al., 1996]. After 3 days the plants were visually examined with the fluorescence microscope. Both DI constructs showed no GFP fluorescence which was readily detectable in the pGJ-gfp infiltrated plants. Therefore it was decided not to generate stable transformants using the DI and mDI GFP constructs.

6.4.3 Agroinfiltration for transient expression of GUS constructs on *N. tabacum and GUS silenced N. tabacum*

N. tabacum plants were agroinfiltrated (5.12) with *ATHV* bacteria carrying the pBar-DIGUSInt and pGJ-GUSInt constructs to evaluate the GUS expression of the 2 plasmids. After 5 days, histochemical GUS staining was performed (5.10.1). Both constructs showed GUS expression in *N. tabacum* (Fig. 50 (A) and (C)) and were utilized for further infiltration experiments with GUS silenced *N. tabacum.* GUS staining of non infiltrated wild type *N. tabacum* and the silenced *N. tabacum* plants did not exhibit GUS expression.

Further infiltration experiments were done with GUS silenced *N. tabacum* plants (Wassenegger, personal communication). Histochemical GUS staining was performed one dpi and five dpi. Leaves stained one dpi did not exhibit GUS expression (data not shown). The resulting GUS stained leaves, which were stained five dpi, can be seen in Figure 50.

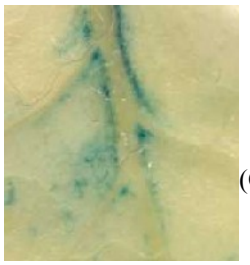
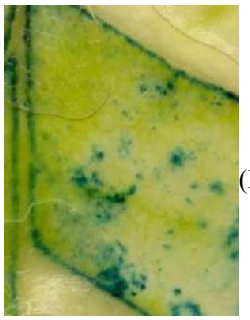
(A)  (B)  (C)  (D) 

Figure 50: Histochemical GUS staining of *Agrobacterium* infiltrated *N. tabacum* leaves 5 days post infiltration. (A) *N. tabacum* infiltrated with ATHV carrying the pGJ-GUSInt plasmid. (B) GUS silenced *N. tabacum* infiltrated with ATHV carrying the pGJ-GUSInt plasmid. (C) *N. tabacum* infiltrated with ATHV carrying the pBar-DIGUSInt plasmid. (D) GUS silenced *N. tabacum* infiltrated with ATHV carrying the pBar-DIGUSInt plasmid.

As has been shown by Eilers [Eilers, 2002] the leaves infiltrated with the DI-GUS construct exhibit a more intensive blue color than leaves infiltrated with the GUS construct. Nevertheless, no differences could be observed between GUS silenced plants and wild type *N. tabacum*.

7 Discussion

Functionality of the viral replicase

The *in trans* complementation system utilizing a replicase deficient mutant of tomato bushy stunt virus (TBSV-M) was able to prove the functionality of the transgenically expressed replicase (biological assay). However, the system was not able to give an indication of the level of activity of the heterologous protein (biological assay). The number of plants susceptible to the mutant virus varied among different experiments and showed only mild viral infection symptoms. The infection however did not result in lethal necrosis as with the wild type virus. The lack of lethal necrosis could be a result of silencing effects due to the presence of the viral replicase in the plant genome. Indication for this was that the plants only showed mild viral symptoms and recovered from the infection after the initial appearance of systemic symptoms.

Earlier studies conducted in our institute [Boonrod et al., 2005] showed that viral RNA, isolated from replicase expressing TR4 plants showing mild viral symptoms upon infection with TBSV-M, possessed mutations restoring the replicase activity. These mutations could only be the result of an active viral replicase present in these plants, since wild type *N. benthamiana* did not show any infection symptoms. This suggests that the replicase expressed in the TR4 plants is active and replicates the viral genome. However, for an efficient infection the viral replicase needs to be expressed from the viral genome. Therefore, only plants which altered the viral genome to produce a functional replicase showed viral symptoms. The different infection ratios of the inoculation experiments (section 6.1) can be explained by this phenomenon because the successful infection depended on random errors made by the transgenic replicase. Although the activity of the recombinant replicase expressed in the TR4 plants was low it was assumed the activity was sufficient for the replication of the DI-RNA for protein expression and the induction of gene silencing.

Protein expression utilizing the DI vector system

In the conducted protein expression studies (section 6.2) no protein could be detected regardless of the gene or the source of the replicase (transgenic expression or helper virus). Reporter genes of different sizes were inserted in the vector to exclude the possibility of size

constraints of the DI vector [Scholthof, 1999]. The reporter genes (GFP, GUS and scFv) had been previously expressed in plants [Meinke et al., 1994] and thus the possibility of a general expression problem with those genes was unlikely.

Furthermore, experiments with several homologous viral genes were not successful, although expression of viral proteins from DI-RNAs has previously been reported for cymbidium ringspot tombusvirus [Burgyán et al., 1994]. Homologous viral genes like the coat protein were utilized to minimize the possibility of negative effects of the RNA secondary structure which might have the potential to interfere with DI-RNA replication. The failure to detect the viral coat protein might be explained by the assay that was applied to detect this protein. Burgyán and coworkers [Burgyán et al., 1994] detected the coat protein by western blot analysis which was not possible in this work because no TBSV coat protein antibodies were available. The ELISA adopted in this work can only detect viral particles not the monomeric coat protein. The assembly of the viral particles is dependent on several factors and the lack of a coat protein gene can not easily be compensated *in trans* [Burgyán et al., 1994]. The authors state that they were unable to detect viral particles if a virus mutant was used as helper virus which had large deletions in the coat protein gene. However, utilization of a different mutant, carrying only point mutations in the coat protein gene which render the protein translated from the genome unable to form viral particles, resulted in the formation of detectable viral particles.

Therefore, it is possible that the coat protein was expressed but our detection assays were not sensitive enough. However, as the aim of the project was to produce large amounts of protein a more sensitive method was not desired.

Replication of DI RNA

Previous publications [Kollár and Burgyán, 1994; Burgyán et al., 1994] demonstrated the instability of some DI/insert combinations. Consequently, replication of our DI constructs was analyzed by different methods to investigate the general ability of the DI to replicate and the possible instabilities of the inserts. Northern analysis of TBSV/DI inoculated wild type *N. benthamiana* with a virus specific probe, failed to detect the replication of the DI-RNA. The helper virus also hybridized with the DI-probe and caused a background signal which made it impossible to distinguish between the DI and the viral background. With a scFv specific

probe no signal could be detected in samples taken from DI-ScFv inoculated plants after the first day post inoculation (dpi). Two possible reasons were considered:

(a) No replication occurred. If so, the signal from the first day would have originated from the inoculum.
(b) The scFv insert was unstable during replication and thus the DIG labeled scFv specific primer was unable to detect the RNA. As the length of the labeled primer was only 20 bp, a deletion of the homologous sequence can not excluded.

Indeed, subsequent Northern blot experiments with a DI-*FtsH* RNA, demonstrated that the insert is gradually deleted from the DI and that only a small portion of the DI-RNA retains the complete insert (Fig. 42). They additionally showed that the DI-RNA moves systemically throughout the plant.

A different system had to be adopted to eliminate the viral background signal and to further analyze the replication of the DI-RNA. Protoplasts isolated from TR4 plants were chosen because they should be able to replicate the DI-RNA without helper virus. The replication can be detected even though the DI-RNA can not move systemically which was not possible in TR4 plants. This method made it possible to monitor the replication without interference of the parental virus and was described previously [Kollár and Burgyán, 1994]. Protoplast transfection experiments prove that the transgenically expressed replicase replicates two of the DI-RNA constructs. For both of these RNAs a band of the corresponding size could be detected in samples taken from protoplasts 24 h after transfection confirming that the RNAs were amplified by the replicase. However, not all the DI constructs were able to replicate. DI-gfp was not detectable after 24 h which could have several reasons like instability of the insert [Scholthof, 1999] or the formation of a secondary RNA structure inhibiting the replication.

Translation of DI-RNA

Since the replication of the DI-RNA constructs and the stability of inserts was not the limitation of our system in all cases, it was assumed that translation of the DI-RNA could be an obstacle. White and coworkers [Wu and White, 1999; Fabian and White, 2004] found that

a 190 bp sequence in the 3' UTR of TBSV enhances translation of DI-RNAs, encoding for proteins, more than tenfold compared to uncapped DI-RNA. TBSV has no 5' cap and no 3' poly-A tail. It instead contains a 3'-terminal RNA sequence that acts as a cap-independent translational enhancer (3' CITE) [Fabian and White, 2004]. This sequence is absent in our DI construct as it is a prototypical short DI [Hull, 2002]. White and coworkers utilized a so called long DI molecule which harbored the 3' CITE sequence. Though a modified DI, possessing the 3' CITE, was successfully constructed, protein expression could not be observed in the inoculation experiments on greenhouse plants. The results of our experiments with the modified DI can however, not be compared with the work from the White group [Wu and White, 1999; Fabian and White, 2004]. They only analyzed transient GUS expression in protoplasts unable to replicate the DI-RNA after a 24 h incubation period. Their system differs from ours in two ways. The replication of the DI-RNA can lead to deletions in the gene and to a decreased expression. The detection assays applied in our system were dependent on the systemic movement of the DI-RNA in the plant. They were not sensitive enough to detect single cells expressing the desired protein. However, as it was the aim of the project to develop a transient expression system for the large scale production of proteins, systemic movement was essential and therefore the detection methods were chosen accordingly.

Our experiments show that the DI-RNA is replicated by the viral replicase, expressed in TR4 plants and by the wild type helper virus. When using transgenic plants the major drawback is the inability of the DI-RNA to move systemically throughout the plant. Only a few cells on each leaf are infected by rub-inoculation and thus rendering the system unsuitable for protein expression. Systemic movement of the DI-RNA was possible when DI-RNA was co-infected with wild type helper virus. However, deletions that occur during DI-RNA replication made protein expression impossible. In conclusion one can say that the DI-RNA system is unsuitable for protein expression.

Induction of gene silencing with the DI/TR4 plant vector system

The TR4 plant/DI-RNA system was unable to induce silencing. The failure of the TR4 plant system could be due to the inability of the DI-RNA to spread systemically throughout the plant without a helper virus since replication of the DI-RNA by the replicase expressed in

the TR4 plants has been shown and silencing could be induced in combination with a helper virus. An indication for this was the fact that co-infection with pTBSVΔΔp19, a virus mutant that did not result in systemic infection, was also not able to silence one of the tested endogenous genes. It was not possible to test whether the TR4 plant system is able to induce GFP silencing, as pTBSVΔΔp19/DI did, because GFP expressing TR4 plants were not available. Furthermore, insufficient replicase activity might be an additional explanation for the inability of the DI/TR4 plants system to induce silencing.

Inoculation with *Agrobacteria* carrying the silencing construct, to promote the silencing induction on the TR4 plants with a stronger inoculum [Gosselé et al., 2002], was not successful. Apparently, the low and local DI replication that was observed in the TR4 plants does not produce enough double stranded RNA that would be required for systemic gene silencing. The co-expression of the viral movement protein in addition to the viral replicase could be a future strategy. However, it is doubtful that such a system would be competitive, in comparison to existing silencing systems [Gosselé et al., 2002; Saitoh and Terauchi, 2002] especially considering high throughput approaches. Therefore, it can be concluded that the TR4 plant system is not suitable for the induction of gene silencing.

Induction of gene silencing with the DI/TBSV vector system

In contrast to the TR4 plant /DI system the use of a DI/TBSV system for the induction of gene silencing has been reported previously [Hou and Qiu, 2003]. However, Hou and Qiu only reported the silencing of a transgene (GFP). Our DI-RNA system was successfully applied to induce gene silencing in combination with different helper viruses like wild type TBSV and different p19 deficient mutants of TBSV. With this system it was possible to silence three different genes, one transgene (GFP) and two endogenous genes (PDS and *FtsH*). The DI-RNA in combination with different viral helper constructs was able to induce silencing to different degrees depending on the helper construct and inoculation procedure. GFP was the gene which was silenced, to some degree, by all of the DI/virus combinations. This was not surprising as GFP and transgenes in general are more prone to silencing than endogenous genes.

Silencing of the *FtsH* protease

Silencing of the *FtsH* protease was only observed when the DI constructs were co-inoculated with pTBSVΔp19. Neither wild type virus nor pTBSVΔΔp19 were able to induce silencing of this gene (section 6.3.9). Even with pTBSVΔp19 silencing symptoms were not pronounced. One may speculate that other proteases compensate the down regulation of this gene, as the *FtsH* gene belongs to a large gene family of metalloproteases [Sinvany-Villalobo et al., 2004; Silva et al., 2003; Sakamoto et al., 2003]. However, efficient silencing of this gene by VIGS has been previously reported [Saitoh and Terauchi, 2002]. Therefore it is likely that the failure of inducing silencing is a deficiency of the DI system. The inserted *FtsH* gene fragment was not stable during replication of the DI-RNA. The majority of the DI molecules detected by Northern blot analysis (Fig. 42) had large deletions and it was not clear how much of the homologous *FtsH* sequence was retained in the DI-RNA. This could also have an impact on the silencing ability of the DI-RNA since length of the silencing inducer plays a role in the induction process [Thomas et al., 2001]. Longer homologous sequences are thought to be better gene silencing inducers. The same publication also states that silencing is additionally dependent on the context of the silencing trigger sequence. Within endogenous genes or transgenes, some trigger sequences, were found to be more efficient than others. Therefore, it is conceivable that the 395 bp long *FtsH* insert, present in the DI construct, comprised a region displaying low silencing capacity. Alternatively, the efficient trigger sequences were deleted during replication of the DI-RNA. This could be a possible explanation for the observed differences between PDS and *FtsH* silencing. For optimization of the vector system one may precisely analyze the deletions of the DI-RNA constructs and subsequently remove deletion hot spots from the constructs. However, this needs to be done for every gene and would, thus be not feasible for a high throughput approach. Experiments in section 6.3.17 indicated that the 5' and 3' terminal sequences of the *FtsH* insert were deleted in the smaller DI molecules (Fig. 42). For each primer pair, one of the primers that was designed to amplify the DI-*FtsH* fragment, matched either the 5' or the 3' end of the inserted *FtsH* fragment (Fig.43). The other primer was complementary to the DI sequence. Using these primer pairs only the complete DI-*FtsH* molecule could be amplified. This was an unexpected result since the Northern blot analysis data revealed that the smaller molecules were present in a higher concentration. One would have expected amplification of not only complete but also

truncated *FtsH* fragments. One may speculate that the primer binding sites in the *FtsH* fragment were preferentially deleted from the DI-RNA. An explanation for this could be that the *FtsH* sequence was inserted between region I and II of the DI-RNA, a site were a large deletion occurs during the evolution of natural DI-RNAs [White and Morris, 1994; Havelda et al., 1997]. However, this site was reported to be the most stable insertion site for heterologous inserts in DI-RNAs by other authors [Burgyán et al., 1994]. RNA secondary structure prediction using the MFOLD program [Zuker, 2003] was done to determine whether a secondary structure was formed which would support the preferential deletion of 5' and 3' sequences of our insert. The analysis did not give an indication of a structure which would support this hypothesis.

Silencing of the phytoen desaturase

The PDS fragment inserted into the DI vector silenced the corresponding gene to a higher degree than the *FtsH* construct. The PDS gene is widely adopted as a control reaction in silencing experiments because it is comparatively prone to silencing and its silencing is easily detectable by the appearance of a white leaf phenotype [Waterhouse and Helliwell, 2003; Constantin et al., 2004]. Additionally the PDS insert is smaller than the *FtsH* insert and therefore might be more stable during replication. Possibly, the size and the fact that PDS is readily silenced are an explanation for the more efficient silencing. As both inserts have been used in other VIGS systems, resulting in pronounced silencing symptoms [Saitoh and Terauchi, 2002; Wassenegger personal communication] it can be excluded that the sequences themselves induce silencing to a different degree. However, the insert stability during DI-RNA replication may be an explanation for the differences in silencing behavior in our vector system.

Differences between the virus mutants

Additional differences between the viral helper constructs TBSV, pTBSVΔp19 and pTBSVΔΔp19 apart from the varied silencing success were studied. The p19 protein is a potent gene silencing suppressor. Therefore, two p19 deficient mutants were created for a more efficient induction of gene silencing. pTBSVΔp19 with a point mutation changing the

ATG start codon to a CTG and pTBSVΔΔp19 with three further mutations introducing two stop codons and one amino acid exchange approximately 200 bp downstream of the start codon. The p19 ORF is situated within the ORF of p22 (Fig. 3). p22 is essential for systemic infection of plants. Mutations had to be designed in such a way that the amino acid sequence of p22 remained unchanged. Variations between the wild type virus and the two p19 deficient mutants were expected [Hou and Qiu, 2003; Qu and Morris, 2002; Qiu et al., 2002] due to the lack of p19 expression. However, differences between the two p19 mutants were surprising since both were unable to express p19. pTBSVΔp19 showed systemic symptoms 2-3 days before pTBSVΔΔp19 when inoculated without DI transcripts. When inoculated with DI transcripts, pTBSVΔΔp19 was not able to infect the plants systemically. An attempt to explain this phenomenon was done and the codon usage of p22 in the two mutants was analyzed. However, the codon usage of both mutants does not differ significantly compared to the average for higher plants or to highly expressed plant genes [Murray et al., 1989]. Therefore, the phenomenon seems to be related directly to the RNA sequence of the constructs. Until now no secondary RNA structure elements have been reported involving the region of the second mutation in p19 (~200bp downstream of the 5' end of sgRNA2) [Fabian and White, 2004]. Results concerning the necessary RNA/RNA interactions to initiate translation of sgRNA2 postulate that the interactions involved are between 5' and 3' end of the sgRNA2 and not the mutated region.

The disposition of RNA virus replicases to a high mutation rate during replication could be an alternative explanation [Hull, 2002]. It enables the viruses to revert to a functional sequence as demonstrated by Boonrod and coworkers [Boonrod et al., 2005] for viral replicase mutants. This ability of RNA viruses was the reason for the construction of the pTBSVΔΔp19 mutant. The second mutation at a different position decreasing the possibility of reverting to a functional sequence. Nevertheless, it was decided to further investigate this difference in infection behavior. The possibility that the pTBSVΔp19 mutant reverted to its original sequence and therefore exhibited a different infection behavior was investigated by isolating RNA from infected plants, reverse transcribing the RNA into DNA, amplifying and cloning the DNA into a suitable vector for sequencing. The sequencing results showed that the mutations were stable and no reversions occurred. Consequently, the differences between the two mutants do not result from differential expression of p19.

There are two further possible explanation for the infection behavior of these two mutants. One theory postulates that the mutations favor the formation of a RNA secondary structure that inhibits the translation of p22 from sgRNA2. Computer aided comparison of the wild type sgRNA2 secondary RNA structure with the two mutants with the MFOLD program [Zuker, 2003] revealed differences between the two mutants and the wild type virus but no significant differences between the two mutants themselves (data not shown). It was decided to abandon this theory as the computer analysis did not support it.

The most likely explanation was that the mutations in pTBSVΔΔp19 inhibit the replication of the viral RNA by the replicase. The replication ability of RNA viruses can be destroyed by point mutations in the viral genome even if the protein sequence is unaltered [Hull, 2002]. There have been no reports about the region carrying the mutation being a control signal. The majority of control signals reported for TBSV or RNA viruses in general lie near the 5' and 3' end of the genome, but other interactions are also possible [Hull, 2002]. This theory is supported by the fact that co-inoculation with DI-RNA had an additional impact on the infection with the virus mutants. DI-RNA have an attenuating effect on symptom development in wild type virus infections [Havelda et al., 1998]. The effect is based on the preferential replication of the DI-RNA compared to the viral genome. This preference could become more pronounced because of the mutation introduced into the pTBSVΔΔp19 construct. As a consequence the titer of the genomic RNA becomes to low for an efficient infection of the plant. The effect is also present in wild type infections, however for our mutant, which has no gene silencing repressor, it becomes so pronounced that systemic infection does not occur. This effect has been described before, though not as severe, [Qiu et al., 2002]. These authors stated that the efficient infection of plants is dependent on the dosage of p19 and the coat protein.

Further research into the infection behavior of our two mutants would be required to get a better understanding of this process.

Introduction of the silencing construct by *A. tumefaciens* infiltration

It was decided to inoculate the silencing inducer by *A. tumefaciens* infiltration, as other experiments with satellite viruses have shown that efficient silencing is to a large extent dependent on the inoculation of the silencing inducer [Gosselé et al., 2002, Helliwell and

Waterhouse, 2005].

Infiltration of TR4 plants with a binary vector DI-PDS construct did not induce silencing symptoms. One can only speculate about the reasons for this since no further investigations were conducted to explain this phenomenon. One hypothesis is that systemic movement of the viral RNA is essential for the successful induction of gene silencing in our system. This is supported by the fact that none of the endogenous genes showed silencing symptoms in inoculated leaves.

Furthermore, infiltration of the silencing inducer in combination with rub-inoculation of helper virus (wild type and p19 deficient mutants) did not induce gene silencing which contradicts findings of Gossele and coworkers [Gosselé et al., 2002]. Their system being similar to ours in regards to separate delivery of the silencing inducer and the helper virus. It was speculated that this could be a further dosage effect. The different findings could be explained in that the large excess of DI-RNA present in the plant, efficiently utilizes all the viral replicase and therefore replication of the genomic RNA is inhibited. This phenomenon was not further pursued and instead the helper virus and the silencing inducer were introduced by *A. tumefaciens* infiltration.

Inoculation of both helper virus and silencing construct by *A. tumefaciens* infiltration

Infiltration of wild type helper virus together with the DI-PDS construct induced silencing 6-7 dpi. This finding contradicts experiments with the wild type virus inoculated as RNA transcripts. This supports the idea that the DI gene silencing system is influenced by an dosage effect. The onset of silencing was 10-15 days earlier than with the TBSVΔp19 mutant which indicates that the infection with the wild type virus was more efficient. However, the silencing symptoms were not as pronounced as with the TBSVΔp19 mutant. The major reason for this is probably the severe stunting of the leaves from the viral infection. This causes a reduced growth of the plants and as a result only small white bleached spots are formed. Severe viral symptoms make this system unsuitable as a VIGS vector as they interfere with the detection of silencing symptoms.

Agrobacterium mediated inoculation with the TBSVΔp19 mutant and the DI-PDS silencing inducer resulted in the appearance of mild viral symptoms approximately 25 dpi. White bleached spots appeared about 30 dpi (Fig. 41). However, the infection was only successful with about 50% of the inoculated plants. The delayed onset of the viral infection is most likely due to the combination of two facts, the missing gene silencing repressor and the high inoculum concentration derived from *A. tumefaciens* infiltration.

For the binary vector constructs it might be possible to enhance the replication by introducing a ribozyme further downstream of the 3' end of the virus. Since the poly-A tail interferes with the replication of the virus [Scholthof, 1999], the ribozyme would remove the poly-A tail and restore the native 3' end of the virus. However, it is unlikely that this modification would remedy all the limitations of our system.

In conclusion one can say that the combination of DI and TBSV can be applied for the induction of gene silencing. However, the system has some limitations which make competing systems like SVISS superior. The limitations are mainly the instability of the silencing trigger sequence and the fact that the silencing symptoms are not as pronounced. The symptoms can not clearly be distinguish from the viral infection symptoms. This may be explained by the fact that TBSV is known to be an aggressive virus [Hull, 2002] and therefore it is possible that it escapes the gene silencing mechanism as described for other viruses [Taliansky et al., 2004]. Escape of the helper virus from silencing means that the silencing mechanism is somehow repressed and therefore the silencing of the target gene may be also effected.

As an outlook, there are some optimizations that could be performed on the DI/TBSV system, like introduction of a weaker gene silencing repressor replacing p19 [Hou and Qiu, 2003]. However, the possible benefits of optimizations like this would have to be evaluated carefully since progress in this field is made continuously and other VIGS systems seem more promising.

Influence of the DI sequences on transient protein expression and gene silencing

Using the two binary vector GFP constructs, made for evaluating the potential of the DI sequences to protect RNA from gene silencing, no GFP expression could be observed.

Several factors could be the reason for this, as the constructs were assembled out of numerous different parts which have not been tested together. The ER-leader peptide had to be altered because a *NcoI* site at the 5' end of the gene was necessary for the insertion into the DI vector. The modification of the 5' end to introduce the *NcoI* site resulted in the addition of Met-Gly at the N-terminal end of the protein. In order to find out whether this has an effect on the cleavage of the leader peptide, the protein sequence was analyzed with the computer program sigcleave [Rice et al., 2000] which predicts the site of cleavage between a signal sequence and the mature exported protein with an accuracy of 75-80 %. The analysis showed that cleavage of the signal peptide was not responsible for the lack of GFP expression. The C-terminal end of GFP was also modified due to an error in the 3' end primer. The error resulted in the loss of the stop codon which had to be reintroduced by a *XhoI* digestion, subsequent fill in of the overhangs and religation. This resulted in the addition of the amino acids Lys-Val-Pro-Arg-Ser-Ser-Thr before the newly introduced stop codon. However, GFP is readily expressed as a N- or C-terminal fusion-protein so the additional amino acids should have no effect on GFP fluorescence. They could however influence the function of the ER retention signal His-Asp-Glu-Leu (HDEL) but this should not cause the protein to be degraded [Ellgaard and Helenius, 2001; Brandizzi et al., 2003] and therefore GFP fluorescence should still be detectable. The most likely reason for the absence of GFP fluorescence is the formation of a RNA secondary structure which inhibits translation of the mRNA. This would also explain the lack of GFP expression in protoplasts transfected with DI-GFP RNA. The analysis of this problem would be beyond the scope of this thesis and it was decided not to use the GFP constructs for further experiments. An already existing DI-GUS construct was used instead.

GUS expression was detected for both the DI-GUS and GUS constructs. The expression level as far as it could be distinguished by histochemical GUS staining was higher in DI-GUS infiltrated plants then in GUS infiltrated plants. However, infiltration of GUS silenced *N. tabacum* plants with GUS and DI-GUS exhibited GUS expression with both constructs. This is contrary to the expected behavior that the GUS construct would exhibit no GUS expression in the silenced plants [Voinnet et al., 1998]. The reasons for the absence of GUS silencing were unknown. The GUS enzyme is reportedly [Cervera, 2005] widely utilized in plant transformation because of its stability and the sensitivity of the detection assay. The high sensitivity of the may be one reason for the inability of our assay to detect the inhibition of GUS expression after *A. tumefaciens* infiltration. The higher sensitivity of the enzymatic

assay compared to the direct detection of GFP was also the basis for green fluorescent protein as the first choice in infiltration experiments on silenced plants since GFP silencing is easily detectable.

When using *A. tumefaciens* infiltration for transient expression of proteins, the role of gene silencing increases greatly within the time course of experiments [Voinnet et al., 2003]. It is therefore possible that at five dpi silencing was occurring in the wild type *N. benthamiana* as well as in the GUS silenced plants. This would be an explanation for the different expression levels observed in our experiments. However, the use of histochemical GUS staining to distinguish between different intensities of GUS expression was not ideal since it is not suitable for the detection of small differences.

In our opinion it would be viable to do more studies regarding the influence of the DI sequences on mRNA stability and protein translation. With a deeper understanding of the effects of DI sequences on mRNA one would be able to differentiate between effects during viral replication [Pantaleo et al., 2004] and others useful for the generation of plant transformation constructs.

It is desirable that experiments with other reporter genes should be performed to get a more general statement about the influence of DI sequences. A different GFP or another unrelated fluorescent protein like RFP would be a good starting point. However, since the experiments would require the generation of transgenic plants this should be done in a new project.

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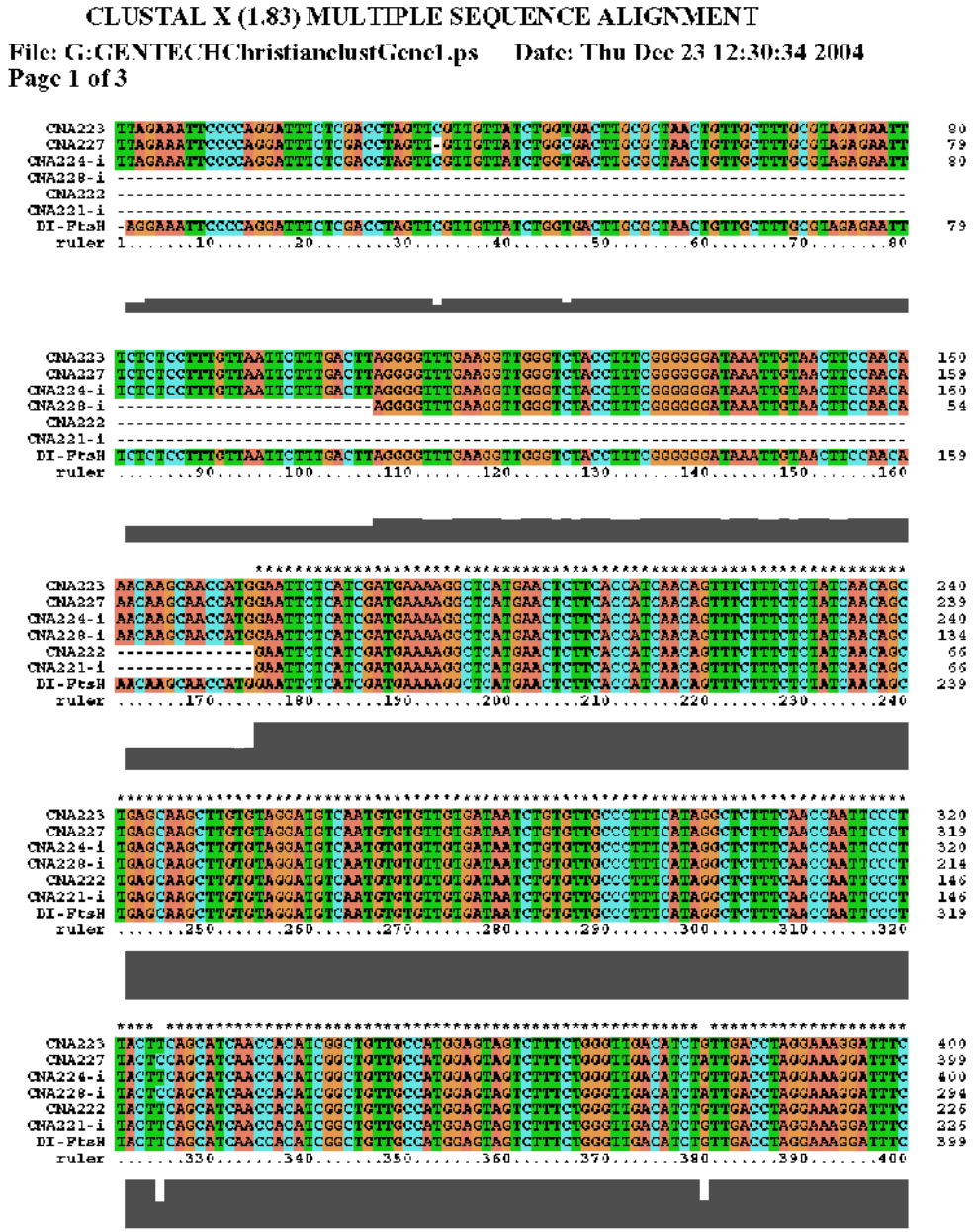
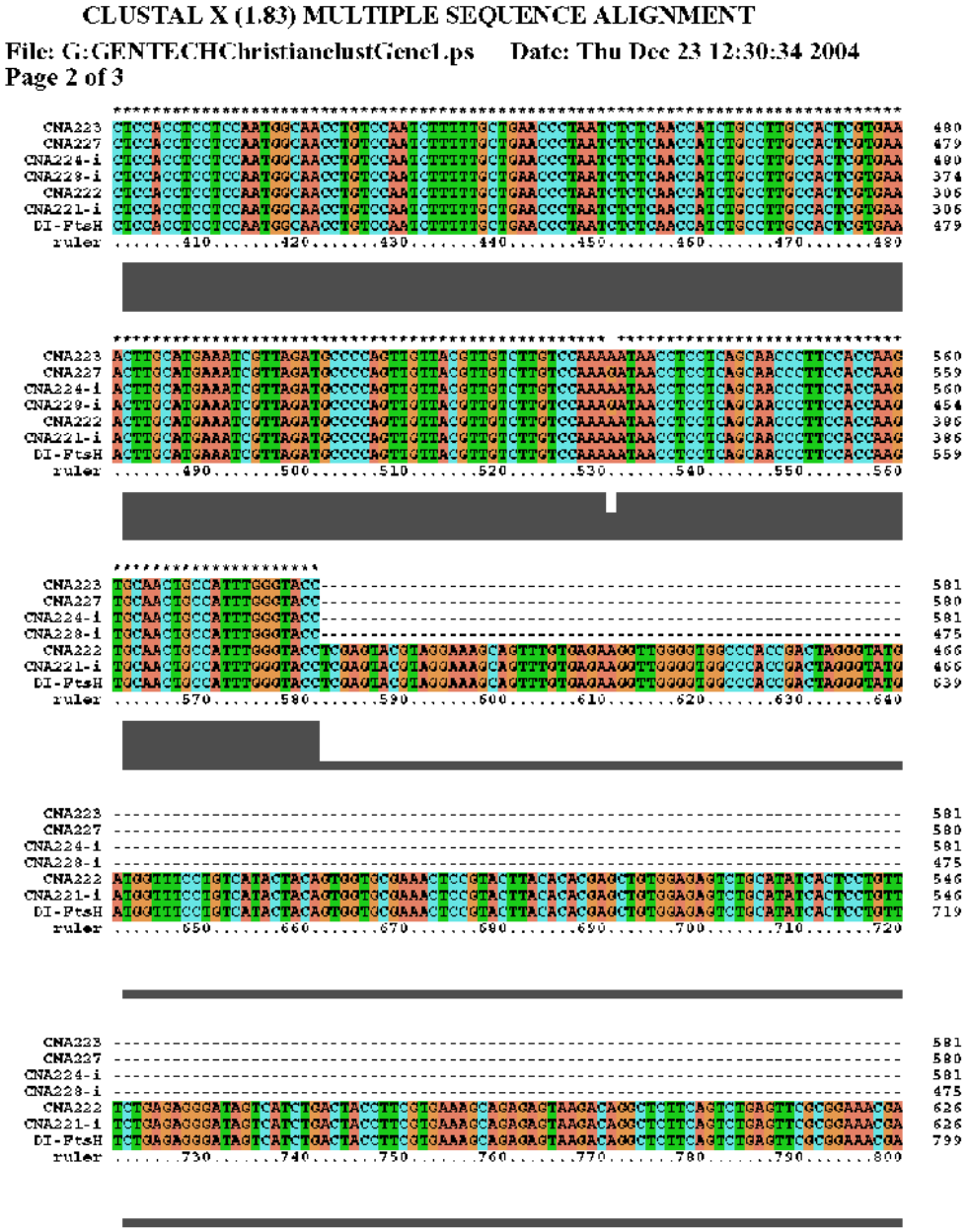
9 Appendix

9.1 ClustalX alignment of the isolated DI-*FtsH* Sequences

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Page 2 of 3
CNA223
CNA227
CNA224-i
CNA228-i
CNA222
CNA221-i
DI-FtsH
ruler

CLUSTAL X (1.83) MULTIPLE SEQUENCE ALIGNMENT

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Page 3 of 3

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    CNA227 ----------------------   580
  CNA224-i ----------------------   581
  CNA228-i ----------------------   475
    CNA222 GTGTGAATCTGACAACGTATGTA   649
  CNA221-i GTGTGAATCTGACAACGTATGTA   649
   DI-PtsH GTGTGAATCTGACAACGTATGTA   922
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9.2 Plasmid map of ppDI-scFv, -mGFP and -CP

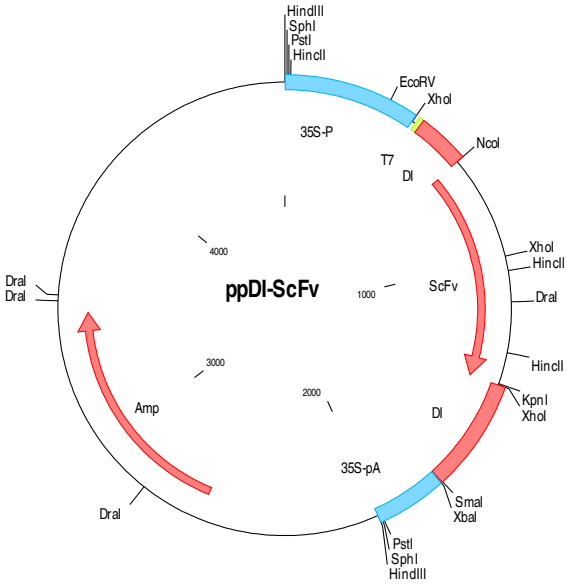

Figure 51: Plasmid map of ppDI-scFv

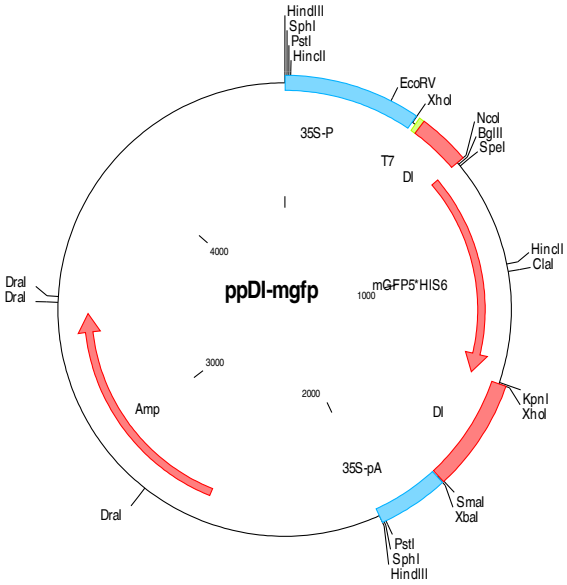

Figure 52: Plasmid map of ppDI-mgfp

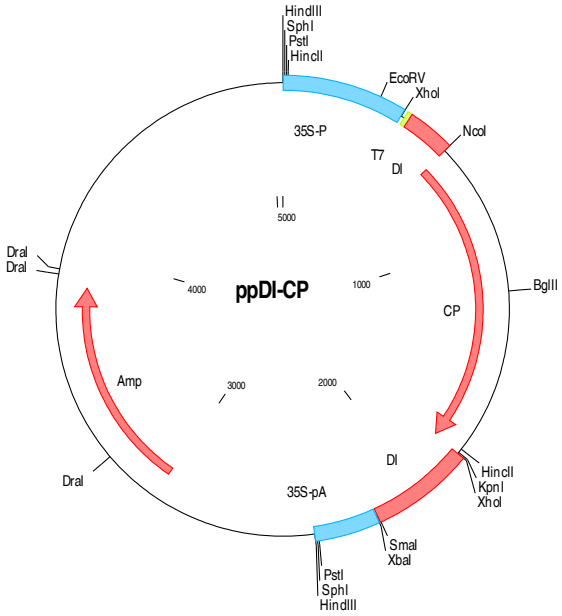

Figure 53: Plasmid map of ppDI-CP

9.3 Plasmid map of ppDI-p22 and ppDI-p22M

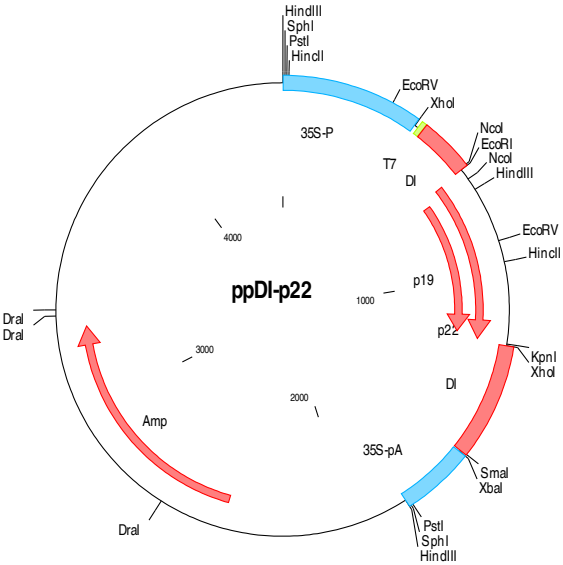

Figure 54: Plasmid map of ppDI-p22

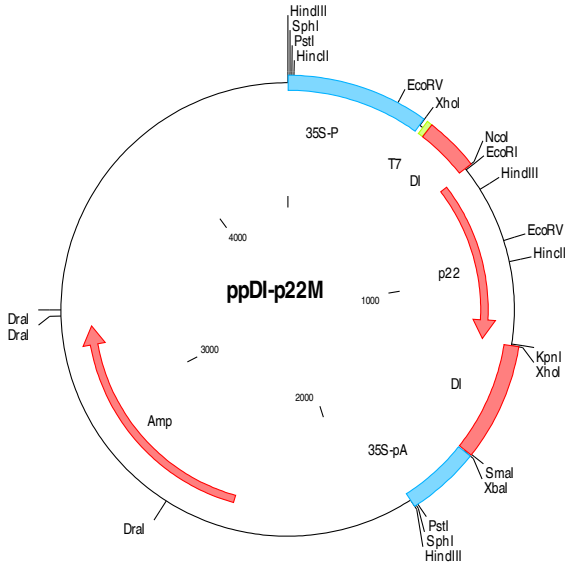

Figure 55: Plasmid map of ppDI-p22M

9.4 Plasmid map of ppmDI

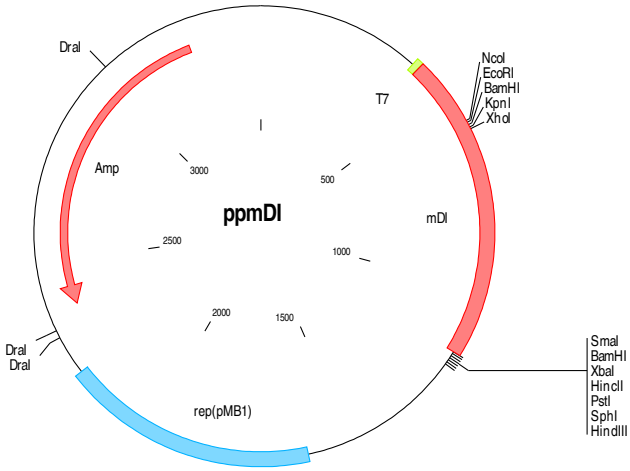

Figure 56: Plasmid map of ppmDI

9.5 Plasmid map of ppmDI-GUS, -GFP, -scFv, -YFP, -CP, -p22

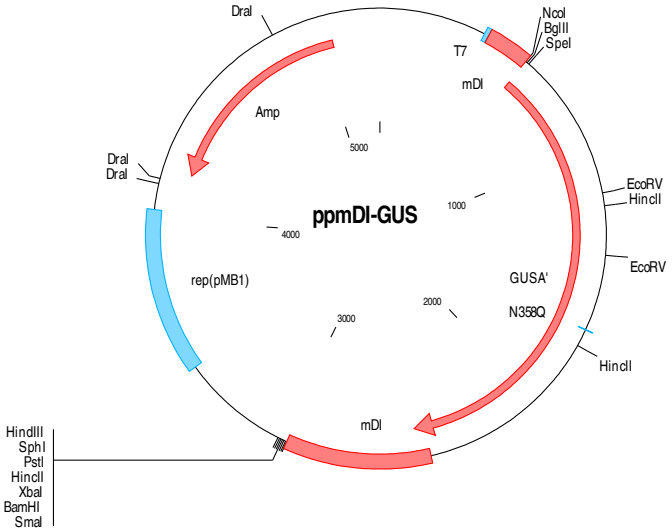

Figure 57: Plasmid map of ppmDI-GUS

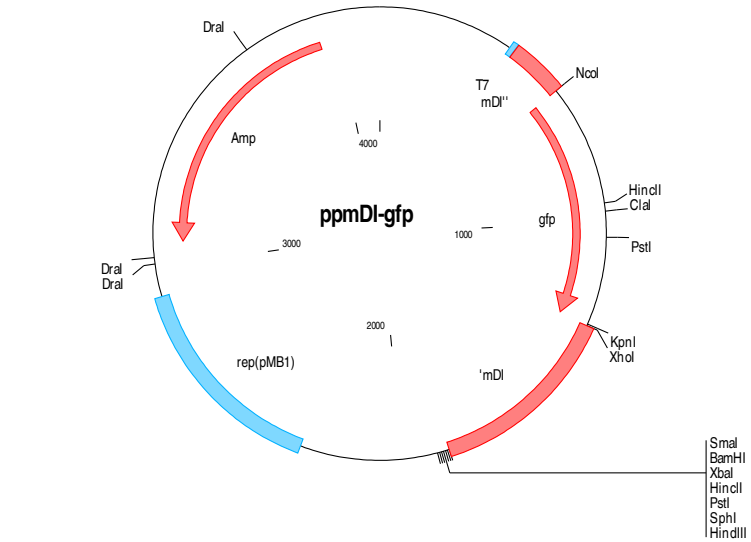

Figure 58: Plasmid map of ppmDI-gfp

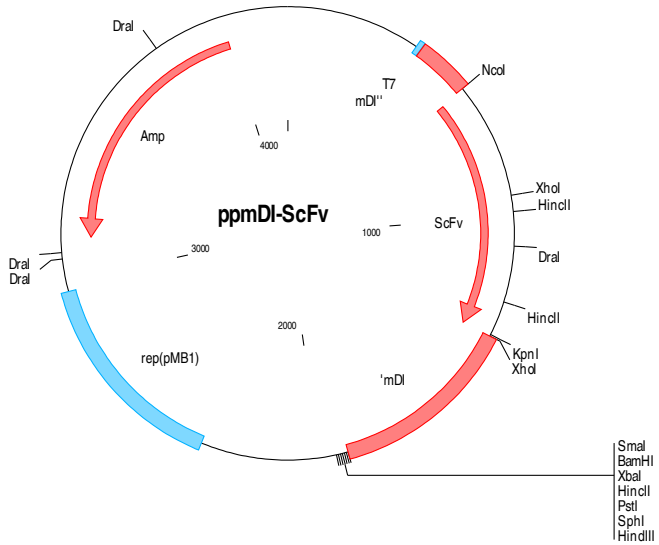

Figure 59: Plasmid map of ppmDI-ScFv

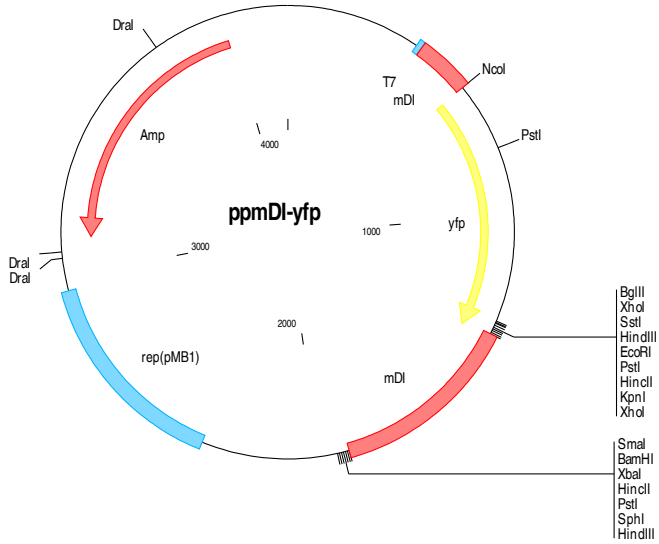

Figure 60: Plasmid map of ppmDI-yfp

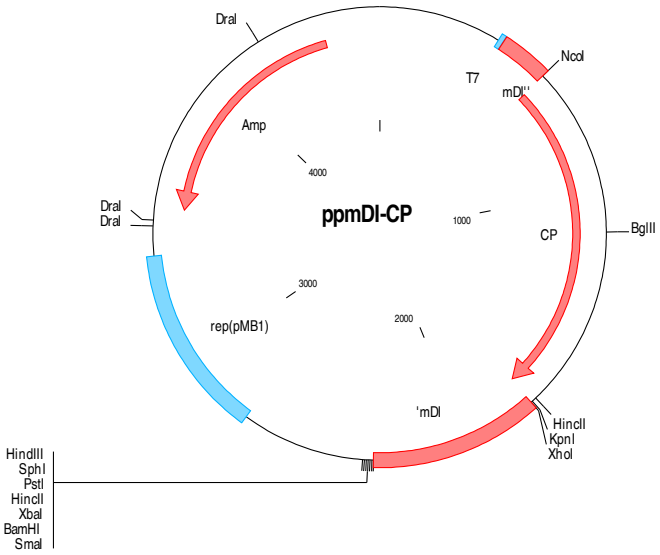

Figure 61: Plasmid map of ppmDI-CP

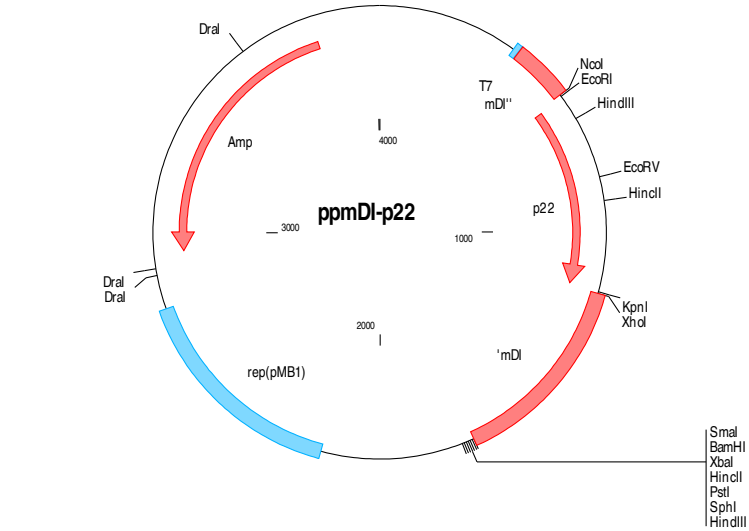

Figure 62: Plasmid map of ppmDI-p22

9.6 Plasmid map of pTBSVΔp19 and pTBSVΔΔp19

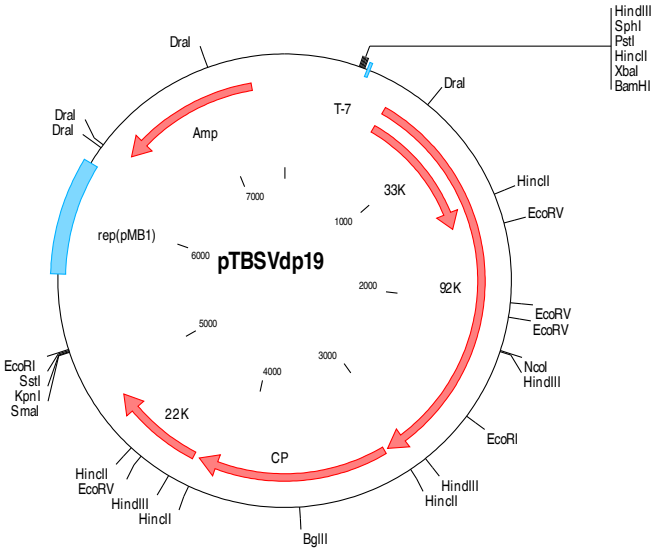

Figure 63: Plasmid map of pTBSVΔp19

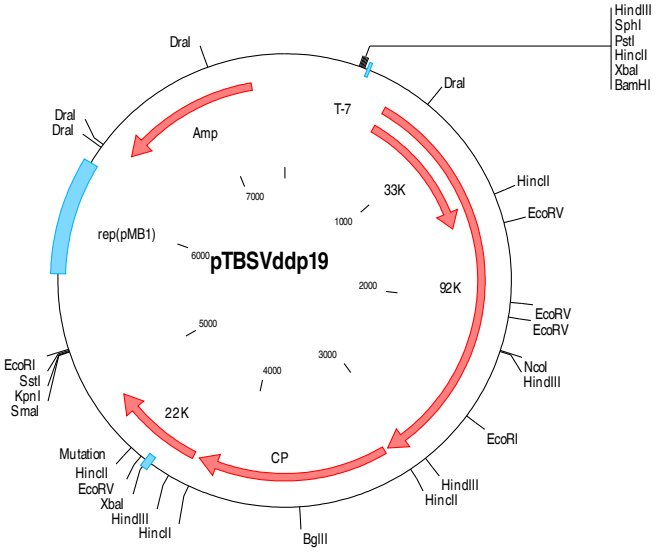

Figure 64: Plasmid map of pTBSVΔΔp19

9.7 Plasmid map of ppDI-FtsH and ppDI(-)3'gfp

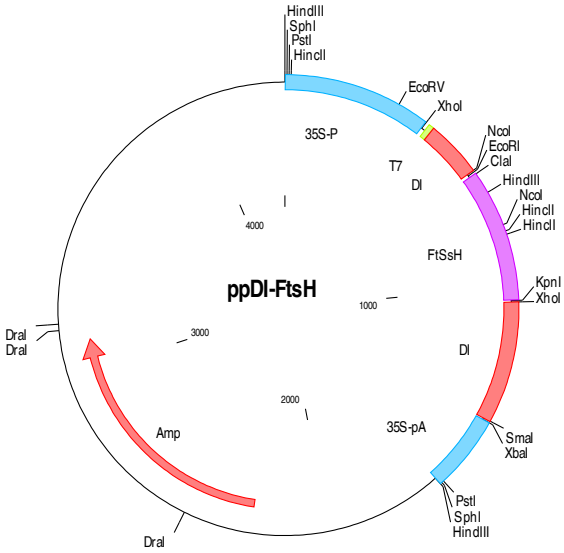

Figure 65: Plasmid map of ppDI-FtsH

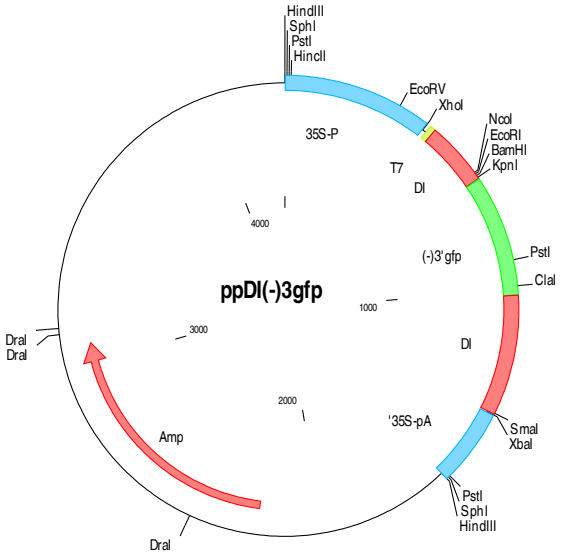

Figure 66: Plasmid map of ppDI(-)3'gfp

9.8 Plasmid map of ppDI-PDS(+) and ppDI-PDS(-)

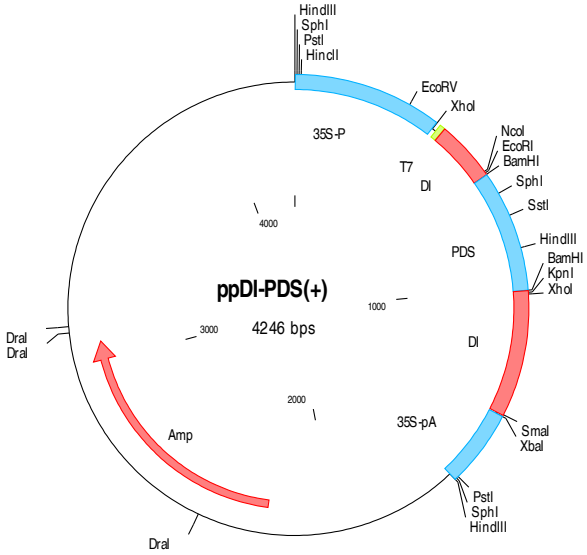

Figure 67: Plasmid map of ppDI-PDS(+)

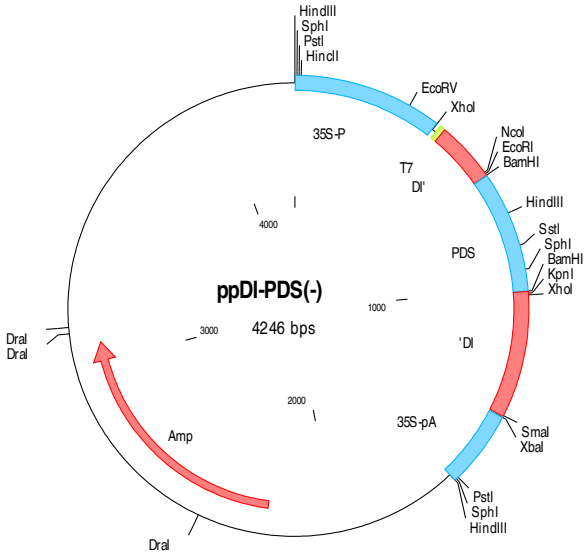

Figure 68: Plasmid map of ppDI-PDS(-)

9.9 Plasmid map of ppdS and dTBSV

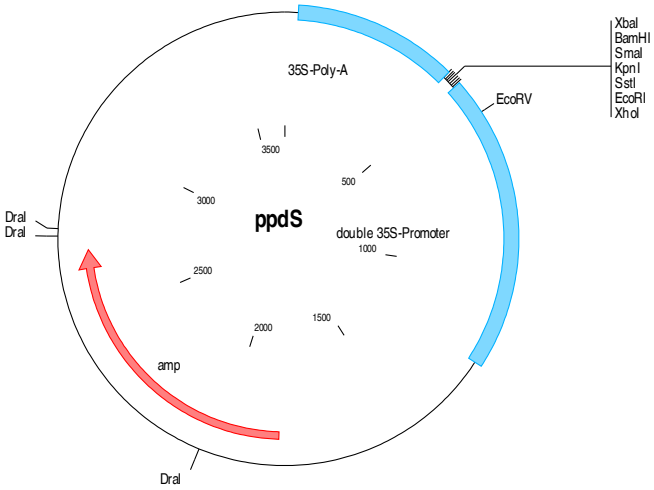

Figure 69: Plasmid map of ppdS

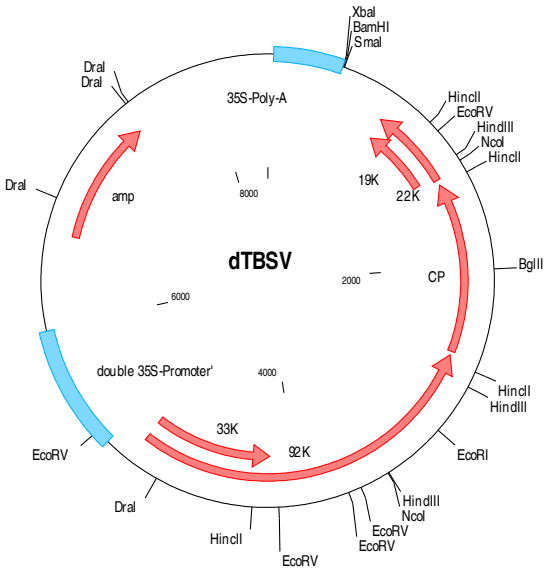

Figure 70: Plasmid map of pTBSV

9.10 Plasmid map of ppzp-TBSV and ppzp-TBSVΔp19

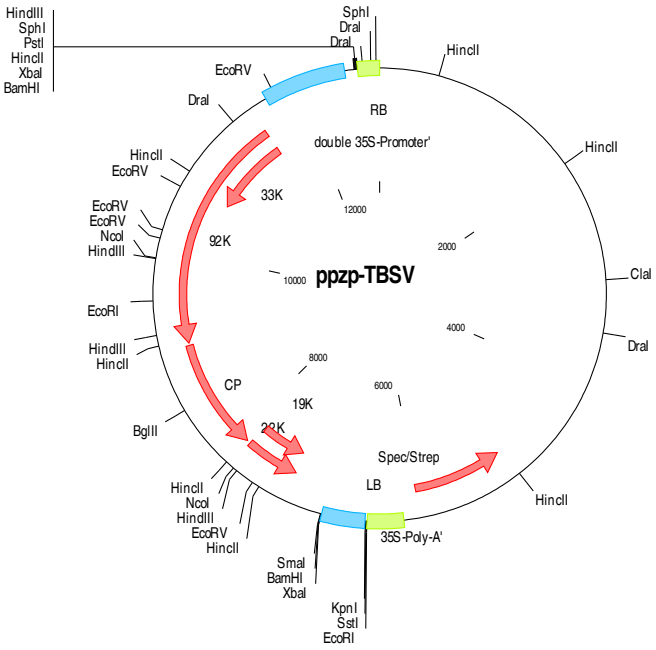

Figure 71: Plasmid map of ppzp-TBSV

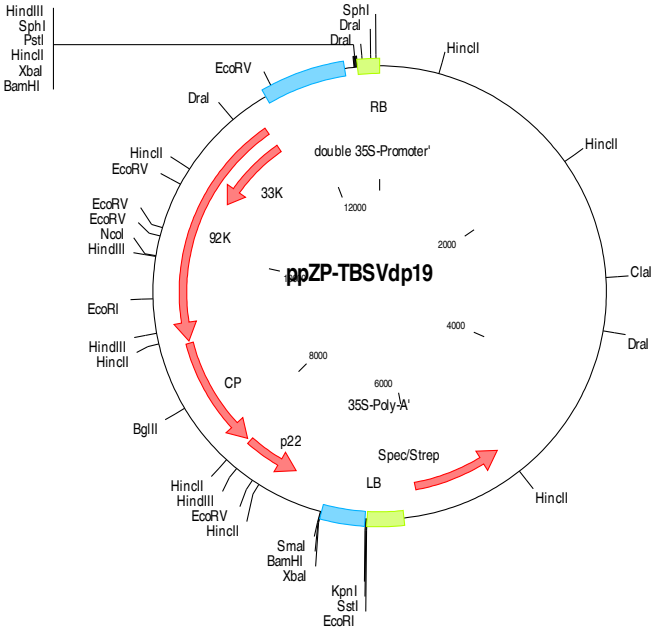

Figure 72: Plasmid map of ppzp-TBSVΔp19

9.11 Plasmid map of ppzp-SDI-PDS(+) and ppzp-SDI-PDS(-)

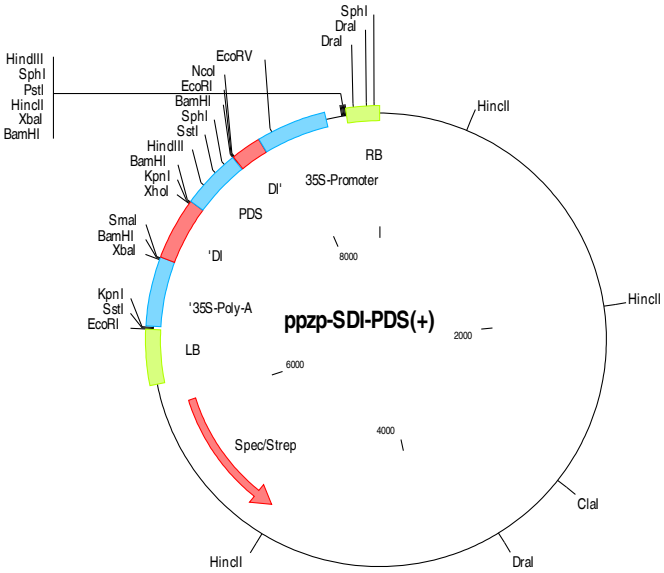

Figure 73: Plasmid map of ppzp-SDI-PDS(+)

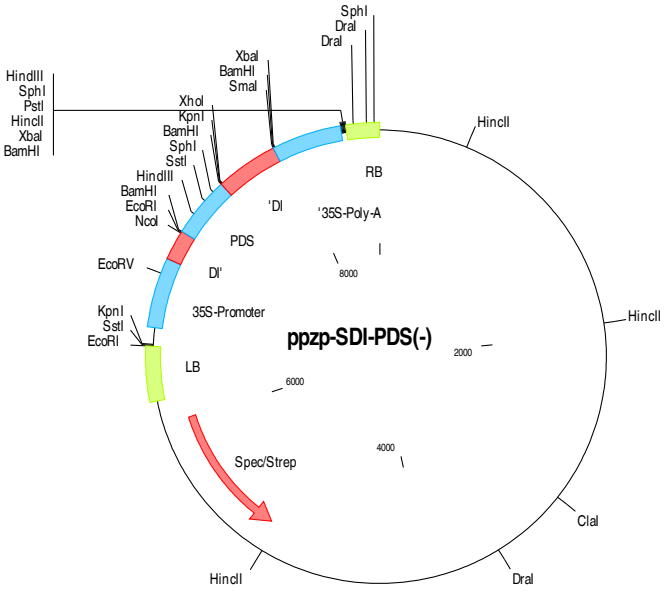

Figure 74: Plasmid map of ppzp-SDI-PDS(-)

9.12 Plasmid map of pGJ-DIgfpERK and pGJ-mDIgfpERK

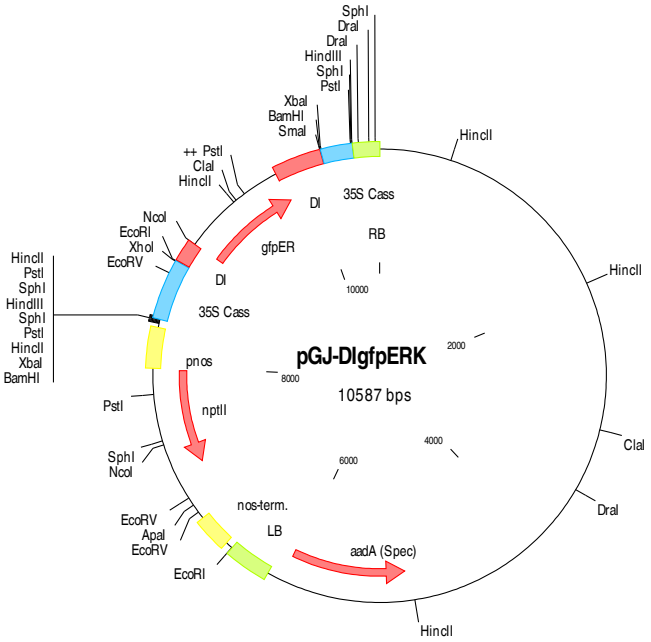

Figure 75: Plasmid map of pGJ-DIgfpERK

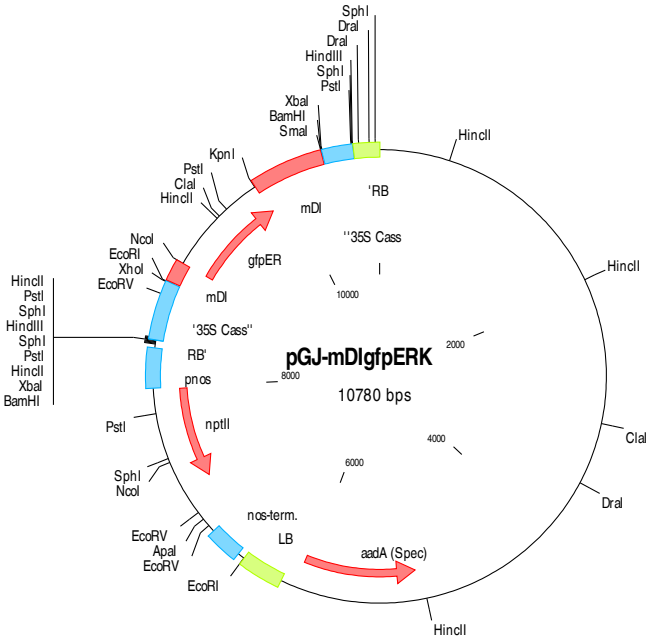

Figure 76: Plasmid map of pGJ-mDIgfpERK