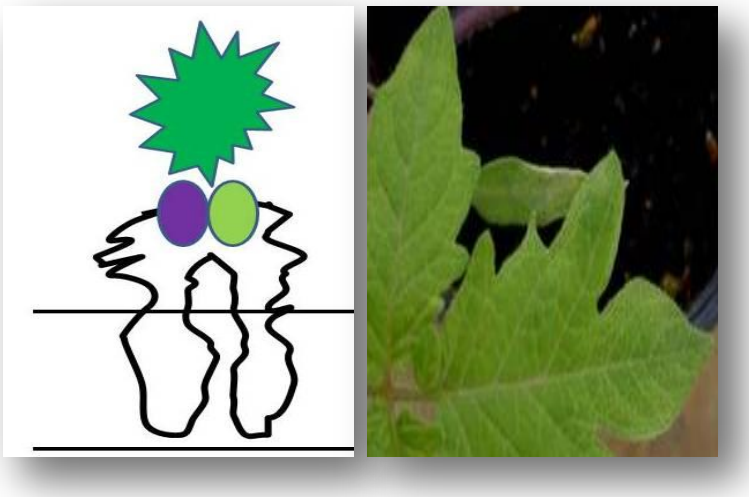
UNIVERSITÄT HOHENHEIM

Faculty of Natural Sciences

Analysis of the structure of Tomato mosaic virus movement protein based on virus host interactions



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Institute of Genetics
Dept. of General Virology

Submitted by

Fariha Tanwir

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Examination committee

Dean Prof. Dr. Heinz Breer
Supervisor and reviewer: Prof. Dr. Artur J.P. Pfitzner
Co-Reviewer: Prof. Dr. Uwe Ludewig
Additional examiner: Prof. Dr. Otmar Spring

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1. Introduction

Viruses are one of the major plant pathogens, drastically influencing crop yield and productivity in the whole world (Kang *et al.,* 2005; Fraser, 1990). Although, there is no statistical data on crop yield losses due to virus attack, but the damage vary from 50 to 80 % and sometimes reaches to 100 % depending upon the severity of virus spread (Roger, 2009). Tomato, potato and cotton are major crops, severely affected due to virus infection in the past (Ahmad *et al.,* 2002).

History of plant viruses starts from 1898, when Martinus Beijerinck found the plant sap from tobacco plants with mosaic disease, remained infectious after passing through a porcelain filter, even when bacterial cells were separated by theses filters. This observation has led to the discovery of the first virus which was named Tobacco Mosaic Virus in 1935. Plant viruses are classified in different groups and sub groups based on their structure, genome organization and way of transmission (Harrison *et al.,* 1971). They consist of a genetic material surrounded by a protein coat. Genetic material can be DNA or RNA, either single stranded (ss) or double stranded (ds). Plant viruses can be transmitted with the help of infectious sap, insect vectors like whitefly, plant hoppers and nematodes. Some plant viruses can also be transmitted by seeds or pollens to the next generations (Matthews, 1982).

Plant viruses are obligatory parasite of different shapes i.e. rod shape or icosahedral (Fig 1). They are dependent on their host machinery for replication (Waigmann *et al.,* 2004). After replication at the point of entry, virus moves to other parts of the plant and cause disease. Plants have also developed a wide range of defence mechanisms to protect themselves against virus infection (Sanseverino *et al.,* 2010).

1.1 Plant resistance against viruses

Resistance is described as a phenomenon by which the plant can stop virus replication or spread at any point during the infection cycle. Plant resistance against viruses is of great importance because of their role in the ecosystem.

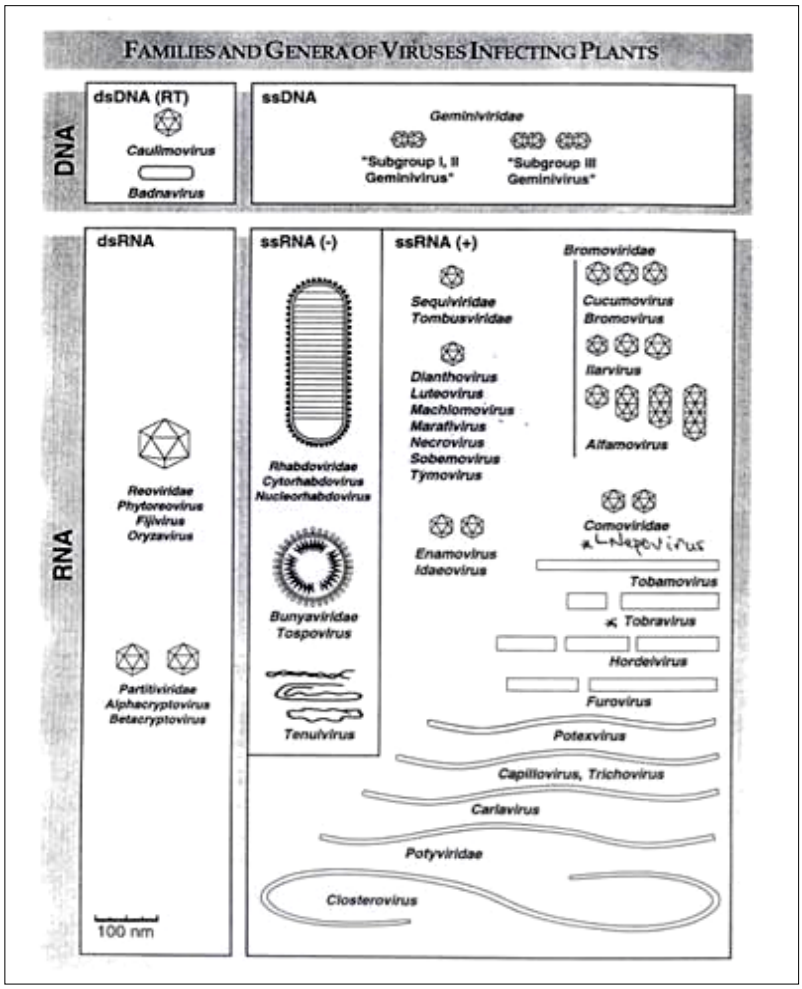


Figure 1. Classification of plant viruses based on genomic material (Roger, 2009)

Plant resistance to viruses is broadly classified into two categories, namely non-host resistance and host resistance. Non-host resistance is a type, where a particular pathogen fails to infect a particular plant species (Maule *et al.,* 2007). Non-host resistance is a more effective and durable defence system (Zellerhoff *et al.,* 2010). Although, a lot of research has been done to understand the mechanism of non-host resistance but very little is known (Mysore and Ryu, 2004). Synthesis of antimicrobial compounds and the physical properties of plants (thick cell wall, waxy cuticle) are major factors for hindering the virus infection in non-host plant species (Soosaar *et al.,* 2005). Host resistance is phenomenon, where a particular genotype of a plant species has heritable resistance against a certain virus. Studies on host resistance against viruses are of great importance for the understanding of host virus interaction and the

resistance pathways (Fraser, 1990).

Host resistance against plant viruses is further classified as natural or engineered resistance. The engineered resistance can be achieved through pathogen derived sequences or post-transcriptional silencing (Goldbach *et al.,* 2003). Natural resistance against viruses is achieved through the presence of resistance genes (*R* genes) in plants. Each *R* gene gives resistance against a specific pathogen (Soosaar *et al.,* 2005).

Presence of R genes mediates two types of phenotypic responses (Fig 2) on virus infection in plants. One of the most prominent pathways initiated through R genes on viral attack is the hypersensitive response (HR) that has been demonstrated to be effective against viruses as well as fungi and bacteria (Martin et al., 2003). The HR is characterized by necrotic lesion formation at the site of infection (Fig 2A) through programmed cell death (Dempsey et al., 1993), and results in isolating the attacking pathogen from the rest of the plant and thus stopping any further spread. The HR has been observed in plants such as A. thaliana, tobacco and soybean (Dempsey et al., 1993; Culver et al., 1994; Mohr and Cahill, 2003). The second phenotypic effect of R genes is the activation of systemic acquired resistance (SAR) that occurs in the cells distant from the point of infection (Fig 2B, Soosaar et al., 2005).

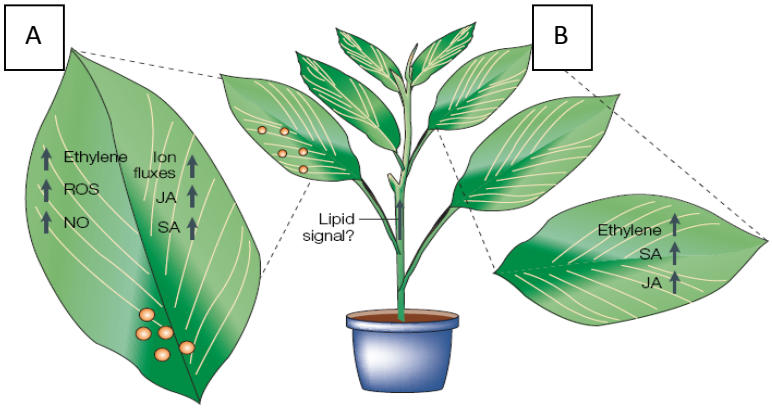


Figure 2. Local and systemic resistance mediated by resistance (*R*) genes. **A**| During resistance, several signaling molecules are locally induced. **B**| Subsequent to the HR, systemic acquired resistance is induced in distal uninfected tissue. The systemic signal is currently unknown, but is thought to be lipid-derived. JA, jasmonic acid; NO, nitric oxide; SA, salicylic acid; ROS, reactive oxygen species. Obtained and modified from Soosaar *et al.,* 2005.

The *R* genes are hypothesized to encode protein that directly or indirectly recognize the specific *Avr* gene products. The predicted characteristics of the *R* proteins include i) constitutive expression in healthy and unchallenged plants, enabling them to detect and defend themselves against pathogen attack, ii) the ability to trigger signalling pathway(s) that initiate the plant defence responses upon the pathogen(s) invasion, iii) rapid evolution in readiness for specific pathogen isolates (Hammond-Kosack *et al.,* 1997).

The recent studies on cloning of genes for resistance against diverse pathogens from a variety of plants have revealed that many *R* proteins contain a centrally located nucleotide-binding site (NBS) and a carboxyl-terminal leucine rich repeat (LRR) protein motif (Saraste *et al.,* 1990). The NBS– LRR genes have been cloned from a variety of plant species. In complete sequence analysis of *A. thaliana* genome more than 160 NBS-LRR-encoding genes have been identified (The Arabidopsis Genome Initiative, 2000). The NBS motif may serve as an ATP- or GTP-binding site. Within the motif, a highly conserved “P-loop” functions in phosphate binding (Traut, 1994). The LRR regions are typically 10~40 repeats of a motif of ~24 amino acids which are highly variable. The LRR domains are implicated in protein-protein interaction and ligand binding (Kobe and Deisenhofer, 1994). Research on defence mechanism against virus infection in rice, flax and tomato suggests that the LRR-encoding region is involved in determination of the gene-for-gene interaction specificity (Thomas *et al.,* 1997; Ellis *et al.,* 1999; Jia *et al.,* 2000).

R genes are categorized in five different classes (Fig 3) based on their function and the presence of different domains (Bent, 1996; Van Ooijen *et al.,* 2007). The major class of *R* genes is CNL (CC-NB-LRR) class, which comprises of resistance proteins containing coiled-coil domain (CC), nuclear binding domain (NB) and leucine rich repeat (LRR). Second important class is TNL (TIR-NB-LRR) which consists of proteins with Toll-interleukin receptor like domain, a nucleotide binding site and a leucine-rich repeat. RLP class is the third important class of *R* genes encoding for receptor like proteins comprising of serine-threonine kinase- like domain and a leucine rich repeat. RLK is fourth class of *R* proteins with kinase domain and leucine-rich repeat (Kin-LRR). Fifth class of *R* genes includes all other resistance genes which have been recognized for

giving resistance against pathogens through different molecular pathways (Buschges *et al.,* 1997; Brandwagt *et al.,* 2000).

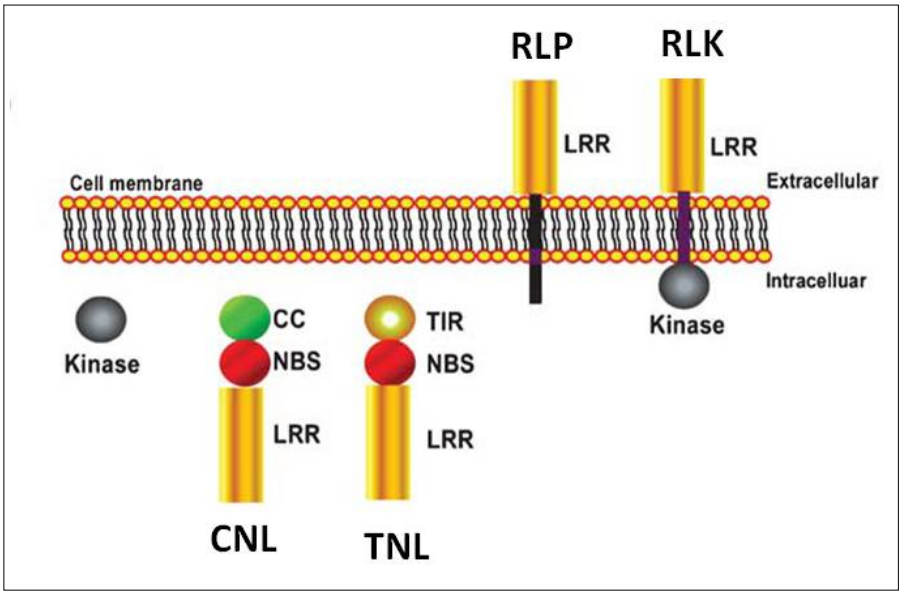


Figure 3. Representation of the location and structure of the five main classes of plant disease resistance proteins (obtained from Kang *et al.,* 2005). CNL and TNL are intercellular protein, present either in cytoplasm or in nucleus. Whereas RLP and RLK are located in cell membrane, with LRR domain present on outer side of the cell.

In 1940s H. H. Flor proposed “gene for gene model” for understanding the resistance pathway of *R* genes. He studied the interaction between flax and the fungal pathogen flax rust (*Melampsora lini*), and found that pathogens contained a variety of molecules, encoded by dominant avirulence (*Avr*) genes, which triggered the defence responses in plants carrying the corresponding *R* gene (Fig 4).

Further studies have shown that recognition of virus *Avr* factors by plant factors initiate the whole cascade of plant defence response (Martin *et al.,* 2003). As a result of these responses, plants may develop different visual symptoms like the hypersensitive response (HR) that leads to the formation of necrotic lesions in infected leaves. In some cases, plants do not show any visible symptoms (Loebenstein, 1972). In the absence of

the *R* or *Avr* genes, no recognition occurs and the pathogen can infect the host plant and cause disease.

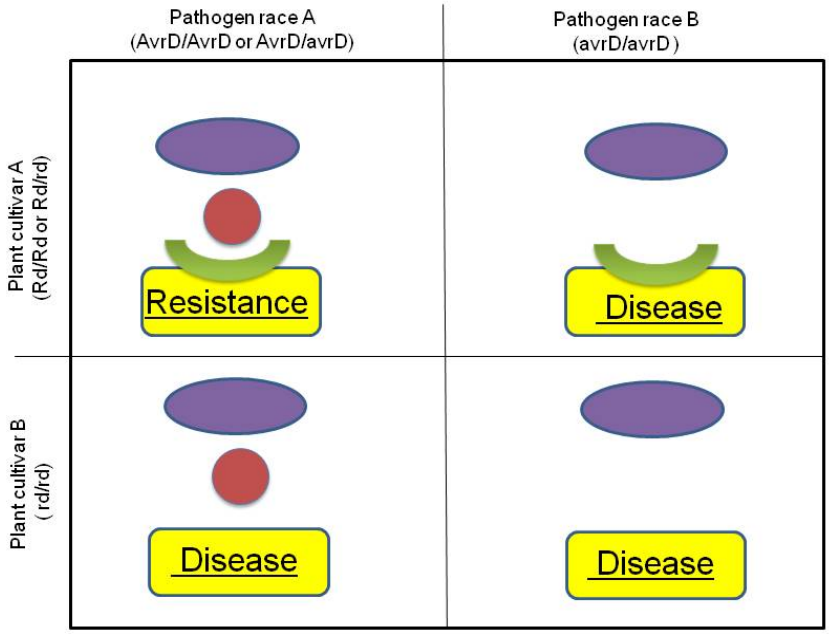


Figure 4. Various types of genetic interactions in the "gene for gene" model.

The gene for gene model proposed by Flor, was not able to explain the interaction of virus *Avr* factors with plant *R* genes. Further understanding of virus resistance mechanisms suggest that in many cases no direct physical interactions between *R* and *Avr* proteins occur. This led to the alternative indirect perception model as shown in figure 4, proposed by Van der Biezen and Jones, named as guard hypothesis (Van der Biezen and Jones 1998). According to this model the *R* proteins (guard) are permanently associated with the host factors (guardees), required by pathogens for infection. During infection, these guardees are modified by pathogen *Avr* proteins. This modification triggers the guard to initiate the whole signaling cascade of resistance mechanism (Fig 5).

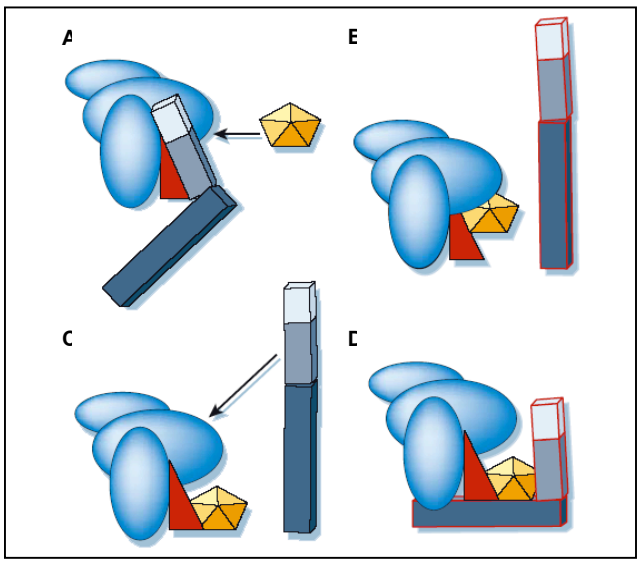


Figure 5. Schematic diagram of Guard hypothesis (obtained from Dangl and Jones 2001). A) A cellular complex of proteins (blue), which includes both the 'guardee' molecule (red) and an NB-LRR protein (grey, shaded from the N terminus through NB and LRR domains), is a target for a bacterial type III effector of disease (orange). B), Binding of the type III effector to its targets results in disassociation and activation of the NB-LRR protein and thus disease resistance. C) Alternatively, the NB-LRR protein may not be part of the target complex until after type III effector binding. D) Recruitment to the type III effector/target complex would then activate the NB-LRR protein.

RIN4 (RPM1-intreacting protein 4) of *Arabidposis thaliana* was found an essential cellular protein for resistance against *pseudomonas syringae pv. tomato* (Mackey *et al.,* 2002; Axtell *et al.,* 2003; Shao *et al.,* 2003). Yeast two hybrid analysis of Turnip crinckle virus has shown the interaction of TCV CP with TIP (TCV-interacting protein) in *Arabidopsis*. It supports the Guard's hypothesis, where the HRT acts as guard and TIP behaves like a guardee (Caplan and Kumar, 2006).

The first cloned *R* gene was the *N* gene from tobacco conferring resistance against tobacco mosaic virus (Whitham *et al.,* 1994). The *N* gene is a member of NBS-LRR class of resistance genes, containing a toll interleukein-1 receptor domain at the N terminus (TIR-NBS-LRR). The remaining antiviral *R* genes also belong to NBS-LRR class but contain a coiled coil domain at N terminus. Potato Rx1 and Rx2 are the

examples of CC-NBS-LRR type of *R* genes that confer resistance against potato virus X (PVX) without inducing the HR response (Bendahmane *et al.,* 2000). The other members of this subclass include *Tm-2* and *Tm-2*^{2} genes resistant against tomato mosaic virus in tomato (Lanfermeijer *et al.,* 2003). Two antiviral *R* genes have been identified in *Arabidopsis* named as HRT and RCY1. HRT and RCY1 give resistance against turnip crinkle virus (TCV) and yellow strain of Cucumber mosaic virus (CMV-Y) (Caplan and Kumar, 2006). Several *R* genes, identified so far, belonging to different classes and recognizing different viral avirulence factors are summarized in table 1.

Resistance proteins (*R* proteins) identify different types of viral proteins as avirulence factors to give resistance against viruses. For example, *R* proteins in tobacco and tomato have the ability to recognize TMV coat protein (CP), ToMV movement protein (MP) and ToMV replication protein (Rep), respectively. The *N* protein of tobacco identifies the helicase domain of tobacco mosaic virus and provides resistance against a wide range of *tobamoviruses* (Tobias *et al.,* 1982). The plants containing *N* genes elicit the resistance response on expression of the helicase domain shows, that this domain is sufficient to induce *N*-mediated response (Erickson *et al.,* 1999). Similarly, the *N´* gene has been shown to recognize the TMV CP to confer resistance (Saito *et al.,* 1989). The allelic resistance genes in tomato *Tm-2* and *Tm-2*^{2} have been shown to interact with MP of ToMV (Meshi *et al.,* 1989; Weber *et al.,* 1993).

Gene	Plant	R protein Structure	Virus	*Avr* determinant
N	*Nicotiana* sp.	TIR-NBS- LRR	Tobacco mosaic virus	Replicase
Rx1	*Solanum tuberosum*	CC-NBS-LRR	Potato virus X	Coat protein
Rx2	*Solanum tuberosum*	CC-NBS-LRR	Potato virus X	Coat protein
HRT	*Arabidopsis thaliana* ecotypeDijon-17	CC-NBS-LRR	Turnip crinkle virus	Coat protein
RCY1	*Arabidopsis thaliana ecotype C24*	CC-NBS-LRR	Cucumber mosaic virus strain Y	Coat protein
Sw-5	*Lycopersicon* sp.	CC-NBS-LRR	Tomato spotted wilt virus	RNA dependent RNA polymerase
Y-1	*S. tuberosum*	TIR-NBS-LRR	Tomato spotted wilt virus	?
Tm-22	*Lycopersicon* sp.	CC-NBS-LRR	Tomato mosaic virus	Movement protein

Table 1. Cloned plant resistance (*R*) genes and the viral avirulence factors, recognized by these *R* genes. (CC- coiled-coil, LRR- Leucine rich repeat, NBS- nuclear binding site, TIR- Toll interleukin-1 receptor). Obtained and modified from Soosaar *et al.,* 2005.

1.1.1 Tomato mosaic virus and resistance genes

Tomato mosaic virus (ToMV) is a member of family *tobamoviridae* and belongs to the genus *tobamovirus*. Tobacco Mosaic Virus (TMV) is a type member of this family. Both tobacco mosaic virus and tomato mosaic virus share similar characteristics like structure, genome organization and way of transmission. ToMV causes yellow mosaic symptoms on the leaves and tomato fruits resulting in a drastic reduction in crop yield (Mathews, 1991). It has a rod shaped structure, as shown in figure 6A, about 300 nm length and 18 nm radius (Casper, 1963). ToMV has its genetic material enclosed in a protein coat as shown in figure 6B. Both ToMV and TMV share high levels of sequence homology. Tomato mosaic virus encodes four different proteins important for virus replication and movement (Fig 7; Table 2, Meshi *et al.,* 1992). The two proteins involved in replication are encoded by the genomic RNA where as the two involved in virus packaging and movement are translated by sub-genomic RNAs. The virus particles are very stable and remain infectious for many years after extraction.

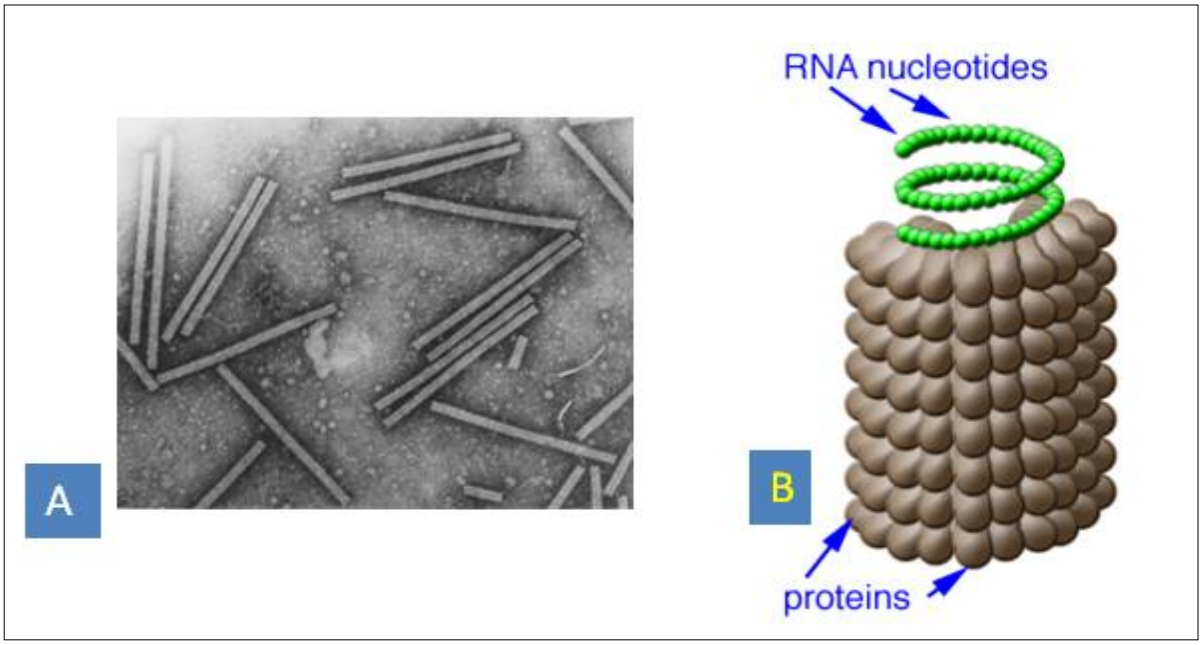


Figure 6. Structure of tobacco mosaic virus. A) Particle shape of Tobacco mosaic virus under electron microscope. B) Coat protein structure enveloped around the RNA molecule.

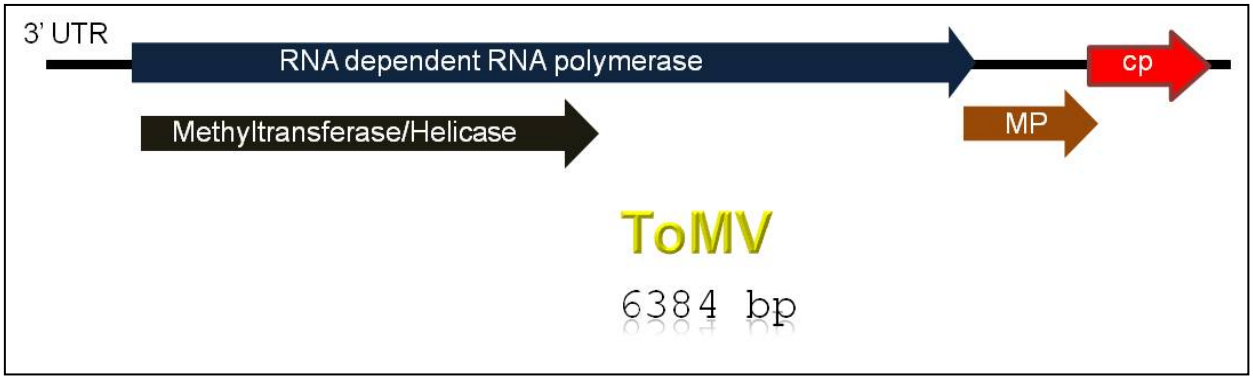


Figure 7. Genome Organization of Tomato mosaic virus. UTR: Un-translated region. 130kDa/Methyltransferase/helicase. 180kDa/RNA dependent RNA polymerase. 30kDa/Movement protein. 18kDa/Coat protein.

ORF	Function
180kDa /RNA dependent RNA polymerase	Replication
130kDa/ Methyltransferase/Helicase	Replication
30kDa/ Movement protein	Cell to cell and long distance movement of virus particles
18kDa/Coat protein	Packaging of viral RNA

Tabel 2. Different proteins and their functions encoded by tomato mosaic virus.

Tomato mosaic virus, because of its drastic effect on tomato yield, has always been under discussion for finding the resistance sources in the field has been an for finding resistance sources in the field. Three different sources of resistance have been found against tomato mosaic virus namely *Tm-I, Tm-2* and *Tm-2*^{2} in wild *Solanum*

species. From these wild species, the resistance sources have been transferred in the cultivated species using intensive breeding programs (Pelham, 1966; Pelham, 1972). *Tm-l* was mapped on chromosome 5 and has been found to interfere with the replication of ToMV in isolated protoplasts and also in the differentiated tissues (Fraser and Loughlin, 1980). This characteristic of *Tm-l* is different from other conventional resistance genes like *N* gene, which is effective only in differentiated tissues for defence reaction (Pfitzner and Pfitzner, 1990). The intensity of resistance effect of *Tm-l* is also dependent on the homozygosity or heterozygosity of the gene in the isolated protoplasts and plants. The *Tm-l* homozygous plants showed the reduction of virus replication to 1% compared to the heterozygous plants have shown only a reduction of 10 % (Yamafuji *et al.,* 1991).

Soon after the introduction of *Tm-l* resistance tomato in the field, *Tm-l* resistance breaking virus strains have evolved in nature. These resistance breaking strains have shown high replication rates in *Tm-l* homozygous tomato plants as compared to the wild type virus (Motoyoshi and oshima, 1979). Sequence analysis of different *Tm-l* resistance breaking strains have revealed that all the mutations responsible for the resistance breaking phenotype are present in the 180kDa/130kDa replication associated proteins as shown in figure 8 (Strasser, 2002).

130.0	INRVTGFPYPA--//--RCP	ADVTHFLNQRYEGHVMCTSS
130.1	INR ITGSPYPA--//--RCP	ADVTHFLNERYEGYVMCTSS
130.Lta1	INRVTGFPYPA--//--RCP	ADVTHFLNERYEGYVMCTSS

Figure 8. Amino acid exchange in 130kDa Protein of different *Tm-l* resistance breaking viruses identified so far. (wild type ToMV 130.0 (Ohno *et al.,* 1984 and two *Tm-l* resistance breaking ToMV strains 130.1 Strasser, 2002 and 130.Lta1 Meshi *et al.,* 1988). The amino acid exchanges are depicted in red.

For the understanding of the resistance mechanism of *Tm-l*, Ishibashi and his colleagues in 2007, have shown that *Tm-l* inhibits the ToMV replication after translation

of viral RNA but before formation of viral replication-complex on the membrane by binding directly or indirectly to replication protein.

The second important gene for resistance against ToMV, named as *Tm-2,* was first isolated from Hawaiian breeding lines (Hall, 1980). The *Tm-2* gene was found to be more effective for resistance against ToMV as compared to *Tm-I* and is present on chromosome 9. The first identified *Tm-2* gene source was linked to an undesirable recessive gene, "netted-Virescent" (*nv*) causing stunting and yellowing in the homozygous conditions (Clayberg, 1959). Afterwards, a new source was found for *Tm-2* in *S. peruvianum* which did not contain the *nv* gene linkage (Laterrot and Pecaut, 1969). Soon after the discovery of *Tm-2* resistance gene, another resistance source was discovered against ToMV named as *Tm-2*^{2} (Alexander, 1963). Both *Tm-2* and *Tm-2*^{2} resistance genes are allelic in nature and differ at seven nucleotides positions resulting in four different amino acid at protein level as shown in figure 9 (Lanfermeijer *et al.,* 2005). Both *Tm-2* and *Tm-2*^{2} are CC -NBS- LRR type of resistance genes.

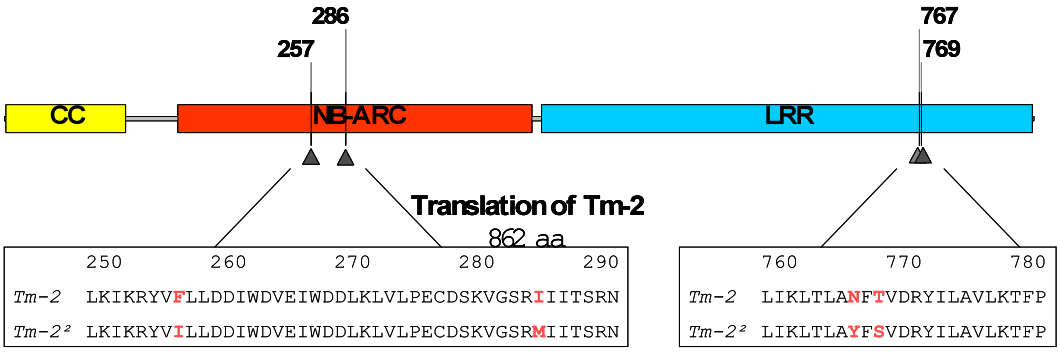


Figure 9. Structure of *Tm-2* and *Tm-2*^{2} resistance genes. Amino acid differences are depicted in red letters (Gerhardts, 2008)

Although the *Tm-2* and *Tm-2*^{2} are more stable resistance genes as compared to *Tm-I* under field conditions, but the emergence of resistance breaking viruses have been reported from time to time (Pelham, 1972; Strasser, 2002; Meshi *et al.,* 1989). Different resistance breaking strains from all over the world were sequenced and analyzed for the changes responsible for resistance breaking phenotype. The sequence analysis of these strains have shown that the amino acid exchanges responsible for resistance breaking

properties of these viral strains are present in 30kDa movement protein ORF (Calder and Palukaitis, 1992; Strasser, 2002).

Amino acid analysis of *Tm-2* and *Tm-2²* resistance breaking viruses (Fig. 10) shows that one amino acid exchange (aa 133 Glu > Lys) is present in all *Tm-2* resistance breaking strains. This finding emphasize on the role of this region of 30kDa MP in the recognition of *Tm-2* gene. Interestingly, a second amino acid exchange has been found in all these strains on different positions important for the stability of structural change from the other amino acid exchange (Pfitzner, 2006).

The amino acid exchanges responsible for *Tm-2²* resistance breaking phenotype has also been found in 30kDa MP but at different positions (aa 130 Lys > Glu, aa 238 Ser > Arg, aa 244 Lys > Glu).This finding has confirmed the evolution of *Tm-2²* from the *Tm-2* resistance gene.

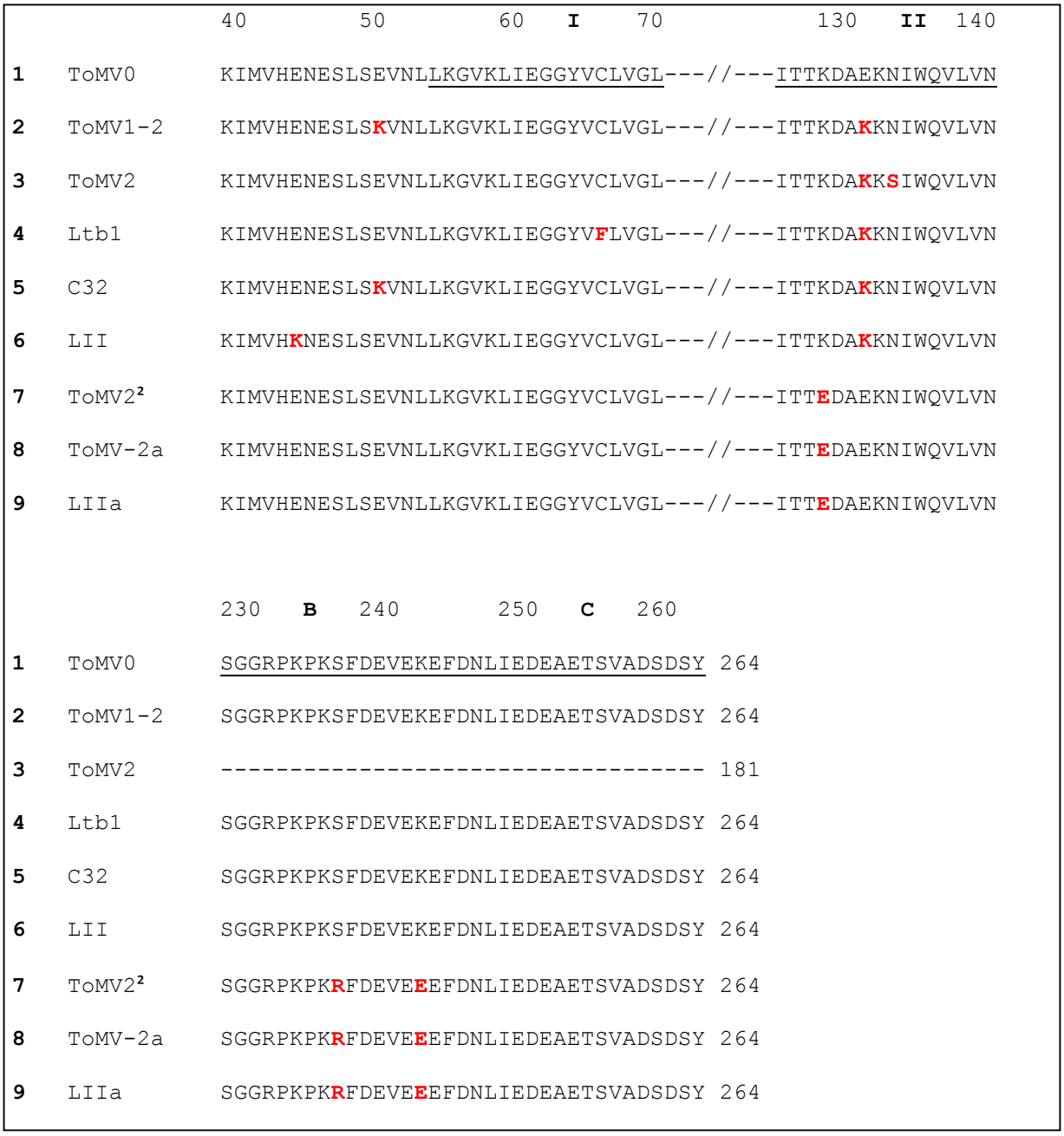


Figure 10. Partial amino acid sequence of movement protein of different tomato mosaic virus strains identified so far, obtained from Gerhardts 2008. Amino acid exchanges in the resistance breaking viruses are depicted in red.

Different efforts have been made to understand the resistance mechanism of *Tm-2* and *Tm-2²* gene and their interactions with movement protein of tomato mosaic virus. Regardless of the allelic nature of *Tm-2* and *Tm-2²*, both genes behave very differently from each other under field conditions. Although, a lot of *Tm-2 and Tm-2²* resistance breaking viruses has been reported, but *Tm-2²* resistance breaking viruses have been found unstable and ineffective under field conditions. Different studies have shown that the *Tm-2* and *Tm-2²* interact with the tomato mosaic virus movement protein for resistance (Meshi *et al.,* 1989; Weber *et al.,* 2004) in different manner. Based on these results, Pfitzner in 2006 has proposed a new model for the interaction of resistance genes with the MP of tomato mosaic virus as shown in figure 11. According to this model, *Tm-2* recognizes N-terminal region of 30kDa MP, where as the *Tm-2²* requires recognition of two different binding sites, one at N-terminus and other at the C-terminus of viral movement protein (Pfitzner, 2006).

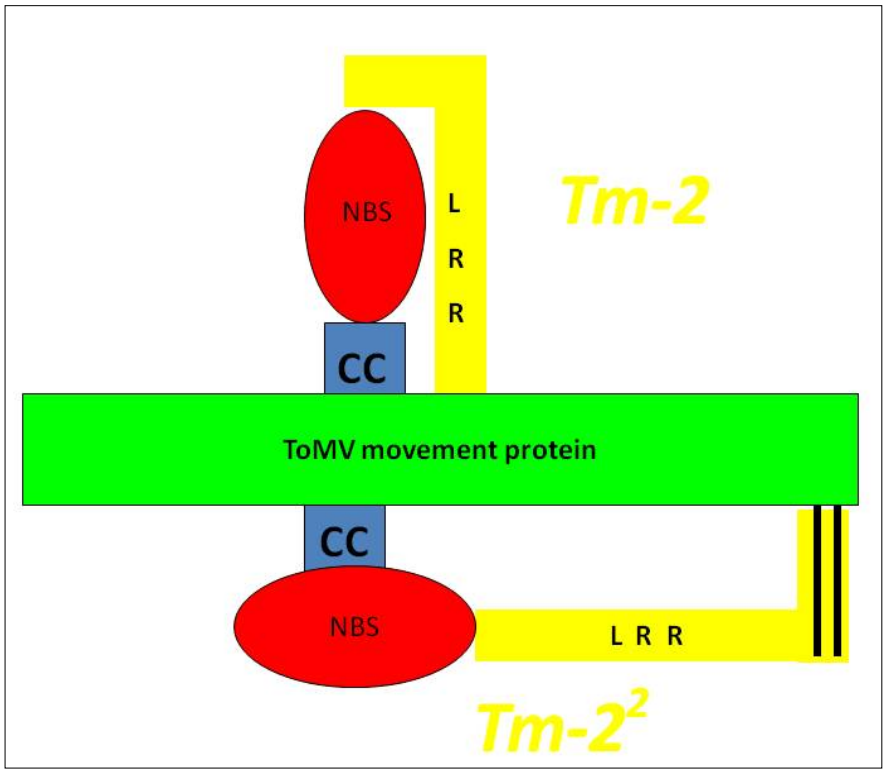


Figure 11. Proposed model of the *Tm-2* and *Tm-2²* interactions with ToMV movement protein (Pfitzner, 2006)

1.1.2 Tomato mosaic virus movement protein and its interactions with resistance genes.

Plant viruses require one or more proteins for cell to cell and long distance transport named as movement proteins (MP). Tomato mosaic virus encodes a 30kDa protein, which is responsible for the movement of viral particles from one cell to the other. Much work has been done on the MPs of tobamoviruses i.e. TMV, to understand their role in virus spread, localization of these proteins in the plant cells and effect on cellular structure. GFP tagged movement protein fusions of TMV have shown that the MP is localized in the plasmodesmata and increases the size exclusion limit for cell to cell transport of virus particles. Brill and his colleagues in 2000, have developed a structural model of the TMV MP as shown in figure 12. The model was based on the ability of MP mutants to transport viral particles, and on mass spectrometry of peptides from protease digested MP after membrane insertion in dog pancreatic microsomes. According to this model, the TMV MP is an integral membrane protein localized in ER with its N and C terminus part exposed in the cytoplasm, where as the middle ~70 amino acids are in the ER lumen. Since then, this structural model has been implicated for the MPs of all tobamovirus MPs. As tomato mosaic virus is closely homologous to TMV, therefore, this model was thought also to be true for ToMV. Different efforts have been made to understand the interactions of ToMV MP and the resistance gene products in the light of this model. The amino acid exchanges in the MPs of *Tm-2* and *Tm-2*^{2} resistance breaking viruses (highlighted with red and green arrows in figure 12), have shown that the structural model of TMV MP proposed by Brill and his colleagues in 2000, is unable to resolve the importance of these mutations (Weber *et al.,* 1993; Weber and Pfitzner, 1998; Pfitzner, 2006; Meshi *et al.,* 1989) and raised many question against this model.

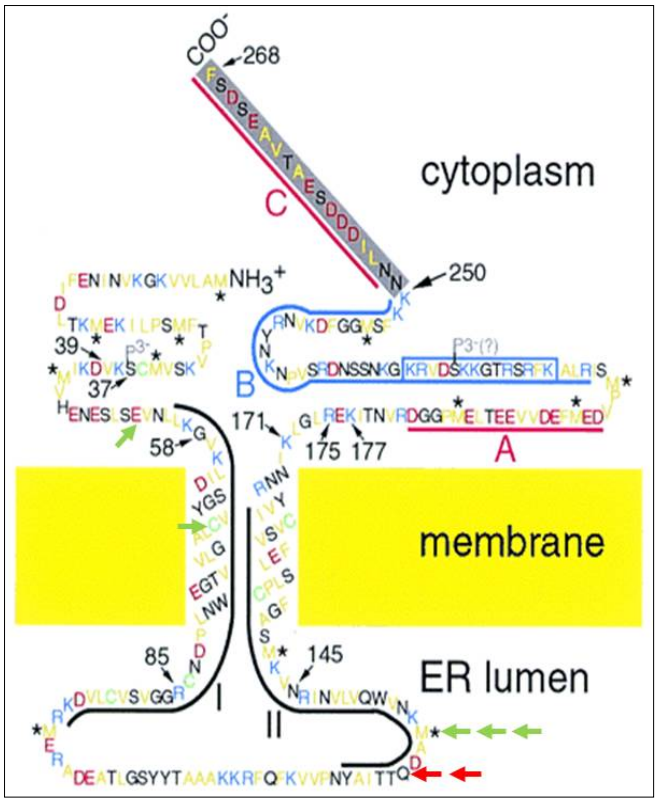


Figure 12. Topological model of TMV MP according to Brill *et al.,* 2000. in this figure the hydrophobic amino acid residues are yellow, basic residues are blue, acidic residues are red, and Cys residues are green. Domains I (residues 56–96) and II(residues 125–164) are regions that are conserved among tobamovirus MPs and are outlined in black; domains A (residues 183–200) and C (residues 252–268) are acidic and are outlined in red; and domain B (residues 206–250) is basic and is outlined in blue. The amino acid exchanges found in *Tm-2* resistance breaking viruses have been depicted by green arrows where as the *Tm-2*^{2} resistance breaking amino acid exchanges are highlighted with red arrows.

One important question on this model was that, because the MP of tomato mosaic virus has shown previously to interact with *Tm-2* and *Tm-2*^{2} resistance gene products and amino acid exchanges responsible for resistance breaking phenotype are present in the middle part of MP, that has been proposed to be present in the ER lumen (Fig. 12), whereas the CC-NBS-LRR resistance genes like *Tm-2* and *Tm-2*^{2} are mainly cytoplasmic proteins as shown in figure 3. The question, thereby, arises that how a protein present in the cytoplasm can recognizes the protein part existing in the ER lumen to give resistance against tomato mosaic virus?

1.1.3 Hypothetical model of 30kDa MP of tomato mosaic virus

The raised question was first answered by Gerhardts in 2008, who suggested a new model, as shown in figure 12, for the structure of the tomato mosaic virus movement protein, based on the results from viral resistance in tomato (Gerhardts, 2008). The hydrophilic profile of the ToMV MP as shown in figure 13A demonstrates that the amino acids from position 100 to 150, have significantly higher hydrophilic values, and are presumably located in the cytoplasm. According to this proposed model (Fig. 13B), the ToMV movement protein is an integral membrane protein with two membrane loops. Whereas, its middle part, containing the *Tm-2* and *Tm-2*^{2} resistance breaking amino acid exchanges, lies in the cytoplasm. Presence of the middle part of MP in the cytoplasm makes the important amino acids available for the interactions of the resistance genes with the MP. In this research, we have tried to prove this hypothesized model in yeast (*S. cerviceae*) and plants (*N. benthamiana*) to understand the interactions between ToMV MP and the resistance genes *Tm-2* and *Tm-2*^{2} for giving resistance.

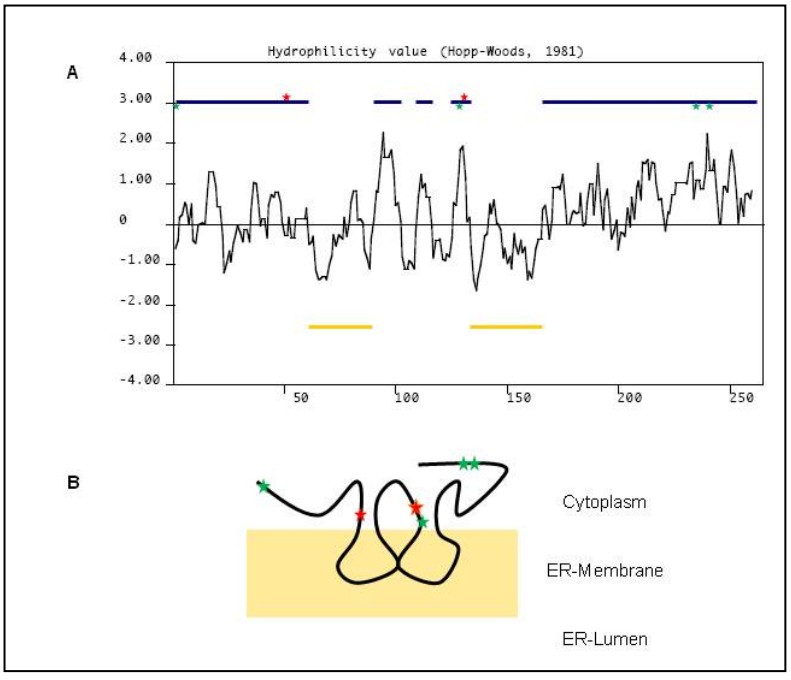


Figure 13. Hydrophobic profile of Tomato mosaic virus movement protein and the alternative structure proposed by Gerhardts, 2008.

1.2 Tomato mosaic virus and Virus induced gene silencing

Virus induced gene silencing is a technique based on RNA silencing pathway in plants for the transient knock down of a desirable gene. During 1990s, RNA silencing (RNAi) was discovered in plants, animal and fungi (Baulcombe, 2000; Matzke *et al*., 2001) as a phenomenon of gene regulation that controls the transcription level of endogenous protein by either suppressing transcription (Transcriptional gene silencing- TGS) or by activating a sequence- Specific RNA degradation process before translation starts (Post transcriptional gene silencing- PTGS). In nature, the RNAi phenomenon was used as a cellular defence mechanism against virus infection and for the regulation of gene expression (Agarwal *et al.,* 2003). RNAi is a multistep process, as shown in figure 14, where the dsRNA molecules are processed in small RNA interfering molecules (siRNA) with the help of dicer (RNAase III endonuclease). These short RNAs are taken by an RNAase complex (RISC), to activate the degradation of ssRNA in a sequence specific manner (Lu *et al.,* 2003).

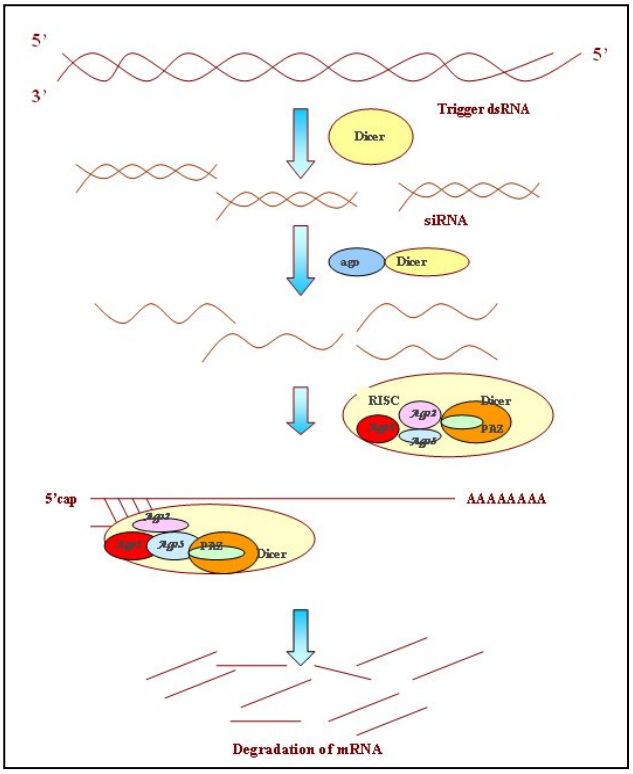


Figure 14. Diagrammatic description of RNAi mechanism (Khanna *et al.,* 2007).

During the infection of RNA viruses, the viruses replicate in the plant cell and produce double stranded RNAs as the replication intermediates as shown in figure 15. These dsRNAs are recognized by plant defence machinery and are cleaved in siRNAs. These siRNAs catalyse the degradation of dsRNA molecules and gives resistance against the virus infection.

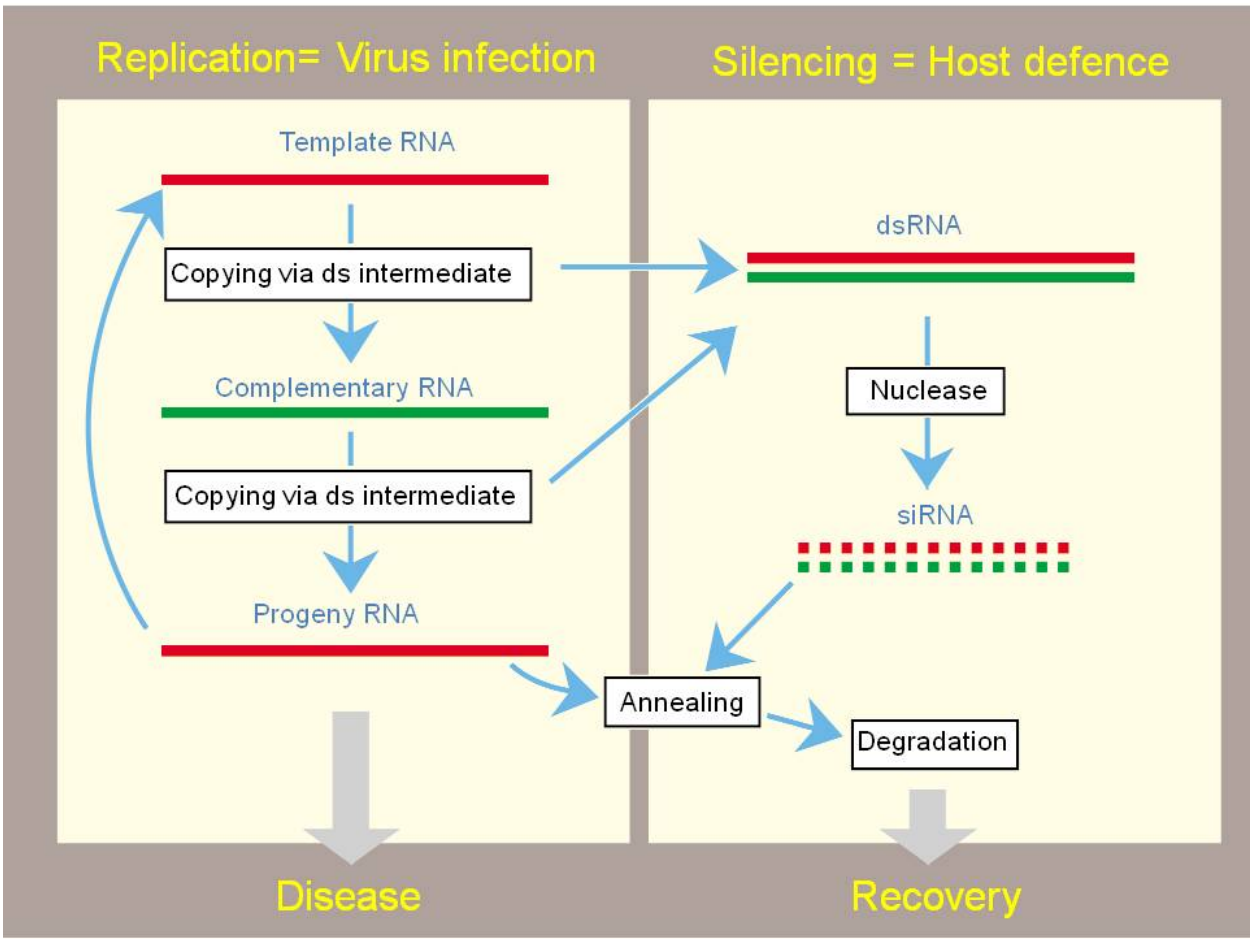


Figure 15. Diagram illustrating the role of base-pairing in the replication of an RNA virus (left panel) and in the silencing response of the host (right panel).

endogenous gene sequences. The dsRNAs will be produced as a replication intermediates during virus replication, that will target the viral genome in the infected cell

to control virus multiplication. If a non viral gene is inserted in the viral genome, it will be silenced during this process (Gould *et al.,* 2007).

Different viruses have been successfully used as silencing vectors, these include both RNA and DNA viruses. Tobacco mosaic virus (TMV) is the first example of RNA viruses (Kumagai *et al.,* 1995) and then later the Potato virus x (PVX) has been successfully used to exploit the silencing process (Ruiz *et al.,* 1998). Phytoene desaturase was the first plant gene, which was silenced using these virus vectors giving rise to photo-bleaching effect on *N. benthamiana.* Similarly, a chlorophyll synthesis enzyme and cellulose synthase enzyme has been silenced successfully (Kjemtrup *et al.,* 1998). Although, both vectors were used successfully for gene silencing, but both systems have some advantages and disadvantages. PVX based silencing vectors were more stable than TMV based silencing vectors, where as TMV has a broader host range as compared to the PVX. Similarly, the strong appearance of viral symptoms makes it difficult to interpret some PTGS phenotypes. Based on these reasons the search for a suitable virus vector is still going on for studying gene function of desirable plant species.

1.2.1 Applications of virus induced gene silencing in plants

Most of the research to study the application of VIGS vectors has been done on *N. benthamiana*, a model plant because of high range of virus susceptibility of this plant. *N. benthamiana* is a favourite choice, because symptoms appeared in this plant are more pronounced and persistent than in other plants. Because of high success of this silencing mechanism, VIGS vectors have been developed based on other viruses and applied to the crops of economic importance to understand gene functions or other important candidates of resistance mechanism. For example, with a barley stripe mosaic virus vector, VIGS of PDS has been demonstrated in monocot plants i.e. barley

(Holzberg *et al.,* 2002). In addition, TRV vectors have been shown to support VIGS in Arabidopsis (Dalmay *et al.,* 2000) and tomato (Liu *et al.,* 2002) as well as in *N. benthamiana*.

Over the past few years, VIGS has been used extensively for identifying the function of unknown genes in various plant species. Fu *et al.,* 2005 has used a TRV based system to identify the genes involved in the ripening of tomato fruits. VIGS has also been used to identify the genes involved in virus resistance pathways in plants. The application of viral vectors have made it, a method of choice, to reveal the factors involved in signalling pathways and to understand the mechanism of virus resistance in plants. The discovery of host factors, including proteins, membranes and nucleic acids involved in viral replication cycles are providing fundamental clues about the molecular basis of viral susceptibility. Chen *et al.,* 2007 has used a *tomato yellow leaf curl china virus* (TYLCCNV) DNA ß based silencing vector to demonstrate the role of TOMI in TMV replication in *N. benthamiana.* TOMI is a putative trans-membrane protein which is associated with the replication complex of TMV and is responsible for the efficient replication of TMV. Silencing of TOMI in *N. benthamiana* has resulted in a complete inhibition of TMV (Chen *et al.,* 2007). Similarly, Peart *et al.,* 2002 has found *EDS1* orthologue, an essential component of the resistance pathway initiated by the presence of N gene. Further studies, have shown that the resistance produced by R genes of the TIR-NBS-LRR type is EDS1 dependent, where as the CC-NBS-LRR group of R genes do not require EDS1. It shows that each group of resistance genes awoke a different signalling pathway to give resistance against pathogens.

Although a lots of VIGS vectors are being used to understand the gene function and candidate genes involved in various pathways, none of them have been used successfully to understand the resistance mechanism of *R* genes of CC-NBS-LRR group. Important members of this group are *Tm-2* and *Tm-2*^{2} resistance genes that are important against tomato mosaic virus. Very little is known about the resistance mechanism of these *R* genes and many questions related to the function of these genes and various host factors involved in the resistance pathway.

For this purpose, tomato mosaic virus based gene silencing vectors were developed for tomato and analysed for their efficiency to produce gene silencing in *N. benthamiana* and tomato. These silencing vectors can be used to identify the role of putative host factors involved in *Tm-2* and *Tm-2*^{2} resistance pathways.

2. Materials

2.1 Chemicals and Enzymes

Standard chemicals were purchased from Roth (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), Duchefa (Harlem, Netherlands), Serva (Heidelberg, Germany) and MP (Heidelberg, Germany). Restriction enzymes and DNA modifying enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany). *Taq* DNA polymerase was supplied from MBI Fermentas and Genaxon (Ulm, Germany).

Ultrapure water (18.3 MΩ/cm), filtered through a Herco-filtration system (Herco, Freiburg) was used for all solutions, unless marked otherwise.

2.2 Biological Materials

2.2.1 Plants

- *Solanum lycopersicum* L. cv. Craigella GCR 26 (-/-)
  GCR 236 (*Tm-2*/*Tm-2*)
  GCR 254 (*Tm-1*/*Tm-1*; *Tm-2*/*Tm-2*)
  GCR 267 (*Tm-2²*/*Tm-2²)*
  Obtained from the „Institute for Horticultural Research“, Littlehampton, England

- *Solanum lycopersicum* L. cv. Moneymaker (MM) (-/-)
  Obtained from seed company (Hild GmbH, Marbach)

- *Nicotiana tabacum* L. cv. Samsun nn (-/-)
  NN (*N*/*N*)

- *Nicotiana benthamiana* L. (-/-)
  Obtained from „Tabacco Institute“, North Carolina, USA
  and from Prof. K.-W. Mundry, University of Stuttgart

2.2.2 Plant viruses

ToMV0, wildtype virus strain with no resistance breaking phenotype.

Strain of Tomato mosaic virus were obtained from P. Gimbley, „Institute for Horticultural Research“, Littlehampton, England, UK.

2.2.3 Bacterial strains

- *Escherichia coli* DH5α
  Obtained fron the labortary stock (Hanahan, 1983)
  Genotype: F'Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44λ^- *thi*-1; *rec*A1; *gyr*A96 *rel*A1

- *Escherichia coli* XL1blue
  Obtained from the labortry of Prof. Dr. Anne Spang, University of Basel
  Genotype: F'*::*Tn*10* (Tet^r) *proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15 recA1 endA1 gyrA96* (Nal^r) *thi-1 hsdR17* ($r_k^-m_k^-$) *glnV44 relA1 lac*

- *Agrobacterium tumefaciens* LBA4404
  Obtained from laboratory stock (Hoekema *et al.,* 1983)

2.2.4 Yeast strains

Saccharomyces cerevisiae Cen.Pk2

Genotype: MATa/α, *ura3-52/ura3-52, trp1-289/trp1-289, leu2-3, 112/leu2-3, 112, his3-Δ1/his3-Δ1*

2.3 Vectors

2.3.1 pBluescript II KS(+)

pBluescript vector (Stratagene USA) has a size of 2961 bp (Fig 16). The pBluescript phagemids are designed for the cloning, sequencing, in-vitro-mutagenesis and in-vitro transcription of the desired gene. It contains a multiple cloning site (MCS) in

the coding region of *Lac-Z* gene for the blue white screening of recombinant clones. This plasmid contains an Ampicilline resistance gene for selection in *E.coli*.

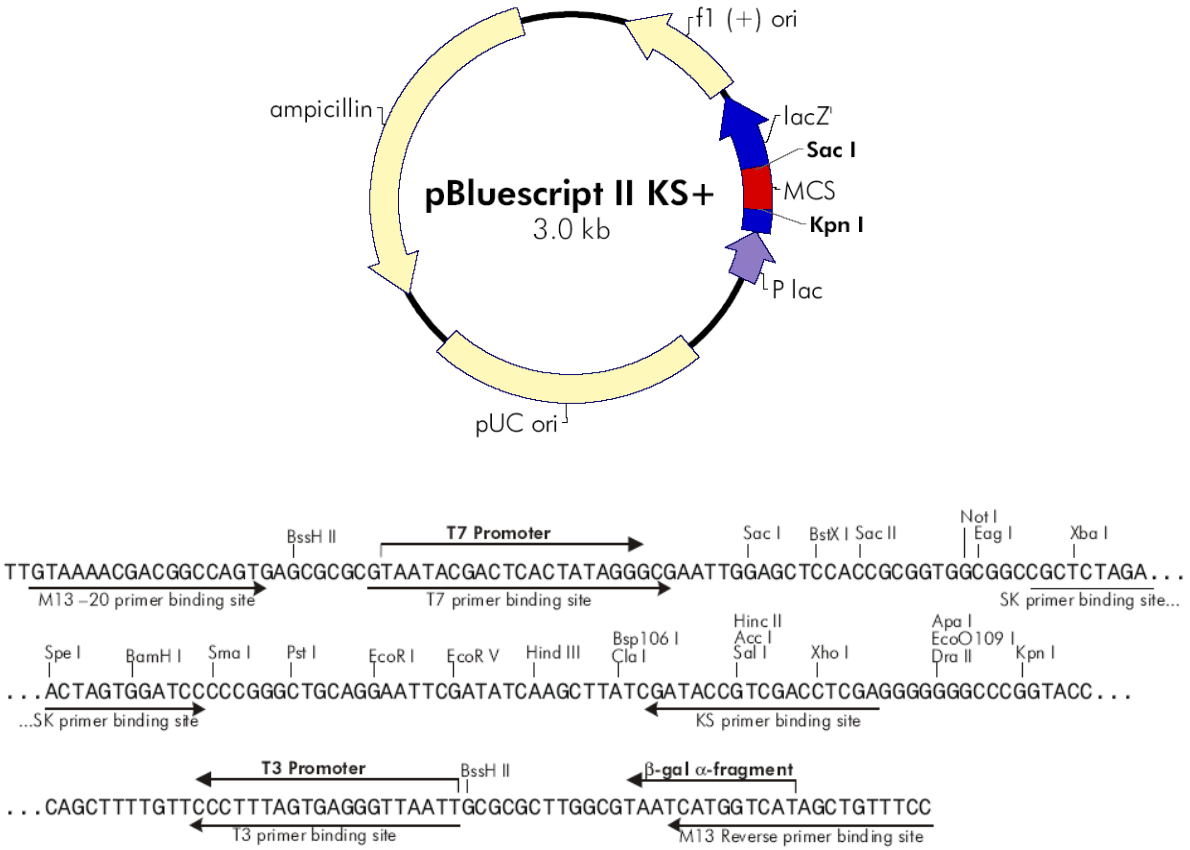


Figure 16. Schematic diagram of pBluescript vector and the detailed description of multiple cloning site

2.3.2 pBS / T-Vector

T-vector used during this research was prepared in the lab. For the construction of T-vector, pBluescript II KS+ (Stratagene USA) was linearized by restriction with EcoRV (Eco 321) enzyme. The linearized vector DNA was treated with terminal deoxynucleotidal transferase (TDT) to add ddTTP at the ends. This vector is useful for the cloning of the PCR products amplified with *Taq* polymerase enzyme because of the addition of an Adenine at the 3' ends of the PCR product.

2.3.3 pBin19 Vector

pBin19 is a binary vector of 11777bp (Fig 17). This vector contains Kanamycine as a selection marker and a MCS present in the coding region of Lac Z gene, therefore allowing blue white selection of recombinant vector. The vector contains the *E.coli* ori and two boundary sequences from the T-DNA region of the Ti plasmid. These two boundary sequences (left border -LB and right border-RB) recombine with plant chromosomal DNA, inserting the DNA segment between them to the plant DNA. Therefore, it is possible to use this vector not only in *E.coli* but also for the plant transformation.

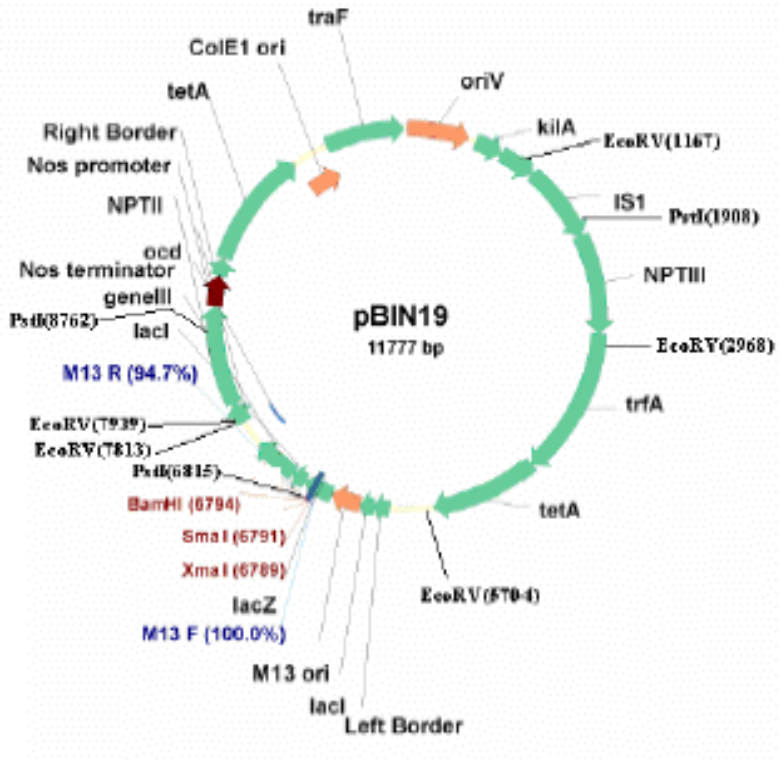


Figure 17. Schematic diagram of pBin19 binary vector showing important features of vector.

2.3.4 pSLN Vector

pSLN vector was originally developed by H. Weber (1993). The vector contains a pUC18 backbone. A full length cDNA of Tomato mosaic virus (ToMV) was cloned in the MCS fused with 35S Cauliflower mosaic virus promoter and nopaline terminator with the

help of SphI and SacI restriction enzymes (Fig 18). It has Ampicilline resistance gene for the antibiotic selection.

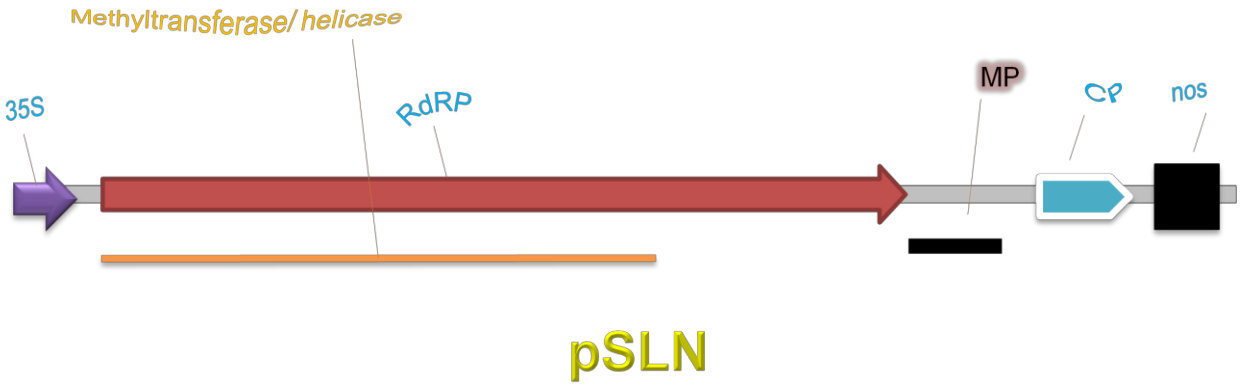
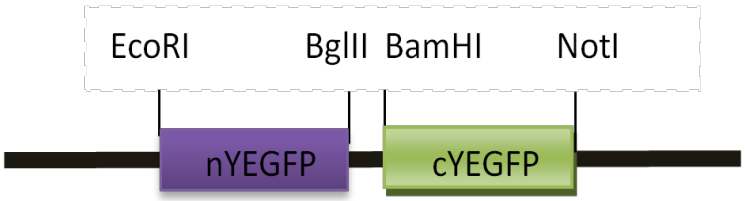


Figure 18. Schematic diagram of pSLN vector containing tomato mosaic virus cDNA along with 35S promoter and nos terminator.

2.3.5 pyEGFP vector

pyEGFP was designed by Dr. U. Pfitzner, Department of general virology, university of Hohenheim. The vector is based on pUC18 vector and contains the two halves of yEGFP (yeast enhanced GFP) splited by two restriction sites BamHI and BglII as shown in figure 19. The N-terminus consists of 1-157aa of YEGFP and was amplified with primers containing EcoRI and BglII to clone in the MCS of pUC18. The C-terminus of yEGFP contains 158-238aa and was amplified with the help of primers containing BamHI and NotI to clone it behind the other half of YEGFP in the pUC18 vector.



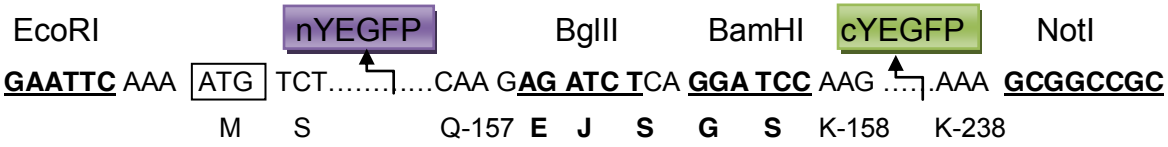


Figure 19. Schematic diagram of pyEGFP vector and the detailed description of restriction sites introduced through PCR.

2.3.6 pESCura/his Vector

pESCura/his vectors were obtained from Stratagene (La Jolla, CA 92037 USA). The vector size is approximately 6.6 Kb. It contains two multiple cloning sites for the cloning of desired gene fragments. It has Ampicilline resistance gene for *E.coli* and *URA3/HIS3* ORF for selection in yeast cells.

2.4 "Kits"

2.4.1 Plasmid preparation

GeneJET™ plasmid miniprep kit (K0503) was used for the isolation of high quality plasmid DNA. For this purpose, plasmid isolated from an overnight grown culture of *E.coli* was passed through a column. The DNA obtained after elution was used for sequencing.

2.4.2 DNA-elution from agarose gels

E.Z.N.A.™ gel extraction Kit from the company Omega bio-tek.

2.5 DNA-Ladders

Standard DNA-Markers used during this research were:

GeneRuler™ 100 bp DNA Ladder (#SM0241, MBI Fermentas, St. Leon-Rot):

1031 bp / 900 bp / 800 bp / 700 bp / 600 bp / **500 bp** / 400 bp / 300 bp / 200 bp / 100 bp / 80 bp

GeneRuler™ 1 kb DNA Ladder (#SM0311, MBI Fermentas, St. Leon-Rot):

10 kb / 8 kb / 6 kb / 5 kb / 4 kb / 3.5 kb / **3 kb** / 2.5 kb / **2 kb** / 1.5 kb / **1** kb / 750 bp / 500 bp / 250 bp

(Bold letters indicate thicker bands)

2.6 Protein-Molecular weight marker

2.6.1 Prestained Protein Marker

Prestained Protein Molecular Weight Marker (#SM0671, MBI Fermentas):

180 kDa / 130 kDa / 100 kDa / **70 kDa** / 55 kDa / 43 kDa / 34 kDa / 26 kDa / 16 kDa / 10 kDa

The reference protein at 70kDa was coupled with red stain.

2.7 Antiserum

2.7.1 Detection of ToMV

For the detection of coat protein from ToMV, a polyclonal antibody anti-TMV-coat protein was used. This antiserum was used as a dilution of 1:5000 in blocking solution. For the detection of this primary anti- TMV-coat protein antibody, an anti rabbit antibody coupled with HRP (horse radish peroxidise) was used (α-rabbit IgG-HPR, Rockland) in a dilution of 1:5000.

2.7.2 Detection of GFP

For the detection of GFP protein, the polyclonal anti-GFP antibody (Santa Cruz Biotechnology) was used as a dilution of 1:5000.

2.8 Oligonucleotide (Primer)

2.8.1 Oligonucleotide

During this research, the oligonucleotides were obtained from the company Invitrogen™ Life Technologies. Different restriction sites were added in the primer sequences the cloning of the PCR products in different vectors.

MP5BglII	GGAGATCTCAATGGCTCTAGTTGTTAAAGG
MP3BamHI	GGGATCCATACGAATCAGAATCCGCGACCG

MP131BglII	GGAGATCTCAGAAAAGAACATATGGCAGG
MP5EcoRI	GGAATTCAAAATGGCTCTAGTTGTTAAAGG
MP130MfeI	CCAATTGTGCATCCTTTGTTGTAATACC
TGB2-5BglII	GGAGATCTCAATGTCCGCGCGCAGGGCCATAG
TGB2-3BamHI	CGGATCCATGACTGCTATGATTGTTACCAC
cyEGFP-5	CGGAATTCAAAATGAAGAATGGTATCAA
cyEGFP-3	GGCGGCCGCTTTGTACAATTCATCC
5EcoRImGFP4	CGAATTCAAAATGAGTAAAGGAGAAGAAC
5BamHImGFP4	GGATTCAAGAAGAATGGAATCAAACTTAAC
3NotImGFP4	CGCGGCCGCTTTGTATAGTTCATCCATGCC
3BamHIBglIIImGFP4	CGGATCCTGAGATCTCTTGTTTGTCTGCCATG
Tm-1a (Forward)	GCCTAGGGGCAAGCCTATTATTCGGGCTGG
Tm-1b (Rev 200)	CGTTAACCTCCAGCCAGAACTGCCACTTCC
Tm-1c (Rev 500)	GGTTAACGCCCCATATGAGCAACTATGATG
Tm-2a (Forward)	GCCTAGGCATTGCAACCCCTAGAATCCGAG
Tm-2b (Rev 200)	CGTTAACGTACTCTGTTCCACGCATGTTC
Tm-2c (Rev 500)	GGTTAACCACCAAATGCATCATGCGCGGTG
PDS4942F	GCACCTGCCCCCGAAAGAATAAGAAATGATG
PDS5485R	CGTGGACGGCACATACATAGGTATGCTACAC
PDS5234R	CGTGGACGCTGGAGCGGCAAACACAAAAGCATC

2.8.2 Oligonucleotide for Sequencing

For the sequencing of the cloned gene fragments, the plasmid DNA was sent to the company MWG Biotech, Ebersberg. The sequencing was done with following primers

M13 uni (-21)	TGT AAA ACG ACG GCC AGT
M13 rev (-29)	CAG GAA ACA GCT ATG AC

3. Methods

3.1 Standard molecular biology methods

Standard molecular biology methods were used according to the instructions from supplier (enzymatic reactions and kits) and according to Sambrook and colleagues (1989).Therefore new and altered methods according to the need of experiments are described below.

3.1.1 Bacterial growth medium

All media and stock solutions for bacterial growth and further work were prepared according to Sambrook and colleagues (1989).

Growth medium and buffer.

- SOB
  2 % Trypton / Pepton
  10 mM NaCl
  2.5 mM KCl
  pH was adjusted to 7.0 , medium was autoclaved and cooled before further use.
  10 mM $MgCl_2$ (0.2 μm sterile filtered)
  10 mM $MgSO_4$ (0.2 μm sterile filtered)

- SOC
  SOB-Medium
  20 mM Glucose (0.2 μm sterile filtered)

- TB-Buffer
  10 mM PIPES (Piperazin-1,4-bis(2-ethansulfonacid))
  55 mM $MnCl_2$
  15 mM $CaCl_2$
  250 mM KCl

All components, except $MnCl_2$, were mixed together and pH was adjusted to 6.7 with KOH. After adjusting the pH, $MnCl_2$ was added to the solution. The solution was aliquotized in 80 ml and 20 ml volumes, after sterile filtration with 0.2 µm syringe sterile filter (Corning Costar, Bodenheim)

- LB-Medium
  10 g Pepton
  5 g yeast extract
  10 g NaCl
  add 1 l H_2O
  pH 7.5 with NaOH
  Autoclave for 15 minutes

- Agar plates
  LB-Medium
  + 1.5 % Micro-Agar (Duchefa Biochemie, Haarlem, NL) was added to the medium and then autoclaved for 15 min.

- Antibiotic concentrations:

Ampicilline:	Stock solution: 50 mg/ml in H_2O
	100 µg/ml (f. c.) in liquid medium
	50 µg/ml (f. c.) in Agar plates
Kanamycin:	Stock solution: 100 mg/ml in H_2O
	100 µg/ml (f. c.) in liquid medium
	50 µg/ml (f. c.) in Agar plates

  For blue-white selection, 500µl of 20% X-Gal and 100 µl of 100mM IPTG was added in 250 ml of LB + Agar medium for LB plates.

3.1.2 Transformation of *E. coli* according to Inoue and Co-workers (1990)

3.1.2.1 Preparation of *E.coli* competent cells

- **Tfb I (sterile filtered)**

  30 mM potassium acetate

  50 mM $MnCl_2$

  100 mM KCl

  10 mM $CaCl_2$

  15 % glycerol (w/v)

- **Tfb II (autoclaved)**

  10 mM Na – MOPS pH 7.0

  75 mM $CaCl_2$

  10 mM KCl

  15 % glycerol (w/v)

E. coli strain (XL1blue/DH5α) was restreaked form a glycerol stock onto LB plates with 10 mM $MgSO_4$. The plate was incubated over night at 37° C. A single colony was used to inoculate a 5 ml LB/ $MgSO_4$ culture and was grown at 37° C and 250 rpm over night. Then the culture was transferred into 100 ml LB/ $MgSO_4$ and incubated till the OD_{546} reached 0.5. The culture was transferred onto two ice cold 50 ml tubes and centrifuged at 6000 rpm at 4° C for 10 minutes. The medium was decanted and the cells resuspended in 40 ml Tfb I each. Then another centrifugation step at 6000 rpm followed. After removal of the Tfb I, the cells were resuspended in 8 ml Tfb II. The cells were aliquotized at 100 µl, frozen in liquid nitrogen and stored at – 70 ° C.

3.1.2.2 Transformation of bacterial cells by "heat shock" method

Competent bacterial cells were shortly thawed on ice. 10 µl of the ligation reaction or 1ng of the DNA was mixed in 100 µl of the competent cells and the reaction mixture was incubated for 30 minutes on ice. After incubation, the cells were heat shocked at 42 °C for 30 seconds and shifted immediately to ice for 2 minutes. 900 µl of SOC medium was added in each reaction mixture and incubated at 37 °C and shaking at a speed of 250 rpm for 60 minutes. The bacterial cells were shortly centrifuged to

pellet the cells. Supernatant was discarded and cells were suspended in 100 µl of SOC medium. The suspended cells were spread on appropriate selective plates. The plates were incubated at 37 °C over night and stored at 4 °C for further use. Desired colonies were inoculated in 5ml liquid medium with the help of sterile tips for plasmid preparation (See 3.1.4).

3.1.3 Transformation of *E. coli* through Electroporation

3.1.3.1 Preparation of electrocompetent cells

50 ml of SOB-medium (without Mg^{2+}) was inoculated with *E.coli* cells taken from glycerol stock in a 300 ml sterile flask and incubated over night at 37 °C and 250 rpm. 2.5 ml of this overnight culture was used to inoculate 250 ml of SOB (without Mg^{2+}) and grown further until an OD_{550} of 0.8 at 37 °C (See 3.1.1).

The grown culture was placed on ice and centrifuged for 10 minutes at 4000 x g and at 4 °C. The pellet was resuspended in 100 ml of ice-cold wash buffer (10 % Glycerin, autoclaved) and centrifuged again for 15 minutes (4000 x g, 4 °C). The supernatant was discarded and the last step was repeated three times. After the last wash, the supernatant was discarded and the bacterial pellet was resuspended in the remaining liquid. The cells were aliquotized in cooled Eppendorf tubes and immediately freozen in liquid nitrogen. The electrocompetent cells were stored at – 70 °C until further use.

3.1.3.2 Electroporation

The electroporation was done with Gene Pulser™ and Pulse Controller from the company Bio-Rad, Munich according to the manufacturer's instructions (Gene Pulser Electroprotocol).

40 µl of the electro-competent cells were mixed with 1-2 µl of the DNA in an ice cold electroporation cuvette (Gene Pulser Cuvette, gap with 2 mm; Bio-Rad, Munich). The electroporation was done at 2.0 kV with a capacity of 25 µFD, resistance of 200 Ω and with a time constant of 4-5 milli-seconds. Directly after the electric shock, 1 ml of the SOC medium was added to the cuvette (See 3.1.1) and the cells were transferred

into a new Eppendorf tube. The regeneration of the cells was done for one hour at 37 °C and 225 rpm. The transformed cells were spread on the selective medium agar plates (as described before in 3.1.1) and incubated at 37 °C for bacterial growth.

3.1.4 Transformation of *Agrobacterium tumeficians* (LBA4404/GV 3101) through Tri-parental mating

For the transformation of plasmid DNA in *Agrobacterium*, tri-parental mating was used. 5 ml of the overnight culture were used from three different parents.

- *E.coli* containing the binary vector (i.e pBin19) with respective antibiotics
- pRK2013 (*E.coli* strain with helper plasmid)
- *Agrobacterium* (LBA4404/GV3101) in LB medium with selection antibiotics

Cultures were centrifuged to pellet the cells for 5 minutes at 6000 rpm. After centrifugation, the *E.coli* strains were suspended in 5 ml of 10 mM $MgSO_4$ and the *Agrobacterium* cells in 1 ml of 10 mM $MgSO_4$. 50 µl from each suspension was pipetted in the middle of an LB plate (without antibiotics), mixed together and spread carefully. The LB plate was incubated overnight at 30 °C. Next day the bacterial cells were washed from the plate and suspended in 1 ml of the LB medium. The suspension was diluted to 1:100 and spread onto selection medium. The plates were incubated at 30 °C for two days and then the single colonies were picked for liquid cultures.

3.1.5 Plasmid preparation from bacterial cells (*E.coli/Agrobacterium*)

- Solution I

  25 mM Tris / HCl pH 8.0

  10 mM EDTA / NaOH pH 8.0

  100 µg/ml RNase A (90 U/mg)

  50 mM Glucose

- Solution II

  200 mM NaOH

  1 % SDS

- Solution III

  3 M Na-Acetate pH 4.8 (Acidic) in 1x TE

- 1x TE

  10 mM Tris / HCl pH 8.0

  1 mM EDTA / NaOH pH 8.0

A single bacterial colony, taken from agar plate, was used to inoculate 5 ml LB medium and grown overnight. 5 ml were taken from the overnight culture and the bacterial cells were pelleted in a 1.5 ml Eppendorf tube after 1 min centrifugation at 12000 x *g*. The supernatant was discarded and the pellet was suspended in 100 µl solution I. After that 200 µl solution II was added and gently mixed by inverting 2-3 times. The mixture was incubated no longer than 5 minutes at room temperature. 150 µl ice cold solution III was added to the mixture and incubated on ice for 5 minutes. The proteins and the genomic DNA were isolated by centrifugation at 11000 rpm in a table centrifuge for 10 minutes at 4 °C. The supernatant was transferred carefully to a new Eppendorf tube without taking up the proteins. 1 ml of the 96 % ethanol was added to the supernatant and incubated for 5 minutes or longer on ice. After the centrifugation (as described before) the supernatant was discarded and the DNA pellet was washed with 500 µl of 70% ethanol. DNA pellet was air dried and resuspended in 50-100 µl of the 1x TE buffer, depending upon the DNA quantity.

3.1.6 Phenol extraction of DNA

Cleaning of the nucleic acid from remaining proteins can be achieved with Phenol chloroform extraction. For this purpose, the DNA pellet dissolved in TE (50-100 µl) was mixed with an equal volume of phenol in a 1.5 ml Eppendorf tube. Both the DNA and phenol were mixed carefully by inverting the tube several times. To isolate the water and organic phase the mixed solution was centrifuged for 5 minutes at 14000 x g. The water phase containing the DNA solution was transferred to a new Eppendorf tube and mixed with equal volume of chloroform: isoamylalcohol (24:1). After mixing it carefully, the tube was centrifuged for 5 minutes at maximum speed in a tabletop centrifuge. The water phase was again moved into a new Eppendorf and mixed with C:I. After

centrifugation, the water phase was transferred to a new Eppendorf tube and the DNA precipitation was carried out (See 3.1.7) and resuspended in 1x TE.

3.1.7 Precipitation of DNA

The precipitation of dissolved DNA with less salt concentration can be done by adding 1/10 volume of 3 M sodium acetate (solution III, See 3.1.4) and 2.5 volume of the 96 % Ethanol p.a. Samples were mixed carefully and incubated for almost 30 minutes at - 20 °C. After the incubation, they were centrifuged for 10 minutes with 12000 x g at 4 °C. The DNA pellet was washed with 70 % ethanol and air dried. The dried DNA pellet was dissolved in 1 x TE.

3.1.8 Sequence specific Restriction of DNA

Plasmid DNA can be restricted with specific restriction enzymes, according to the instructions from the company using specific restriction buffers. 0.2 to 1 µg of the DNA was restricted using 5 units of the enzyme and incubated for approximately two hours at 37 °C.

3.1.9 Separation of DNA fragments

The separation of restricted DNA fragments can be executed using electrophoresis on a 1% agarose gel. For this purpose a specific amount of agarose (Biozym, Oldendorf) is melted in 1x TAE buffer. For the visualization of the DNA under a UV lamp 10µg/ml Ethidium Bromide is added to the solution after cooling down to 55 °C. The DNA samples were mixed with 1:10 of the 10x blue dye or 1:6 with 6x orange dye and loaded onto the gel. The electrophoresis was done at 60 to 100 Volts. The sizes of the DNA fragments were observed by loading a Ladder (See 2.5) on the agarose gel along with the samples.

- 50x TAE-buffer
  242 g Tris
  100 ml 500 mM EDTA pH 8.0
  57.1 ml 100 % Acetic Acid
  add 1 l H_2O

- 10x Blue dye

  65 % Saccharose (w/v)

  30 mg Bromphenolblue (Serva, Heidelberg)

  30 mg Xylencyanol (Serva, Heidelberg)

  2 ml 50x TAE

  add 10 ml H_2O

- 6x O'Dye

  65 % Saccharose (w/v)

  30 mg Orange G (Serva, Heidelberg)

  2 ml 50x TAE

  add 10 ml H_2O

3.1.10 Isolation of DNA fragments from agarose gel

For the cloning of a desired size of DNA fragment into a vector, restriction of 10-20 µg of the DNA was carried with specific restriction enzymes. Linearized Vector DNA was treated with "Calf Intestinal Alkaline Phosphatase" (MBI fermentas, St Leon-Rot) according to the supplier's instructions. After the restriction of the DNA, the samples were loaded on agarose gel for the isolation of different sized fragments (See 3.1.8).The isolation of the desired DNA fragments were done with E.N.Z.A. Gel extraction Kit (Omega) according to the supplier's instructions.

3.1.11 Ligation of DNA fragments

Ligation of DNA fragments with Vector DNA was executed with the help of enzyme T4 DNA Ligase (MBI Fermentas, St. Leon-Rot), which is responsible for the formation of phosphodiester bond between 5'-phosphate and the 3' Hydroxy group of double stranded DNA molecules. In ligation reaction the recommended ratio of vector to insert was 1:3 (for sticky ends) and 1:1 (for blunt ends).

- Ligation reaction: 5-17 µl DNA-Mix: Vector-DNA: 50-400 ng
  Insert-DNA: as described above
  + 2 µl 10x Ligation buffer

+ 2 µl 50 % PEG 4000 (only for blunt-end Ligations)

For sticky-end Ligation: + 2 U T4-DNA-Ligase

For blunt-end Ligation: + 4 U T4-DNA-Ligase

add 20 µl deion. Water

Ligation reaction was carried out for 1 hour at 22 °C in a water bath or at 4 °C over night. Before the transformation of the ligation reaction in *E.coli*, the DNA ligase was inactivated for 10 minutes at 65 °C (See 3.1.2.2).

3.2 Polymerase chain reaction (PCR)

For the amplification of a specific DNA sequence, the polymerase chain reaction PCR was used according to the protocol from Saiki and Colleagues (1988) and Bej and colleagues (1991). During PCR reaction, required DNA sequence was amplified with a thermostable *Taq* polymerase and synthetic oligonucleotides (primers) to such an extent that it can be seen on the agarose gel. By changing the annealing temperature and ionic concentration of Mg^{++}, specificity of the reaction can be increased. All samples were put on ice in 0.5 ml thin wall reaction tube from Croning costar, Bodenheim (#6530).

Reactions were carried out in a programmed thermal cycler with a preheated lid (Master cycler from Eppendorf) with a program written below. Amplified PCR products were checked for their right size on the agarose gel (See 3.1.8) by gel electrophoresis and were finally cleaned by using a gel elution kit for further cloning (See 3.1.9).

3.2.1 Standard PCR with Taq DNA polymerase

- 20 µl reaction sample

  1-100 ng “template” DNA

  2 µl 10x PCR-buffer

  0.4 µl 10 mM dNTPs

  0.5 µl 10 µM primer 5’

  0.5 µl 10 µM primer 3’

0.1 µl *Taq* DNA Polymerase (Eppendorf, Hamburg)

add 20 µl H_2O

For every reaction, a negative control, with water or 1xTE was added instead of the DNA, was prepared to check for contamination and to avoid false positive results. As a positive control, sample was prepared with vector DNA to be sure if the PCR conditions were suitable for the amplification.

- Standard program:

	2 min	94 °C	Denaturation of the "template"-DNA
	---	---	---
33 cycles:	30 sec	94 °C	Denaturation of the "template"-DNA
	30 sec	var.	Annealing of Primer ("annealing")
	var.	68 °C	Polymerisation (synthesis of double stranded DNA)
	---	---	---
	7 min	68 °C	Elongation of the Reaction
	∞	8 °C	cooling of PCR product until further use

Variable (var.) values of the temperature for primer annealing and also the time duration for double stranded DNA synthesis depend upon the melting temperature (Tm) of primer and the expected length of the amplified DNA fragment (appro.45 sec-1 min per 1Kb).

3.2.2 "Proofreading" PCR

For the cloning of the amplified DNA with a specific DNA sequence; PCR reaction was carried out with "Proof Reading" *Taq* from MBI Fermentas, St. Leon-Rot. This polymerase enzyme has the proof reading activity for corrections of any mismatches occurred during PCR reaction to minimize the mutation rate.

- Reaction mixture:

1-100 ng "template" DNA

5 µl 10x proof reading PCR-Puffer + $MgCl_2$

1 µl 10mM dNTPs

1.25 µl 10 µM Primer 5'

1.25 µl 10 µM Primer 3'

0.5 µl proof reading *Taq* (5 U/µl)

add 50 µl H_2O

For a negative control, with every reaction one sample was prepared with H_2O or 1x TE.

- Program: Proof reading PCR

	2 min	94 °C	Denaturation of "template" DNA
10 cycles:	20 sec	94 °C	Denaturation of "template"-DNA
	30 sec	57 °C	Annealing of Primer ("annealing")
	2 min	68 °C	Polymerisation (second strand synthesis)
25 cycles:	20 sec	94 °C	Denaturation of "template"-DNA
	30 sec	57 °C	Annealing of primer ("annealing")
	2 min	68 °C	Polymerisation (second strand synthesis)
	10 min	68 °C	Elongation reaction
	∞	4 °C	Cooling of PCR product until further use.

3.2.3 "Colony screen" PCR

In case of difficult cloning strategies or presence of a lot of colonies after transformation, to find positive clones directly from bacterial plate or from *Agrobacterium* plate, Colony PCR was used to solve these problems.

10 µl of the sterile water was added to a 0.5 ml PCR reaction tube. With the help of a yellow tip one single colony was picked and streaked onto a new plate with selection antibiotics. The rest of the bacteria in the tip were suspended in the water in the reaction tube.

The newly streaked agar plate with bacterial colonies was incubated overnight at appropriate temperature. The bacterial cells inside the reaction tube were used for PCR, according to the PCR conditions written below.

5 min	96 °C
90 sec	50 °C
90 sec	96 °C

1 min	45 °C	
1 min	96 °C	
1 min	40 °C	
∞	8 °C	

The broken bacterial suspension was used as “template DNA” in a standard PCR reaction (See 3.1.11.1). For primer pairs, oligonucleotides based on the flanking region of the insert or the vector or one primer for the insert fragment and one for the vector, were used.

- Program:

	2 min	94 °C	Denaturation of “template”-DNA
28 cycles:	30 sec	94 °C	Denaturation of “template”-DNA
	30 sec	55 °C	Annealing of Primer (“annealing”)
	2 min	68 °C	Polymerisation (double strand synthesis)
	∞	8 °C	cooling of PCR product until further use

PCR reactions were loaded on agarose gel to check for the amplification of the desired size fragment. Samples, where amplification occurred, were used for liquid cultures in LB medium and tested for the right clone after DNA isolation from overnight culture.

3.2.4 DNA Sequencing

To confirm the DNA sequence of a positive clone, plasmid DNA was isolated using DNA isolation Kit from Fermentas (K0503). Sequencing was carried out by company Eurofins DNA. Samples were prepared according to the instruction of the company (Eurofins DNA).

3.2.5 Determination of DNA concentration

The concentration of purified DNA was tested using a spectrometer. 1 µl DNA (in 1x TE) was mixed with 49 µl H_2O and transferred into an UVette® (Eppendorf, Hamburg, Germany). As reference, a cuvette with 50 µl H_2O was used. The

measurement was executed with an Ultraspec 300 spectrometer (Pharmacia Biotech, Cambridge, UK).

The concentration was calculated as followed:

Concentration = [(absorption λ 260 nm) – (absorption λ 320 nm)] * F * V^{-1}

λ 320 nm = background absorption

F [μg/ml] = factor: 50 for DNA

V = dilution; 1:50

3.2.6 Bacterial stocks

To store bacterial cultures for longer periods of time, 800 μl of a freshly grown overnight culture was mixed with 200 μl of 100% glycerin. The reaction tube was stored at –70 °C.

3.3 Protein analysis through SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated with SDS-polyacrylamide gel electrophoresis after Laemmli (1970). The gels can be used for the specific detection of proteins. The molecular weight can be measured with the use of a protein marker.

- **Solution I (for SDS-Page)**

  18.3 g Tris
  115 µl TEMED (N,N,N,N – Tetramethylethylenediamine)
  add 100 ml H_2O
  pH 8.9 with HCl

- **Solution III (for SDS-Page)**

  6.1 g Tris
  230 µl TEMED

add 100 ml H_2O

pH 6.8 with HCl

- **Acrylamide solution Gel 30 (Roth, Karlsruhe)**

37.5: 1 mixture of 30% acrylamide solution and 0.8 % bisacrylamide

- **1x electrophoresis buffer**

3 g Tris

14.4 g glycin

1 g SDS (Sodium dodecyl sulfate)

add 1 l H_2O

Loading buffer

- **3x SDS probe buffer**

1.875 ml 1 M Tris/pH 6.8

0.6 g SDS

3 g glycerol

30 mg bromophenol blue

add10 ml H_2O

- **5x SDS loading buffer**

313 mM Tris/HCl pH 6.8

10 % SDS

50 % glycine

0.05 bromophenol blue

- **20x DTT (dithiothreitol) (2 M; Fermentas St. Leon Rot)**

The following solutions were prepared fresh before the gel preparation

- **Resolving gel (15%)**

4 ml Gel 30

2 ml solution I (for SDS-Page)

80 µl 10 % SDS

1920 µl H_2O

200 µl 10% ammonium persulfate (APS)

- **Stacking gel**

1 ml solution III

540 µl Gel 30

40 µl 10 % SDS

2420 µl H_2O

100 µl 10 % APS

After addition of APS, the gel must be casted because it catalyses the polymerization of acrylamide.

A glass plate and an aluminium oxide plate separated by a 1 mm thick spacer were held together by several metal brackets. The gel cassette was sealed with 1 % agarose at the bottom. First, the resolving gel was casted and overlaid with 1000 µl water to prevent the hydration of gel. After polymerization of the resolving gel, water was removed using filter papers. Then, the probe comb was inserted and the stacking gel was filled on the top of the resolving gel. After the stacking gel was polymerized, gel cassette was inserted into the electrophoresis chamber (Mighty Small II SE 250; Hoefer Scientific Instruments, San Francisco) and covered with 1 x electrophoresis buffer. The protein probes were prepared as described above and 20 µl were loaded into the stacking gel pockets. The proteins were separated at 120 V and 50 - 75 mA for 1 to 2 hours.

At the end of the gel electrophoresis, the protein gels were either stained with the Coomassie-staining solution (See 3.2.1) or were blotted to the nitrocellulose membrane for the immune-detection (See 3.3.2).

3.3.1 Staining of the protein gels

- Coomassie-staining solution

  100 ml Acetic Acid

  450 ml H_2O

  450 ml Methanol

  2.5 g Coomassie Brillant Blue R-250 (Serva, Heidelberg)

- Destaining solution

  70 ml Acetic Acid

  430 ml H_2O

  500 ml Methanol

After the gel electrophoresis (See 3.3), the stacking gel was removed and the resolving gel was incubated in the coomassie staining solution from 2 to 16 hours. Afterwards, the gel was transferred in the destaining solution for approximately one hour and later into the 5 % Acetic Acid overnight. For the documentation of the SDS gels, the pictures were taken with a digital camera (Olympus Camedia C-7070) or scanned on the computer.

3.3.2 Immuno-detection of proteins ("Western-Blotting")

3.3.2.1 Protein transfer

- **Western Blot buffer**

  5.45 g Tris

  25.9 g glycin

  add 1620 ml H_2O

  + 180 ml methanol

For the specific detection of proteins, they were transferred to a nitrocellulose membrane and detected with specific antibodies. Either, the primary or secondary antibody is linked with HRP (horseradish peroxidase). The protein antibody complex can be detected through peroxidase activity by using a chemiluminescence reaction.

After the SDS-PAGE, the stacking gel was removed and the Western Blot was stacked as followed:

Blot cassette cathode (black)

Fiber pad (pre-soaked in Western Blot buffer)

2 layers of Hybond^{Tm} blotting paper (pre-soaked)

Polyacrylamid gel (resolving gel)

Nitrocellulose membrane (4 x 9 cm) (pre-soaked)

2 layers of Hybond^{Tm} blotting paper (pre-soaked)

Fiber Pad (pre-soaked)

Blot cassette anode (red)

The stacked cassette was inserted into a Criterion^{Tm} Blotter (Bio-Rad, Munich, Germany) and filled with Western Blot buffer. The proteins were transferred for 30 minutes at an amperage of 1 A.

3.3.2.2 Immunodetection

- **TBS**
  20 mM Tris / HCl pH 7.5
  500 mM NaCl
- **TTBS**
  0.5 ml Tween 20
  add 1 l TBS
- **Blocking solution**
  5 % skim milk powder
  Dissolved in TTBS
- **Antibody solution:**

  Diluted 1:100 – 1:10.000 (depending on the antibody) in blocking solution

ECL – Solution (chemiluminescence solution)

- **Solution A (for immunodetection)**
  200 ml 0.1 M Tris / HCl pH 8.6
  50 mg luminal

- **Solution B (for immunodetection)**
  11 mg p-coumaric acid
  Dissolved in 10 ml DMSO

- **H_2O_2 (30 %)**

After transferring the proteins to the nitrocellulose membrane, the blocking solution was added to prevent unspecific binding of antibodies. The membrane was incubated in the blocking solution at 4 °C over night with shaking. Then, the membrane was washed with TTBS and incubated with 5 – 10 ml antibody solution for 2 h at room temperature with shaking. After hybridization, the membrane was washed three times for 10 minutes with TTBS. After that, the membrane was incubated with the second antibody (linked with HPR) for 1 to 2 hours. The following wash steps were repeated three times, 10 minutes each, first with TTBS and then with TBS. The detection of the peroxidase is based on a chemiluminescence reaction. Therefore, the membrane was moved onto a glass plate, covered with the chemiluminescence solution (2 ml Solution A, 0.6 µl H_2O_2 (30%) and 200 µl Solution B) and incubated for 2 minutes. Afterwards, the membrane was wrapped in plastic foil, an x - ray film was laid on top and was exposed for 2 minutes to a few hours (depending on the strength of the reaction) and developed.

3.3.3 Immuno-detection of Dot-blot

For Dot-blot, the protein extract from plants was prepared as described under 3.4.3.2. From this protein extract, 2 µl of the extract was pipetted directly onto the Nitrocellulose membrane porablot NCP (pore size 0.45 µm; Macherey-Nagel, Duren). Each sample was loaded in a specific distance to avoid sample mixing. After drying shortly, the membrane was directly incubated in blocking solution overnight at 4 °C (See 3.3.2.2). Antibody detection was done as described before in 3.3.2.2.

3.4 Plant Work

3.4.1 Plant production and growth in greenhouse

Production of non-transgenic tomato and tobacco plants were carried out under green house conditions with 16h/day light and the temperature between 28 °C day and 22 °C day. The seeds were sown in moist soil type P, Register number 05216 (Gebr. Patzer GmbH, Sinntal-Jossa) in small trays and were incubated with light for germination. Tomato seeds were sown in soil a little deeper. After germination, the small seedlings were transfered in smaller pots in autoclaved soil.

3.4.2 Isolation of Plant genomic DNA

The genomic DNA, from plants, was isolated according to the protocol from Murray und Thompson (1980).

- DNA-Extractions buffer
  350 mM Sorbitol
  100 mM Tris
  5 mM EDTA
  pH 7.5

- Cell-Lysis-buffer
  200 mM Tris
  50 mM EDTA
  2 M NaCl
  2 % Hexadecyl-trimethylammoniumbromid (CTAB)

- Microprep-buffer
  2.5 % DNA-Extractionsbuffer
  2.5 % cell-Lysis-buffer
  1% of 5 % N-Lauroylsarkosin (= Sarkosyl) (w/v)

3 g/l sodiumbisulfite

The Microprep buffer was fresh prepared, directly before use.

50-100 mg plant material was placed in a 1.5 ml Eppendorf. 200 µl of fresh microprep buffer was added in the Eppendorf tube containing plant sample. Plant sample was homogenized with the help of a plastic pistil. After homogenization, 550 µl of micro-prep buffer was added to the sample and vortexed slightly. Sample was incubated for 30 minutes at 65 °C in a water bath with occasional vortexing. After incubation, 750 µl Chloroform: Isoamylalcohol (24:1) were added to the homogenized sample and centrifuged for 5 minutes at 10000 x *g* at 4 °C. Upper water phase was transferred in a new reaction tube and was mixed with equal volume of cold isopropanol, until a DNA precipitate became visible. Then, samples were immediately centrifuged for 5 minutes at 10000 x *g* and 4 °C. DNA pellet was washed with 70 % Ethanol and air dried. The dried DNA pellet was dissolved in 50 µl of 1x TE and incubated at 65 °C for 15 minutes for better resuspension. Plant DNA was stored at – 20 °C and 1 µl of the genomic DNA was used for a standard-PCR reaction (See 3.2.1).

3.4.3 Isolation of proteins from plant leaves

- Plant extractionsbuffer

  100 mM Tris / HCl pH 7,5

  10 mM KCl

  5 mM $MgCl_2$

  400 mM Saccharose

  10 % Glycerin

  1 % Triton X-100

  0.7 µl/ml β-Mercaptoethanol

50-200 mg leaf material was placed in a 1.5 ml reaction tube. 4 ml of plant extraction buffer was added per gram of leaf material and homogenized on ice with a plastic pistil. Homogenized samples were centrifuged for 10 minutes at 11000 x *g* and 4 °C to remove the cell debris. Supernatant was transferred to a new reaction tube. For protein analysis, raw plant extract was loaded on SDS-PAGE (See 3.3). Proteins were analyzed by either staining with Coomassie staining solution (See 3.3.1) or immune-detected with the respective antibody (See 3.3.2).

3.4.4 Inoculation of plants with *Tomato mosaic virus* and isolation of virus particles from plants

3.4.4.1 Inoculation of plants with *Tomato mosaic virus*

The Tomato Mosaic Virus (ToMV) is a mechanically inoculated virus. It means that ToMV virus has ability to enter in the plant from injured epidermis cells. So, it is possible to rub the virus suspension on the leaf surface with the help of fine powdery substance “Carborandum” that damages the upper surface of leaf to inoculate the plant.

For systemic or HR-producing infection in tobacco or tomato plants, 50 µl of each 1:100 or 1:10 dilution of virus containing plant extract in 10 mM Tris HCl pH 7.5 /100 mM NaCl was spread on young leaves of four weeks old tomato plants or eight weeks old tobacco plants. 500 ng of the virus was used to infect, 3-4 leaves of a plant.

3.4.4.2 Isolation and detection of the virus particles from inoculated plants

From tomato or tobacco plants, infected with ToMV, samples were collected from very young upper leaves after two to three weeks of inoculation. Leaf samples were homogenized in homogenization buffer (Plant-extractionsbuffer or 10 mM Tris pH 7.5 / 1 mM β-Mercaptoethanol). One gram of leaf sample was crushed in 4 ml of the buffer. After homogenization, the samples were centrifuged for 10 minutes at 11000 x g and supernatant was collected in a new reaction tube. For the detection of the virus particles, virus extract was loaded on SDS-PAGE (See 3.3) and immune-detected with ToMV coat protein antibody (See 2.7.1 and 3.3.2).

Extract from the systemically infected plants can be used for the inoculation of new plants. For this purpose, a large amount of leaf material was homogenized with 10 mM Tris/ HCl pH 7.5 (2 ml per Gram of leaf material). The homogenate was filtered through four layers of cheese cloth and was centrifuged for 10 minutes at 11000 x g and 4 °C. Supernatant was incubated at 55 °C for 10 minutes and centrifuged again at 11000 x g and 4 °C for 10 minutes to precipitate the proteins. Supernatant was then sterile filtered with 0.45 µm Syringe Filter (Nalgene, Hamburg) and stored at 4 °C.

3.4.5 Transient protein-expression in plants through *Agro-infiltration*

Agro-infiltration in plants, for the transient expression of proteins, was done according to the protocol from Morilla *et al.,* 2007. *Agrobacterium* strain LBA4404 containing a binary vector with the desired gene for expression, from the frozen glycerol stocks, was incubated in 5 ml LB medium containing Rifampicin and other desirable antibiotics. Inoculated medium was incubated at 30 °C for two to three days with vigorous shaking until the OD_{600} reached 0.5-1. Afterwards, the cells were centrifuged for 5 minutes at 5000 x g and 4 °C. The pelleted cells were suspended in 10 mM $MgCl_2$ and 50 µM Acetosyringone for the induction of *vir* genes. This cell suspension was incubated for 2 hours at room temperature in the dark. Afterwards, these cells were infiltrated on the lower side of leaves of 5-6 week old tobacco plants with the help of 2 ml Omnifix® syringe (Braun, Melsungen). Leaf samples were collected for the protein expression 3-5 days after infiltration.

3.4.6 Analysis of Viral RNA

RNA is a very unstable molecule and is susceptible to the RNases. For this reason, every solution used for RNA isolation was prepared with DEPC treated H_2O. Plastic wear and tips were autoclaved to destroy the RNases.

- DEPC-H_2O
  0.1 % Diethyl Pyrocarbonat (DEPC)
  in Herco H_2O

Overnight incubation at 37 °C and autoclaved

3.4.6.1 RNA Isolation from ToMV

For the isolation of RNA from viral particles, 100 µl of the virus isolate (See 3.4.4.2) was mixed with 10 µl of 10% SDS solution and 10 µl of solution III. After slight mixing, 50 µl of the Phenol/Chloroform (1:1) was added immediately. Samples were vortexed and centrifuged shortly at 4 °C. Supernatants were transferred to new Eppendorf tubes. 200 µl of 96 % ethanol p.a. was added to the samples for the precipitation of viral RNA and incubated at - 20 °C for 30 minutes. After incubation, samples were centrifuged at 15000 x g for 10 minutes. RNA pellet was washed with 70 % ethanol and air dried. 20 µl of DEPC treated H_2O was added to suspend the RNA pellet.

3.4.6.2 Reverse transcription of viral RNA

For the analysis of viral RNA, cDNA was synthesized with the help of a specific primer that can bind to viral RNA specifically. Reverse transcriptase was used to amplify the double stranded DNA. This Hybrid RNA/DNA was used in a standard PCR reaction for amplification of viral genes.

- RT-Reaction (pipetted on ice):

  1 µl RNA

  1 µl 10 µM ToMV specific Primer

  10.5 µl nuclease free Water

  Incubation for 5 minutes at 70 °C, and immediate cooling on Ice.

  4 µl 5x RT-Reaction buffer

  2 µl 10 mM dNTPs

  0.5 µl RiboLock™ Ribonuclease Inhibitor (MBI Fermentas, #EO0381)

  5 minutes at 25 °C incubation,

1 µl RevertAid^{TM} M-MuLV *Reverse Transcriptase* (MBI Fermentas, #EP0041)

The complete RT reaction mixture was first incubated for 10 minutes at 25 °C and then for 60 minutes at 42 °C. The reaction was stopped by heating the samples at 70 °C for 10 minutes. For the amplification of viral genes, 1 µl of RT reaction was used in a standard PCR (See 3.2.1) with specific primers.

3.4.7 Fluorescence Microscopy

Plants were screened for GFP expression under the Axiophot fluorescence microscope (ZEISS, Oberkochen, DE; with filter G 365; FT 395; LP 420). Macroscopic detection of GFP fluorescence was performed by using a hand-held, long-wave UV lamp (4 W) and photographed with a Canon Powershot 1 with yellow filter. Images were processed using Paint Shop Pro.

3.5 Molecular biological methods with Yeast (*S. cerevisiae*)

3.5.1 Cultivation of yeast cultures

The yeast cultures were incubated at 30° C, whereas liquid cultures were incubated at 30° C and 250 rpm. The yeast strain CenPK is auxothrophic for essential components of their metabolism, such as adenine, histidine, leucine or tryptophan. On minimal medium without these amino acids CenPK is not able to grow. Through the transformation of plasmids carrying the genes to produce these components, the yeast cells expressing these are able to grow. This enables a selection similar to the antibiotic selection in *E.coli*.

Media and buffers

- **YPAD medium**

  40 g peptone

  20 g yeast extract

  40 mg adenine sulfate

  add 950 ml H_2O

  pH adjusted to 5.8 with H_2SO_4 and autoclaved

  50 ml 40 % glucose (sterile filtrated) was added

  for agar plates + 1.5 % agar added before autoclaving (about 3.75g in 250 ml)

- **SD medium**

  1.7 g yeast nitrogen base without amino acids

  5 g ammonium sulfate

  add 850 ml H_2O

  pH adjusted to 5.9 with NaOH and autoclaved

  50 ml 40 % glucose and 10 ml 100x Drop in solution were later added

  For agar plates 15 – 20 g agar were added before autoclaving

- **40 % glucose solution**

  20 g D-(+)-glucose

  add 50 ml H_2O

  The solution was sterile filtrated with a Syringe filter 0.2 μm

- **100 x Drop – in solutions**

L – adenine (A)	2000 mg/l
L – histidine / HCl monohydrate (H)	2000 mg/l

L – leucine (L)	10000 mg/l
L – lysine / HCl (K)	3000 mg/l
L – methionine (M)	2000 mg/l
L – tryptophan (T)	2000 mg/l
L – uracil	2000 mg/l

3.5.2 Preparation of competent *S. cerevisiae*:

- **Solution A (for yeast transformation)**

10 mM Bicine (N,N – Bis(2 – hydroxyethyl)glycine)

1 M Sorbitol

3 % Ethylenglycol (v/v)

pH 8.35

50 ml YPAD were inoculated with several colonies of CenPK taken from a fresh plate and grown overnight at 30 °C with shaking, till the OD_{546} reached 0.6 – 0.8. The cells were centrifuged at 2500 g for 5 minutes and resuspended in 2.5 ml solution A. Then 100 µl aliquots were made and stepwise frozen at -70 °C. The cells are storable for several months without loss of competence.

3.5.3 Transformation of yeast described by Dohmen et *al.* (1991)

For the transformation of competent yeast cells they were mixed with 0.1 – 1 µg Plasmid – DNA and 50 µg salmon sperm DNA (10 mg/ml) and incubated for 5 minutes at 37 °C and 150 rpm. Then 1 ml solution B was added and incubated for 1 h at 30 °C and 250 rpm. Afterwards the cells were harvested through centrifugation at 12000 x g for 30 seconds. The pellet is washed with 800 µl solution C and centrifuged at 12000 x g for

30 seconds. The pellet is resuspended in 200 µl solution C and 100 µl plated on SD/Drop-in plates. After approximately 4 to 5 days the colonies could be used for other experiments.

- **Salmon Sperm DNA (single stranded DNA)**

  10 mg/ml in TE buffer

The solution was treated with ultrasound (breaking DNA into fragments) allowing easier pipetting. Then it was boiled for 10 minutes and immediately transferred to ice and cooled down.

- **Solution B (for yeast transformation)**

  200 mM Bicine
  40 % Polyethylenglycol 1000 (PEG1000) (w/v)
  pH 8.35

- **Solution C (for yeast transformation)**

  10 mM Bicine
  150 mM NaCl
  pH 8.35
  The pH was adjusted with KOH.

For the transformation of competent yeast cells 0.1-1 µg of the plasmid DNA was mixed with 50 µg of the Salmon sperm DNA and added to the frozen aliquot of competent yeast cells. After that the cells were thawed at 37 °C for three minutes. Soon after the incubation, 1 ml of the solution B was added in the yeast cells, mixed shortly and incubated at 30 °C for 1 hour. The cells were pelleted after incubation at 12000 x g for 30 seconds and were washed with 800 µl of Solution C. After another centrifugation step, the cells were suspended in 100 µl of Solution C and spread on the SDS/Drop-in

plate (See 3.5.1). The colonies were visible after two days and were used for further experiments.

3.5.4 Plasmid preparation from yeast cells

- SCE-buffer
  1.2 M Sorbit
  100 mM Na-Citrate
  10 mM EDTA
  pH 7.0

- SCE/Lyticase-Solution
  10 µl Lyticase (2 U/µl)
  8 µl β-Mercaptoethanol
  Add 1 ml SCE-buffer

- SDS-solution
  2 % SDS
  50 mM Tris / HCl pH 8.0
  10 mM EDTA

For the plasmid isolation from yeast cells, 5 ml of a yeast culture was centrifuged for 5 minutes at 5000 x g. The yeast pellet was washed with 1 ml of H_2O and pelleted again.

The pellet was suspended in 200 µl of SCE/Lyticase solution and incubated at 37 °C for 40 minutes with shaking. Then 200 µl of the SDS solution was added and incubated at room temperature for 10 minutes. After the addition of 100 µl of 5 M potassium acetate, samples were incubated for 30 minutes at 4 °C.

Precipitated proteins and the genomic DNA were isolated by centrifugation at 1000 x g for 10 minutes. The supernatant was transferred to new tubes and with 400 µl of isopropanol. The samples were centrifuged again as before to pellet the DNA. Then the DNA pellet was air dried and dissolved in 50 µl of 1xTE.

3.5.4.1 Verification of yeast clones

Mini-prep DNA isolated from yeast was diluted to 1:50. The diluted DNA was transformed in *E.coli* (See 3.1.2). The plasmid DNA was isolated from the transformed *E.coli* colonies and sent for the sequencing to the company Eurofinsdna. Sequencing samples were prepared according to the instructions from the company.

3.5.5 Protein isolation from yeast cells

5 ml of the yeast culture was used to isolate the protein for immune-detection. Yeast cells were pelleted by centrifugation at 5000 x g for 5 minutes in Eppendorf tubes. The pellet was washed with H_2O and centrifuged again as before. Then the yeast pellet was suspended in 100 µl of 1x SDS loading buffer and cooked for 5 minutes at 95 °C. The samples were centrifuged for 5 minutes at 5000 x g to precipitate the cell debris. The supernatant containing the proteins was transferred to a new reaction tube and analyzed via SDS gel (See 3.3).

3.6 Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) is a new technique to study protein-protein interactions. It has been used for inter and intra molecular protein interactions (Hu and Kerppola, 2003; Hu *et al.,* 2002). In this technique two non-fluorescent parts of GFP (green fluorescent protein) or other derivates of GFP (Yellow fluorescent protein-YFP; Red fluorescent protein-RFP) were fused either with the putative interacting proteins or with interacting domains of one protein.

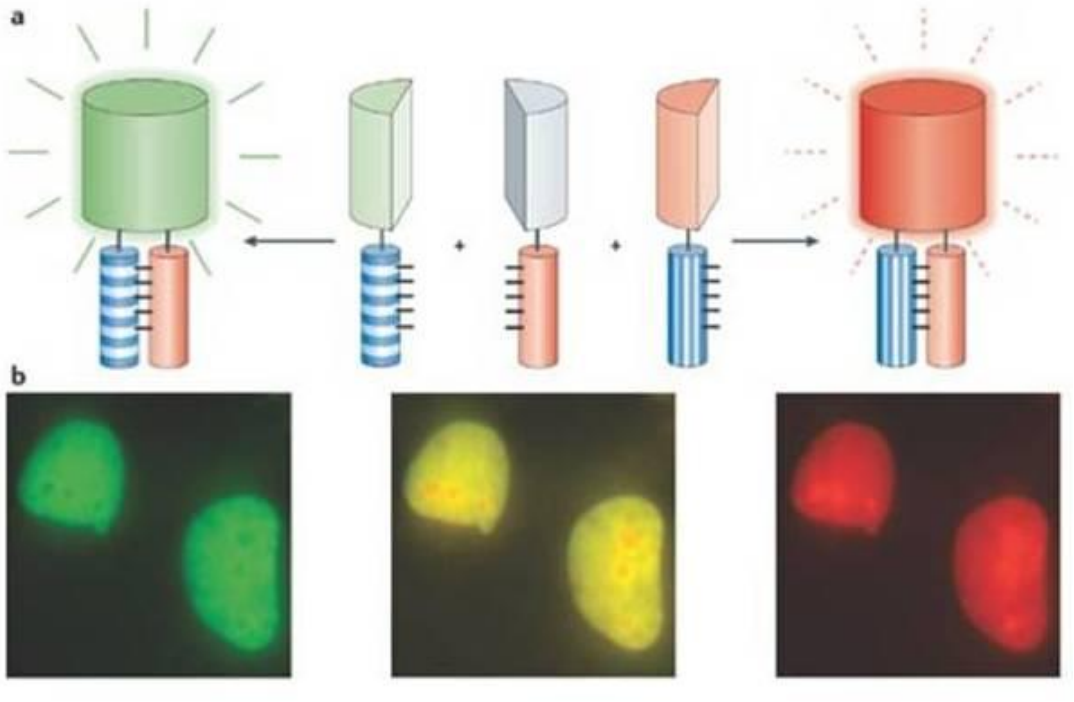


Figure 20: a | Multicolour bimolecular fluorescence complementation (BiFC) analysis is based on the formation of bimolecular fluorescent complexes with different spectra through interactions between proteins that are fused to different fluorescent-protein fragments. These complexes can be independently visualized by using different excitation and emission wavelengths. **b** | These three panels show multicolour BiFC analysis of two different complexes in the same cells. The images on the left and right were acquired using different excitation and emission wavelengths (shown in false colours), whereas the image in the center is an overlay of these images (Kerppola, 2006).

Upon expression of these proteins in vivo, the interacting proteins come close to each other, thus bringing the two parts of GFP together to reassemble and emit fluorescence (Fig. 20).

BiFC has several advantages over other techniques used for studying protein interactions. These include the absence of background signal, high specificity and high stability of the reconstituted chromophore complexes (Hu and Kerppola, 2003; Hu *et al.,* 2002). This process can be carried out in living cells and provides information on both the interaction and its sub cellular localization. The split GFP fragments are relatively small and therefore unlikely to interfere with protein complex formation.

4. Results

4.1 Structural analysis of ToMV 30kDa movement protein in yeast and plants

Bimolecular fluorescence complementation (BiFC) or the Split GFP is a technique for studying protein-protein interactions in eukaryotes, since 2002, when Kerppola and colleagues demonstrated the utilization of this system for analyzing protein interactions in mammalian tissue culture cells. BiFC/split GFP system use two non-fluorescent parts of GFP or its derivatives fused with two different proteins, which are supposed to interact with each other. During the interaction of fused proteins, the non-fluorescent fragments of GFP come together in close proximity and make up the fluorescent molecule. Bracha-Dori *et al.,* 2004, has demonstrated the usefulness of this system also in tissues and at sub-cellular level in plants. Advantages of split GFP system over other techniques are high stability, high specificity and absence of background signals. Interactions between α and β subunit of Arabidopsis protein farnesyltransfrease (PFT) has been demonstrated using split YFP system.

Split GFP system has also been utilized for the analysis of protein structures and localization of various protein domains in the cell (Zamyatinin *et al.,* 2006). Zamyatinin and colleagues have proved the usefulness of this system for analysis of viral protein structures and localization of different protein domains in *N. benthamiana* cells.

Based on successful incorporation of split GFP system for the assessment of membrane associated protein topology, we have developed a system based on GFP protein splitted in two non-overlapping fragments of GFP spliced at aa 156 as shown in 2.3.5. For analyzing the structure of ToMV 30kDa MP, different deletion mutants were fused with split GFP vector. Deletion mutants of ToMV 30kDa MP were designed in such a way that part of MP, containing *Tm-2* and *Tm-2*^{2} resistance breaking mutations at aa position 131 and 133, was exposed for influencing the localization of MP and reconstruction of fluorescent molecule (Fig 21). Experiments were performed both in yeast (*S. cerviceae*) and plants (*N. benthamiana*) to prove the biological

relevance of our hypothesized model in a eukaryotic model system as well as in host plants.

4.1.1 Construction of pyEGFP vector

For the structural analysis of 30kDa MP in yeast using split GFP system, a vector containing yEGFP (yeast enhanced GFP) was prepared as shown in figure 21. The vector was prepared and kindly provided by Dr. U. Pfitzner, Department of general virology, University of Hohenheim, for this study (See 2.3.5). pyEGFP contains nYEGFP and cYEGFP spliced with the incorporation of restriction enzymes for further cloning of ToMV movement protein and its deletion mutants.

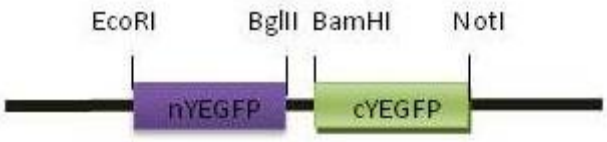
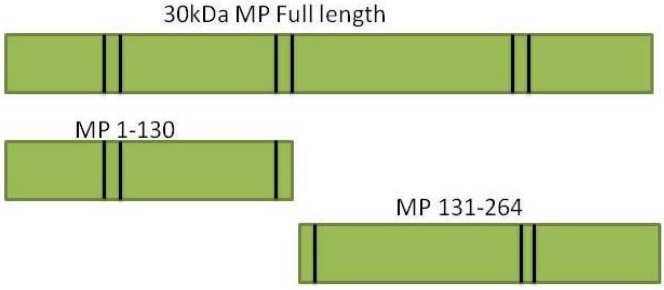


Figure 21. Schematic diagram of pyEGFP vector with restriction sites.

4.1.2 PCR amplification of 30kDa MP and its deletion mutants

For the amplification of tomato mosaic virus 30kDa MP and its deletion mutants, sequence specific primers based on the movement protein sequence were used in a standard PCR reaction (See 3.2.1). Three different fragments of 30kDa MP were amplified, named as 30kDa MP full length (30kDa MP FL), MP1-130 (30kDa MP deletion mutant ranging from aa 1 to 130) and MP131-264 (30kDa MP deletion mutant ranging from aa 131-264) as shown in figure 22A. Suitable restriction sites for the cloning in pyEGFP were introduced in the amplified products on the both ends through PCR primers.



igure 22A. Schematic diagram of ToMV movement protein and its deletion mutants. Black bars represent the amino acid exchanges responsible for resistance breaking phenotype.

As a positive control, TGB2 gene of potato virus X (PVX) was amplified. After the completion of the PCR reaction, 5 µl of the PCR products were mixed with 6x orange loading dye and loaded on the 1 % agarose gel for the analysis of amplified products (See 3.1.9). Samples, loaded on agarose gel, were run for 45 minutes at 70 Volts and exposed to UV. Figure 22B shows the result of PCR where as the expected fragment sizes of amplified products are described in Table 3. Amplified PCR fragments were isolated from agarose gel and cleaned with gel elution kit (See 2.4.2 & 3.1.10). The eluted PCR fragments were cloned in T-vector (See 2.3.2) and sequenced before further cloning (See 3.2.4).

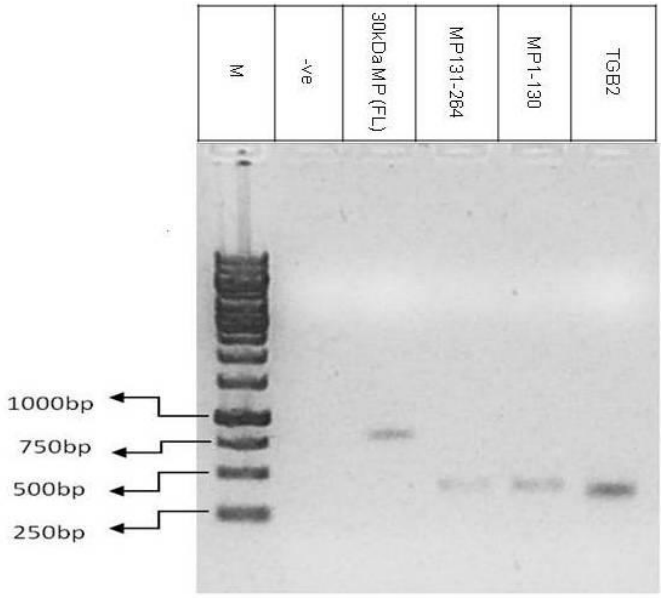


Figure 22B. PCR amplification of 30kDa MP, MP1-130, MP131-264 and TGB2 fragments. **Lane 1**. 1kb ladder, **lane 2**. H_2O control, **lane 3**. 30kDa MP (full length), **lane 4**. MP131-264, **lane 5**. MP1-130 and **lane 6**. TGB2.

Name of amplified fragment	Size in bp
30kDa MP (FL)	784
MP 1-130	384
MP131-264	400
TGB2	343

Table 3. Expected fragment sizes of PCR amplified products.

4.1.3 Cloning of 30kDa MP and its deletion mutants in pyEGFP

Sequences of amplified fragments were analyzed before further cloning to avoid any mismatches. For cloning of 30kDa MP, its deletion mutants and TGB2 fragments in pyEGFP vector, the vector DNA was restricted overnight with BamHI and dephosphorylated with Calf intestinal alkaline phosphatase (CIAP) according to the supplier's instructions. Restricted DNA was loaded on 1 % agarose gel and run for 30 minutes to separate any uncut DNA from restricted DNA. Desired fragment was isolated from the gel and cleaned with the gel elution kit (See 3.1.10) for the ligation of different inserts.

For the cloning of 30kDa MP (full length) and TGB2 fragments in pyEGFP, 30kDa MP and TGB2 fragments in the T-vector were restricted with BamHI and BglII overnight. The restricted BamHI and BglII fragments (TGB2 and 30kDa MP) were separated on 1 % agarose gel and were cleaned with a gel elution kit. These fragments were further ligated in a 3:1 insert to vector ratio of BamHI restricted pyEGFP vector overnight at 4 ºC. Next day the ligation reactions were transformed in DH5α competent cells and selected with Ampicilline for transformed colonies.

For the construction of pyEGFP-MP1-130-N-131-264-C, first MP131-264 was cloned in pyEGFP with BamHI and BglII sites. A positive clone was used further for the insertion of MP1-130 with the help of the EcoRI and MfeI restriction sites present in the MP1-130 amplified fragment. For further analysis of positive clones, five colonies per ligation reaction were grown in liquid medium for plasmid isolation. The isolated plasmid DNA was restricted with NotI and EcoRI for the confirmation of cloned fragments in pyEGFP. Figure 23 shows the restriction analysis of positive clones. NotI/EcoRI restriction results in the isolation of vector backbone (~3Kb) and the yEGFP fusions of amplified fragments. Schematic organization of final yEGFP fused 30kDa MP and its deletion mutants have been shown in figure 24.

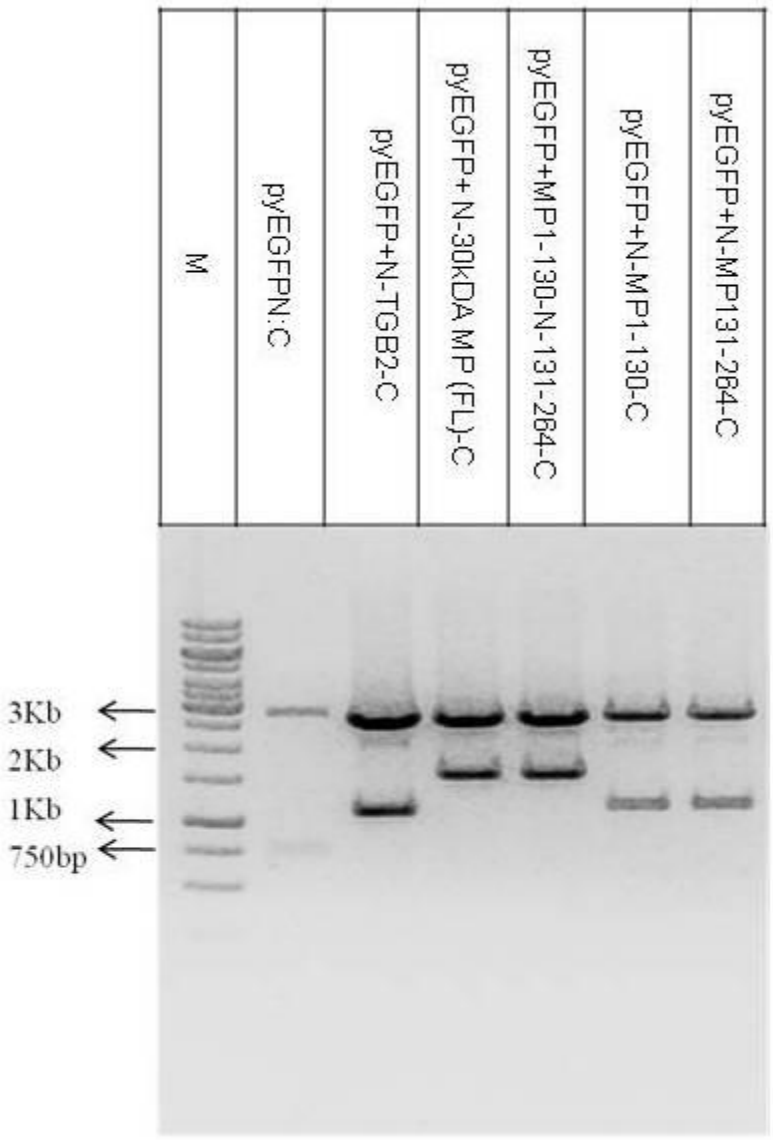


Figure 23. Restriction analysis of pyEGFP constructs with NotI and EcoRI. **Lane 1**. 1Kb ladder, **lane 2**. pyEGFP N:Cvector, **lane 3**. pyEGFP +N-TGB2-C, **lane 4**. pyEGFP+ N-30kDa MP(FL)-C, **lane 5**. pyEGFP+MP1-130-N-131-264-C, **lane 6**. pyEGFP+N-MP1-130-C, **lane 7**. pyEGFP+N-MP131-264-C.

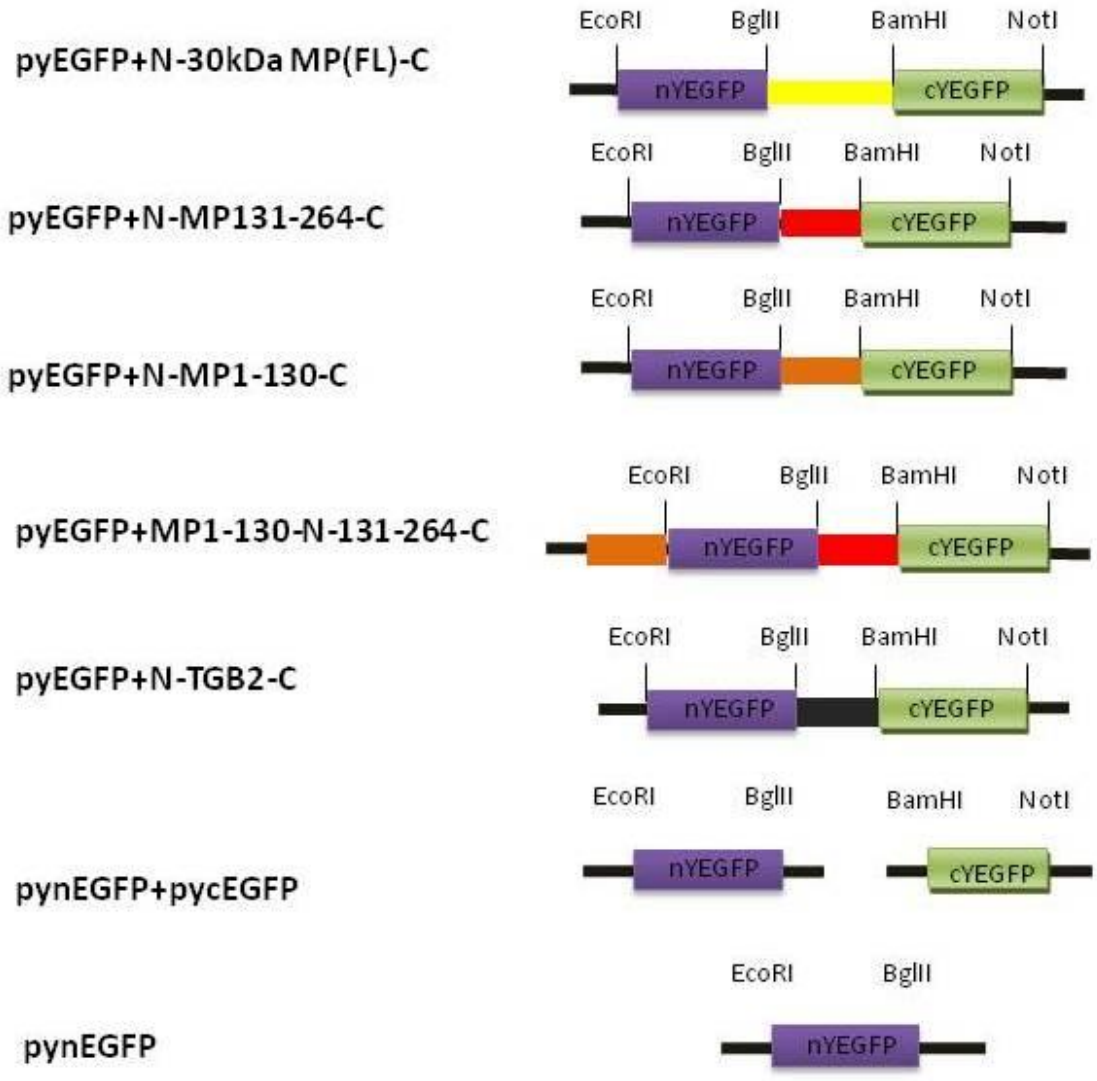


Figure 24. Diagrammatic representation of pyEGFP clones containing the fused 30kDa MP and its deletion mutants. Yellow colour represents the full length 30kDa MP, Red Colour represents the MP131-264, Orange colour represents the MP1-130 and the TGB2 fragment is represented with black colour. As a control, the pynGFP vector was prepared that contains only the N-terminus of the yEGFP. Both parts of the split yEGFP were cloned in two different vectors to see the possible reconstruction of yEGFP without interacting proteins.

4.1.4 Cloning of GFP fused 30kDa MP and deletion mutants in yeast expression vector

yEGFP fused 30kDa MP and its deletion mutants were further cloned in the pESCura vector (See 2.3.6) for expression in yeast (CenPK). For this purpose,

pyEGFP positive clones from 4.1.3 and yeast expression vector pESCura were restricted with EcoRI and NotI.

Restricted DNA was separated on a 1 % agarose gel, desired fragments for MP fusions with yEGFP and the restricted pESCura vector were purified from agarose gel with the help of gel elution kit. Purified DNA was used for further ligation. The ligation reactions were first transformed in DH5α competent cells by heat shock method (See 3.1.2.2). The transformed colonies were grown in LB medium under antibiotic selection for plasmid isolation.

Isolated plasmid DNA was test restricted with EcoRI and NotI for the screening of positive colonies as shown in figure 25. With this restriction, the cassette containing yEGFP and fused MP inserts pop out from the vector backbone to give different size fragments. After restriction analysis, the plasmid DNA of the positive clones were transformed in (See 3.5.2) in yeast, *Sacchromyce cerviceae* (CenPK), for protein expression.

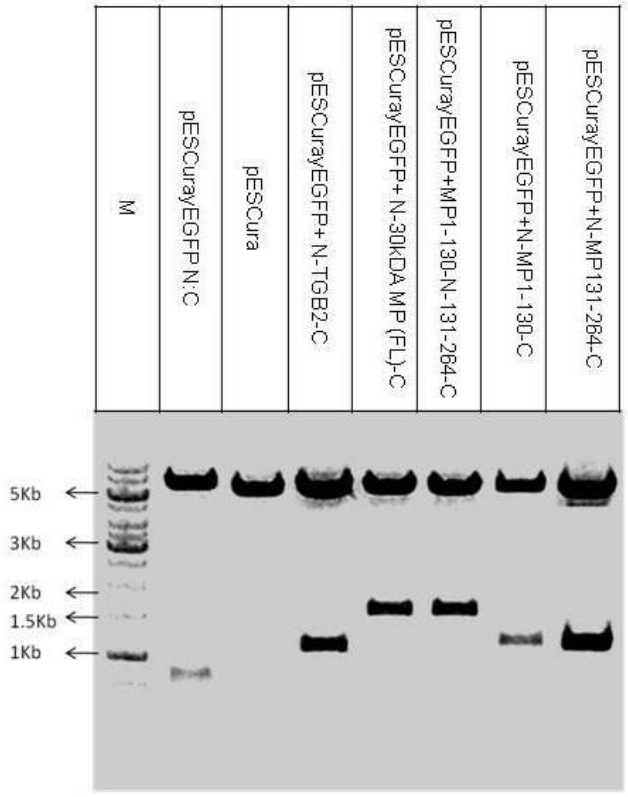


Figure 25. Restriction analysis of pyEGFP MP constructs in pESCura with NotI and EcoRI. **lane 1**. 1Kb DNA ladder, **lane 2**. pESCura yEGFP N:C vector (~750bp), **lane 3**. Empty pESCura Vector (~30bp), **lane 4**. pESCura yEGFP+N-TGB2-C (~1093bp), **lane 5**. pESCura yEGFP+ N-30kDa MP(FL)-C (~1534bp), **lane 6**. pESCura yEGFP+ MP1-130-N-131-264-C (~1534bp), **lane 7**. pESCura yEGFP+ N-MP1-130-C (~1134bp), **lane 8**. pESCura yEGFP+N-MP131-264-C (~1150bp).

4.1.5 Expression of pESCura 30kDa MP and its deletion mutants in yeast

Yeast expression clones containing the yEGFP fused 30kDa MP and its deletion mutants were grown on glucose medium lacking uracil for two days on SD agar plates at 30 ºC. After two days of yeast growth, the single colonies were selected and grown, further, in 5 ml of SD liquid medium with galactose, instead of glucose, for the expression of cloned 30kDa MP and its deletion mutants. Liquid cultures were incubated at 30 ºC and 250 rpm for 48h. Grown yeast cells were pelleted in Eppendorf tubes for protein isolation (See 3.5.2). The extracted protein samples were analysed on SDS gel (See 3.3) and were detected using αGFP antibody. Furthermore, the yeast cells from same cultures were observed under fluorescence microscope for GFP expression and localization of GFP fusion proteins.

By GFP analysis, under the fluorescence microscope using split GFP system, it has been already demonstrated that split halves of GFP, when expressed alone without the fusion of interacting proteins, give no fluorescent signals or very weak signals (Walter *et al.,* 2004). Fusion of GFP halves with interacting proteins bring the two halves together to give rise a fluorescent molecule, that can be observed under microscope. Whereas the fusion of two GFP halves with non-interacting proteins or proteins residing in different cellular compartments inhibit the formation of fluorescent GFP molecule.

Recently, another variant of GFP called ***venus*** has been used to demonstrate the intra-molecule interaction of ICP27, a regulatory protein of Herpes Simplex Virus (Hernandez and Sandri-Goldin, 2010). These studies have shown that the split GFP system can be used successfully to study the protein topology in yeast and plants.

Based on these results, we have used split GFP system for analyzing the topology of ToMV 30kDa movement protein. Various controls have been included in the experiments to avoid any mis-conclusion.

1) The yEGFP expressed in one vector (Fig 26, Slide 1).

   In this case, two parts of the split yEGFP were expressed in one vector and in close proximity to reconstruct the fluorescent molecule.

2) Split yEGFP parts expressed from two different vectors (Fig 26, Slide 2).

   In this case, two parts of yEGFP were cloned in two different vectors and expressed in one cell to observe if the two halves can give fluorescence. N and C halves of yEGFP did not show any fluorescence when expressed from different vectors.

3) N-terminus of yEGFP expressed alone in yeast (Fig 26, slide 3).

   If one half of the yEGFP, NyEGFP was expressed without CyEGFP, no fluorescence signals were observed in our experiments.

4) Expression on pTGB2 in yeast (Fig 26, Slide 4).

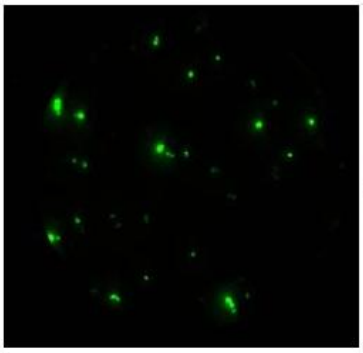
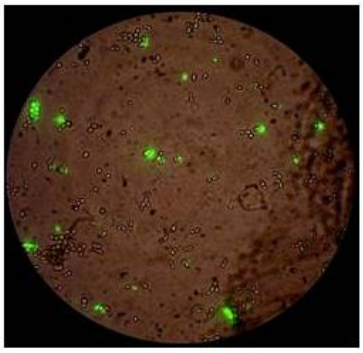
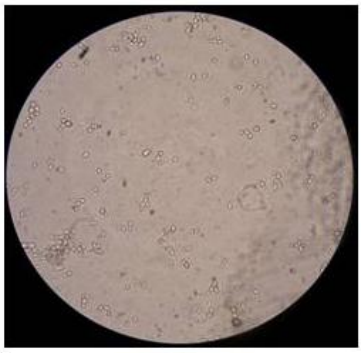
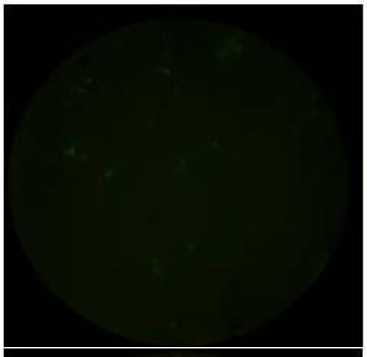
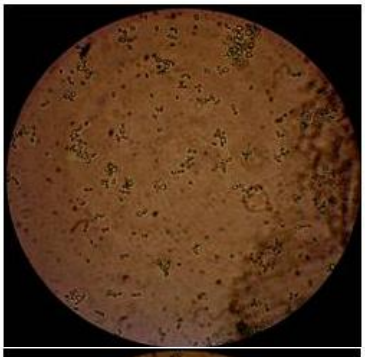
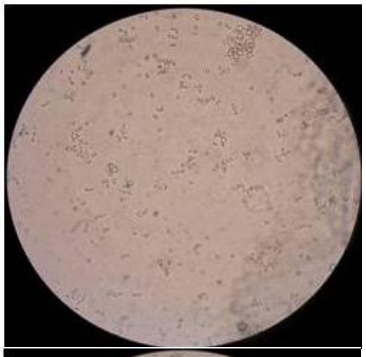
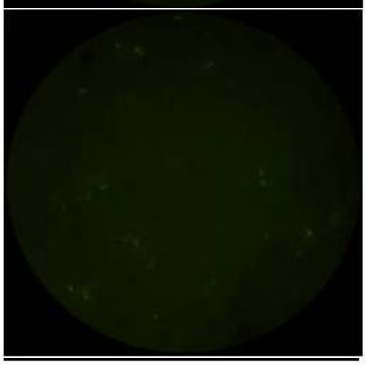
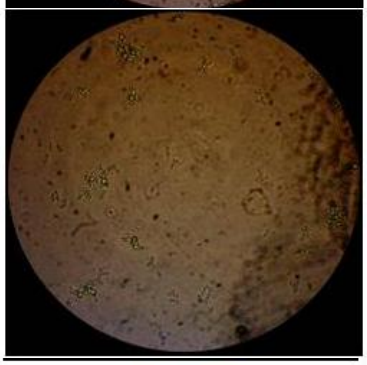
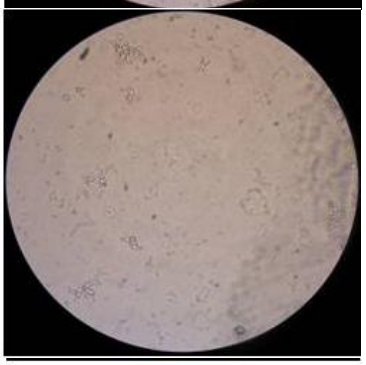
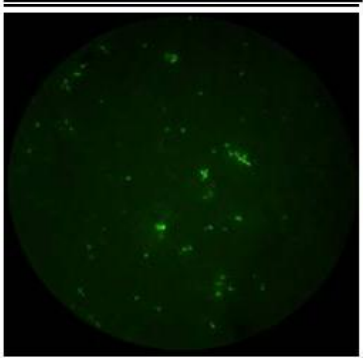
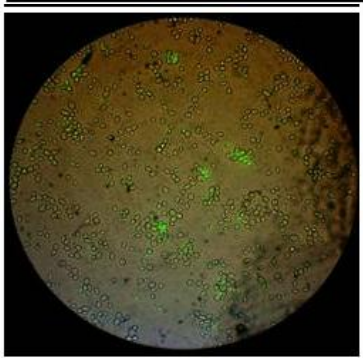
   Triple gene block 2 (TGB2) protein of Potato Virus X (PVX) is an integral membrane protein containing its N and C terminus in the cytoplasm. Zamyatnin *et al.,* in 2006 has used split YFP system, to study the protein topology in plant cells. He used different fusion constructs to show that both N and C terminus of TGB2 are in cytoplasm where as the middle part is present in the ER lumen. During our experiments we have used this protein as a control to prove the functionality of our approach for analysing the topology of membrane proteins. yEGFP fusion with TGB2 showed high level of fluorescence under microscope (Fig 26, Slide 4). In addition, yEGFP fused TGB2 was also detectable on the western blot as a protein band of approx. 39kDa (Fig 27). These results, not only confirm the findings of Zamyatnin and his colleagues, but also showed the feasibility of our system to study membrane associated protein topology.

First construct for the analysis of 30kDa MP structure analysis was the fusion of the full length 30kDa MP with split yEGFP (pESCura yEGFP+N-30kDa MP (FL)-C). Expression of this construct in yeast and analysis under microscope showed a high number of fluorescent cells (Fig 26, Slide 5). This demonstrated that both N and C terminus of 30kDa MP are present at the cytoplasmic side of the cell and in close proximity to build up the fluorescent molecule.

In the next step, we wanted to analyze the middle part of the 30kDa MP. The middle part of the 30kDa MP is significant because all the mutations found in the resistance breaking viruses are found in this part of protein. The placement of this part in the membrane plays an important role on the resistance breaking phenotype of virus. To analyze the localization of this protein part, a second construct was made (Fig 26, Slide 6), whereas deletion mutant of 30kDa MP containing the N terminus (ranging from 1 to 130aa) was fused between the N and C terminus of yEGFP (pESCura yEGFP+ N-MP1-130-C). Expression of this construct in yeast yielded significant number of fluorescent yeast cells. Fluorescent signals indicated that both ends of N terminus deletion mutant (N terminus and the central part of ToMV MP) are embedded in the membrane in such a way that they both are present on one side of the membrane and are close enough to give fluorescent signals. Expressed protein was also detected on a western blot as GFP fusion product of 43kDa (Fig 27).

Expression of the complementary construct containing the 30kDa MP C-terminus (ranging from 131-264aa) fused with split yEGFP also resulted in the significant number of fluorescent yeast cells expressing fused protein (Fig 26, Slide 7), and confirming the proposed model that C-terminus of the 30kDa MP and central part are present in the same cellular compartment

As a final probe, we used a construct where the N-terminus of split yEGFP was inserted at position 130 of 30kDa MP and theC-terminus was fused to the Carboxyl terminus of the MP (MP1-130-N-131-264-C). This fusion construct also showed a yEGFP fluorescence signal under the microscope (Fig 26, Slide 8). The fusion protein showed no significant expression when detected against anti-GFP antibody.

GFP fluorescence (429nm) after excitation at 365nm Magnification 40x	Overlay of Fluorescence and bright field picture	Bright field picture at a magnification at 40x	Name of Expression Construct
			1:pyEGFP N:C
			2:pNyEGFP pCyEGFP
			3:pNyEGFP
			4:pyEGFP N-TGB2-C

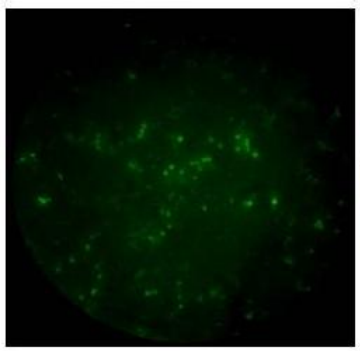
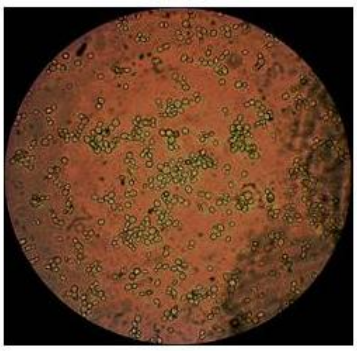
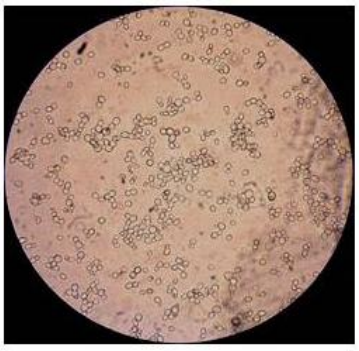
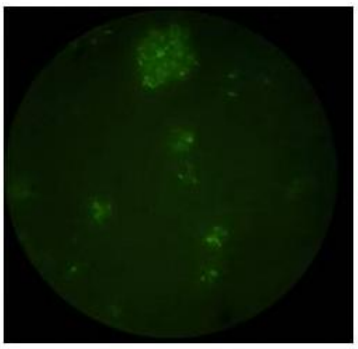
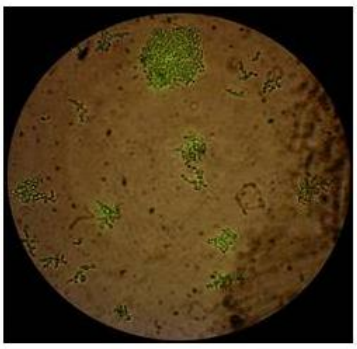
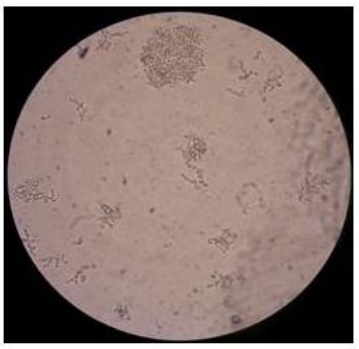
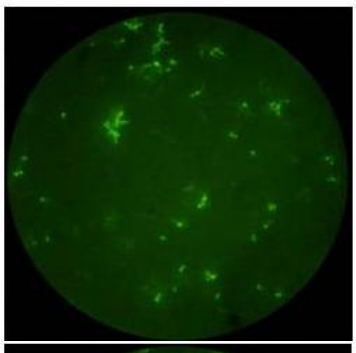
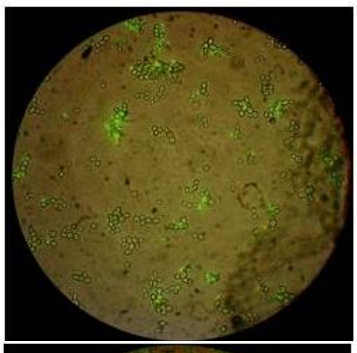
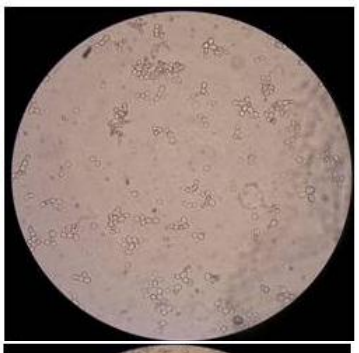
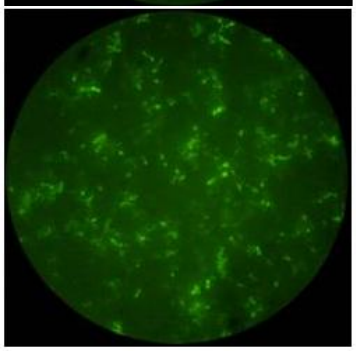
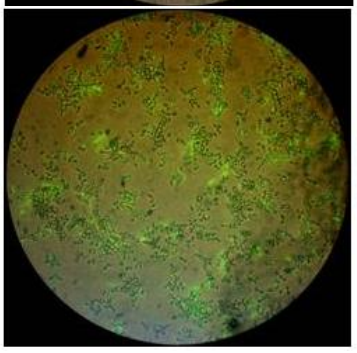
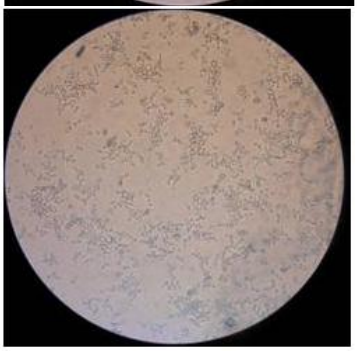
GFP fluorescence (429nm) after excitation at 365 nm Magnificatlon 40x	Overlay of Fluorescence and bright field picture	Bright field picture at a magnification of 40x	Name of Expression Construct
			5:pyEGFP+N-30kDa MP(FL)-C
			6:pyEGFP+N-1-130 MP-C
			7:pyEGFP+N-131-264-C
			8:pyEGFP+1-130-N-131-264-C

Figure 26. Fluorescence microscopy of yeast cells expressing different fusion products.

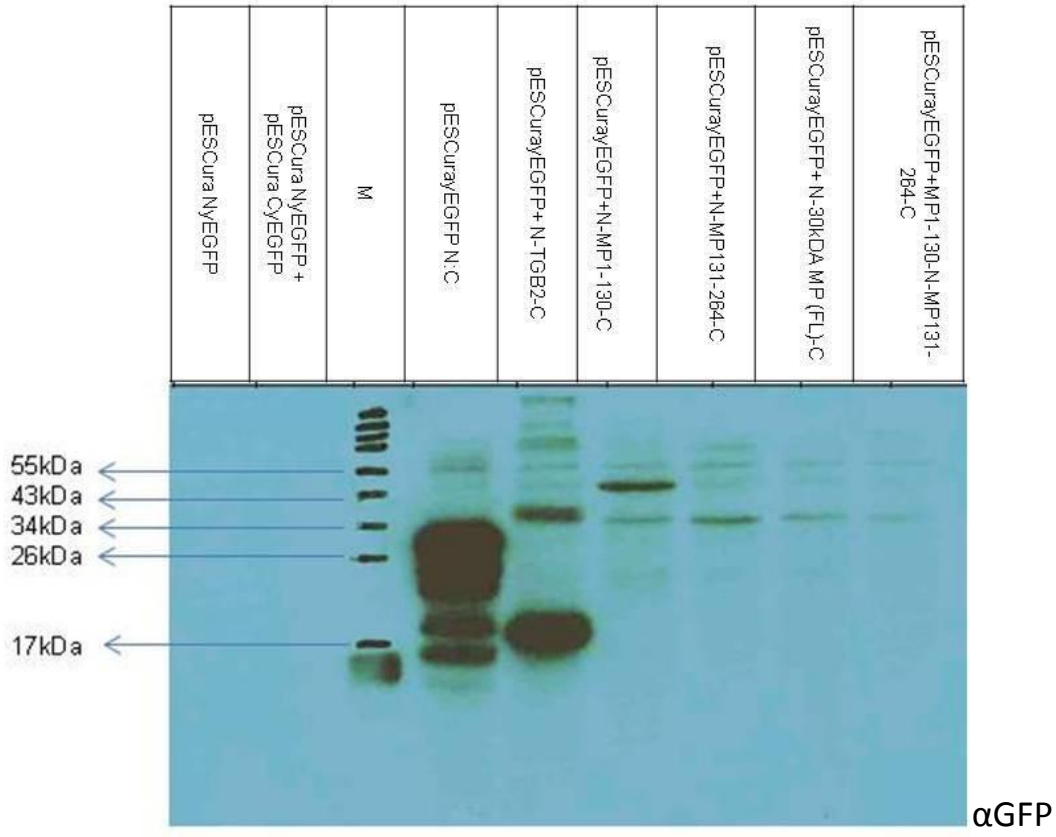


Figure 27. Western blot analysis of Protein extracts from yeast cells expressing different pESCura yEGFP vectors fused with different inserts. αGFP antibody was used for the detection of yEGFP or the yEGFP fused proteins.

4.1.6 Isolation of cytoplasmic and membrane proteins from yeast

GFP fusions of ToMV MP only give information, if parts of this protein lie on same side of the membrane or in one cellular compartment but does not give any information about membrane association of the fusion proteins. Therefore it's essential to demonstrate that the fusions proteins are inserted in the ER membranes. For this purpose, membrane and cytoplasmic protein fractions were isolated after expressing the 30kDa ToMV MP and its deletion mutants in yeast. Yeast pellets obtained from overnight cultures were disrupted in lysis buffer without SDS, with the help of glass beads. 10 mg of the glass beads were added in each tube containing the yeast cells. Pellet was vortexed for 1 minute with incubation for 2 minutes on ice in between to keep

the cells cold. After vortexing the pellet 3 times, cells were centrifuged for 5 minutes at 10.000 x g at 4 ºC to pellet the cell debris and membrane residues. The resulting supernatant was transferred in a new tube and SDS buffer was added in both supernatant and pellet fractions. The both fractions were denatured for 5 minutes at 95 ºC and loaded on 15 % poly-acrylamide gel for protein analysis and antibody detection. Results in figure 28 showed that yEGFP fused 30kDa MP (FL) and its deletion mutants were present in the isolated membrane fractions whereas, free yEGFP, was present in the cytoplasm.

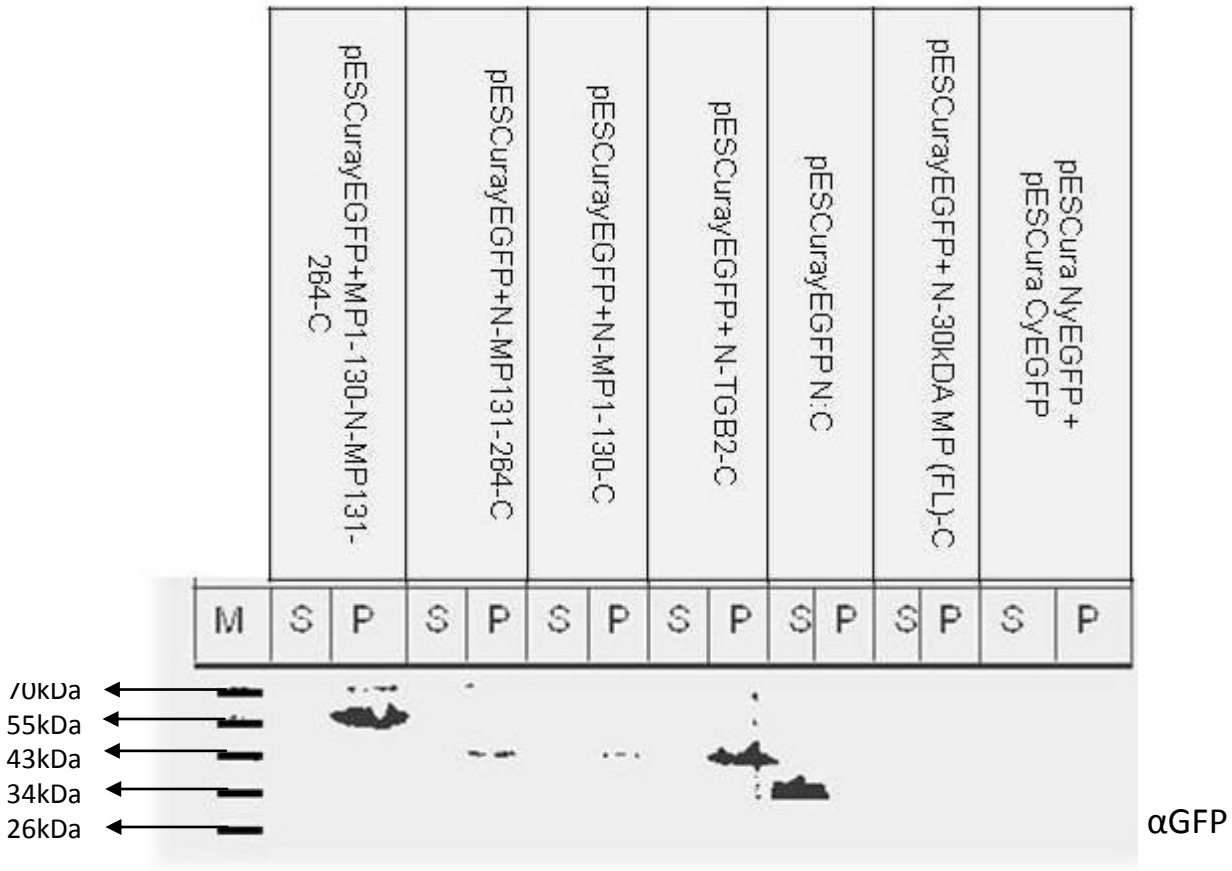


Figure 28. Protein analysis of cytoplasmic and membrane fractions isolated from 30kDa MP and its deletion mutants expressed yeast cells. **Lane 1**. M prestained protein marker SM0671, **lane 2 & 3**. Supernatant and pellet fractions of MP-1-130-N-131-264-C. **lane 4 & 5**. Supernatant and pellet fractions of N- MP131-264-C, **lane 6 & 7**. Supernatant and pellet fractions of N-MP1-130-C, **lane 8 & 9.** Supernatant and pellet fractions of N-TGB2-C, **lane 10 & 11**. Supernatant and pellet fractions of yEGFP N:C, **lane 12 & 13**. Supernatant and pellet fractions of N-30kDa MP(FL)-C, **lane 14 & 15**. Supernatant and pellet fractions of NyEGFP and CyEGFP.

4.2 Structural analysis of ToMV MP in *Nicotiana benthamiana*

Although, expression of MP-split GEP fusions in yeast has given us important information about the folding of ToMV 30kDa MP, major cell components are missing in yeast, as compared to plant cells. In particular, plasmodesmata are major target sites of the MP in plants, which are absent in single cell organisms i.e. yeast. Therefore confirmation of yeast data on the structure of 30kDa MP was done by performing similar experiments in *N. benthamiana*.

4.2.1 Construction of pBSKmGFP4 N:C

To perform these experiments in plants, a plant based split GFP vector was constructed. For this purpose, modified GFP protein was used that is mainly located in cytoplasm when expressed in plant cells named as mGFP4 (Haseloff *et al.,* 1997). The idea behind using this modified GFP was to see any localization effect of GFP when fused with 30kDa MP and its deletions. For the construction of a split GFP vector, mGFP4 was split in two parts, N and C terminus. The N part of mGFP4 consists of 1-157aa where as C part of mGFP4 contains 158-238aa. The N and C terminus parts of mGFP4 were amplified with primers (See 2.8.1) containing restriction sites for site directed cloning in pBSK vector (See 2.3.1).

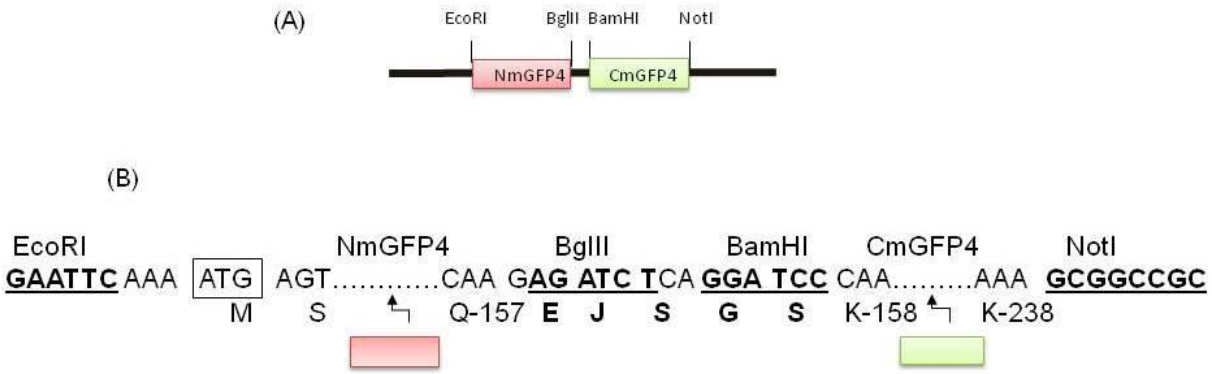


Figure 29. Construction scheme of pBSKmGFP4 vector. **A)** Schematic diagram of pBSKmGFP4 with the restriction sites introduced through PCR. **B)** Nucleotide sequence and the amino acid sequence of the vector showing start codon ATG and addition of nucleotides to avoid frame shift mutations.

4.2.2 Cloning of 30kDa MP and its deletion mutants in pmGFP4 N:C

For the cloning of 30kDa MP fragment and its deletion mutants in pmGFP4 N:C, amplified fragments cloned in the T-vector were restricted with enzymes introduced with the help of PCR primers and cloned in pmGFP4 N:C as described before in 4.1.3. The cloned fragments were confirmed by EcoRI/NotI restriction and agarose gel analysis for their right size and orientation (Fig 30).

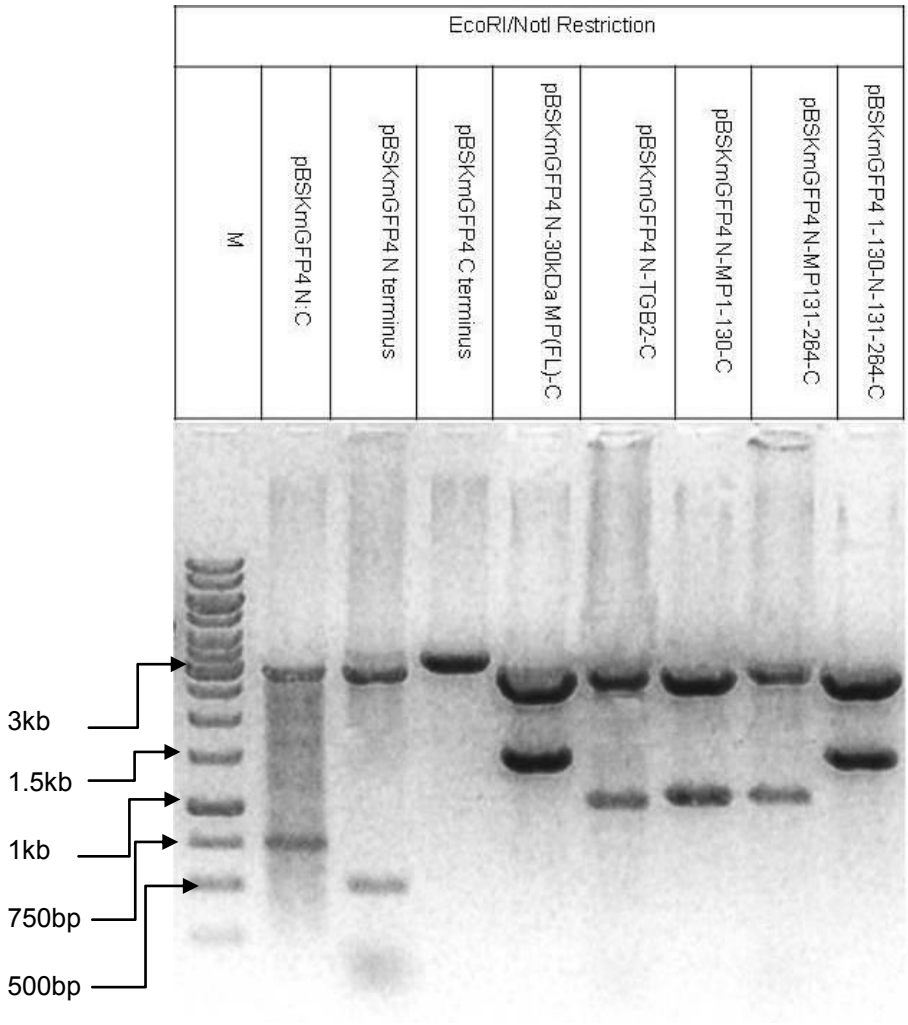


Figure 30. Restriction analysis of cloned 30kDa MP and its deletion mutants in pBSKmGFP4 N:C vector with EcoRI and NotI. **lane 1**. 1kb Ladder, **lane 2**. pBSKmGFP4 N:C vector (~750bp), **lane 3**. pBSKmGFP4 N-terminus (~471bp), **lane 4**. pBSKmGFP4 C-terminus (~270bp), **lane 5**. pBSKmGFP4 N-30kDa MP(FL)-C (~1534bp), **lane 6**. pBSKmGFP4 N-TGB2-C (~1093), **lane 7**. pBSKmGFP4 N-MP1-130-C1(~1134), **lane 8**. pBSKmGFP4 N-MP131-264-C(~1150bp), **lane 9**. pBSKmGFP4 1-130-N-131-264- C (~1534bp).

4.2.3 Cloning of pBSKmGFP4 N:C fused 30kDa MP and its deletion mutants in plant expression vector

For the expression of mGFP4 fused 30kDa MP and its deletion mutants in *N. benthamiana*, whole cassette (mGFP4 fused MP and deletion mutants) was cloned in pBin19 vector (See 2.3.3) under the control of 35S CaMV promoter and nos terminator. For this purpose, pBin19 vector containing 35S promoter and nos terminator (pTAK vector) was restricted with SacI and SmaI overnight at 37 ºC. SacI/SmaI restricted vector DNA was separated on 1 % agarose gel, desired fragment was cut out from the gel and cleaned with gel elution Kit.

pmGFP4 N:C clones with 30kDa MP and its deletion mutants were also restricted with SacI and SmaI and cleaned on the gel. This restrcited DNA was ligated in the pTAK vector (prepared before) in 3:1 insert to vector ratio. The ligation reaction was incubated at 4 ºC overnight and transformed in DH5α competent cells by heat shock method. The transformed colonies were selected for kanamycin resistance. Plasmid isolated from grown colonies was restricted with EcoRI and NotI to confirm the ligation of desired fragments (Fig 31). Expected fragments and their sizes are described below (See table. 4). The plasmid DNA of positive clones were transformed in *Agrobacterium* strain GV3101 (See 3.1.4) for transient expression of mGFP4 fused proteins in *N. benthamiana* through *Agro-infiltration*.

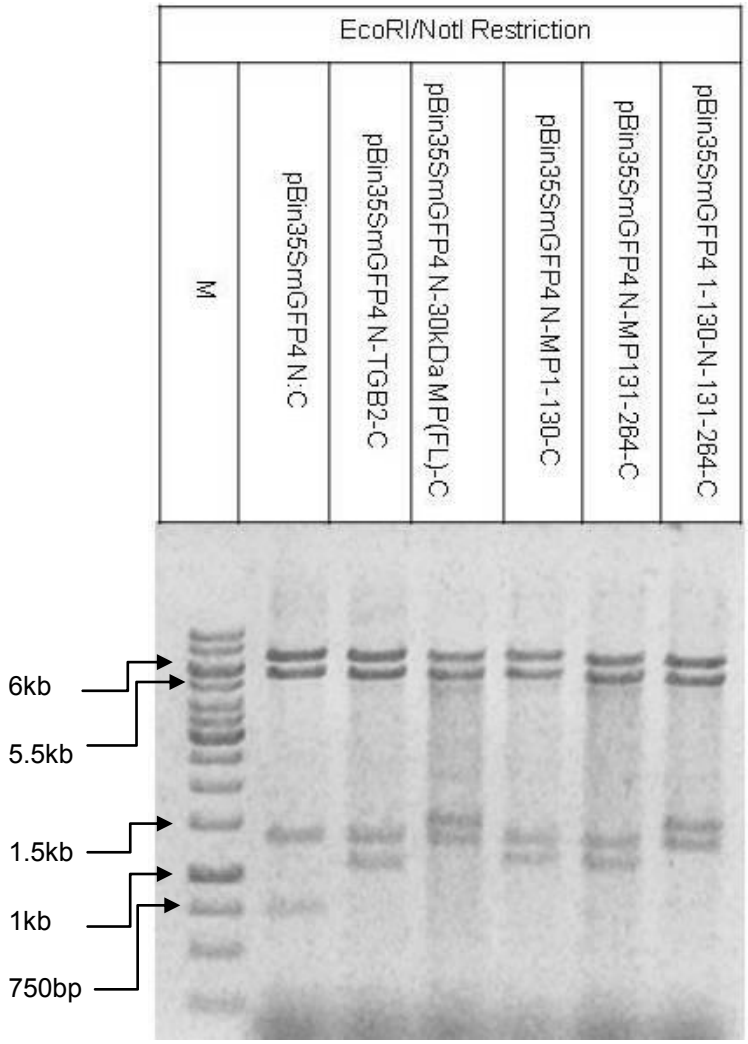


Figure 31. Restriction analysis of pBin35SmGFP4 N:C constructs containing 30kDa MP and its deletion mutants with EcoRI and NotI. **Lane 1**. 1kb Ladder, lane 2. pBin35SmGFP4 N:C vector, **lane 2**. pBin35SmGFP4 N-TGB2-C, **lane 3.** pBin35SmGFP4 N-30kDa MP(FL)-C, **lane 4**. pBin35SmGFP4 N-MP1-130-C, **lane 5**. pBin35SmGFP4 N-MP131-264-C, **lane 6**. pBin35SmGFP4 1-130-N-131-264-C.

Name of construct	Expected fragment sizes
pBin35SmGFP4 N:C	~6kb, ~7kb, ~1.4kb, ~0.75kb
pBin35SmGFP4 N-TGB2-C	~6kb, ~7kb, ~1.4kb, ~1.1 kb

pBin35SmGFP4 N-30kDa MP(FL)-C	~6kb, ~7kb, ~1.4kb, ~1.53kb
pBin35SmGFP4 N-MP1-130-C	~6kb, ~7kb, ~1.4kb, ~1.3kb
pBin35SmGFP4 N-MP131-264-C	~6kb, ~7kb, ~1.4kb, ~1.25kb
pBin35SmGFP4 1-130-N-131-264-C	~6kb, ~7kb, ~1.4kb, ~1.53kb

Table 4. Expected fragment sizes of pBin35smGFP4 N:C constructs after restriction with EcoRI and NotI.

4.2.4 Expression of pBinmGFP4 30kDa MP and its deletion mutants in *N. benthamiana*

For expression of 30kDa MP and its deletion mutants in *N. benthamiana*, transformed *Agrobacterium* colonies with different clones (pBin35SmGFP4 N:C, pBin35SmGFP4 N-TGB2-C, pBin35SmGFP4 N-30kDa MP(FL)-C, pBin35SmGFP4 N-MP1-130-C, pBin35SmGFP4 N-MP131-264-C, pBin35SmGFP4 1-130-N-131-264-C) were grown in LB liquid medium overnight at 30 ºC and 250 rpm until OD_{600} of 0.5-0.8. When all cultures had reached the desired OD_{600}, *Agrobacterium* cultures were transferred in 15 ml plastic tubes, centrifuged at 4º C and 4000 x g for 5 minutes to pellet these cells. The supernatant containing LB medium was discarded and pellet was dried shortly to dispose all LB medium. *Agrobacterium* pellets were resuspended in 50 ml of 10 mM $MgCl_2$ + 50 µM *Acetosyringone*. The resuspended cells were incubated at room temprature for two hours in dark for induction of *vir* genes.

Two hours after incubation, these activated *Agrobacterium* sloutions were infiltrated with the help of 1 ml syringes on lower side of *N. benthamiana* leaves with slight pressure. 1 ml of *Agrobacterium* solution was infiltrated per leaf and three to four leaves per plant were infiltrated for one construct.

Because of low protein expression and high degradation rate of our fused proteins as observed in yeast, we used the ***tomato bushy stunt virus p19*** (Kindly provided by Prof. Dr. Klaus Harter, University of Tübingen, Germany) as a silencing supressor in our experiments. To use this in combination with our clones, p19 was grown under similar conditions as described for other constructs. Just before the infiltration in *N. benthamiana,* p19 *Agrobacterium* strain was mixed with *Agrobacterium* containing expression cassettes of 30kDa MP and its deletion mutants in 1:1 ratio and infiltrated in leaves. To check the effect of p19 on the expression of our protiens, the cultures were infiltrated with and without p19.

After infiltration, plants were incubated under green house conditions for three days. Three days after *Agro-infiltration*, leaf samples were collected. For each infiltration one leaf was taken in an eppendorf tube to isolate proteins for western analysis and frozen immediately in liquid nitrogen to avoid degradation by proteinases and other cell damages. For microscopic analysis of GFP fused MP and its deletion mutants, leaf samples were taken and directly analysed by making epidermal preparations of the samples.

Results showed that mGFP4 protein, when expressed alone in *N. benthamiana* (Fig 32, Slide 1) in the presence or absence of p19, is evenly distributed in the cytoplasm and nucleus. Effect of p19 co-infiltration was significant on the number of flourescent cells and amount of GFP protein expressed in the leaf, as shown in western blot analysis (Fig 33).

Expression of fusion construct pBin35SmGFP4 N-TGB2-C in *N. benthamiana* (Fig. 32, Slide 2), showed that localization of mGFP4 was changed from cytoplasm to the cellular membranes. Aggregates of GFP fused protein was also visible near plasmodesmata under light microscope. The results showed that TGB2 is a membrane localized protein as also seen in yeast and split GFP system can be used succesfully for this type of analysis.

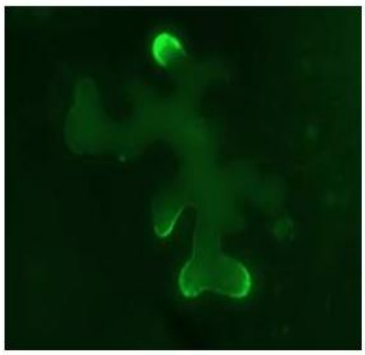
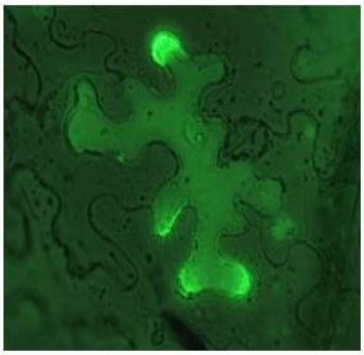
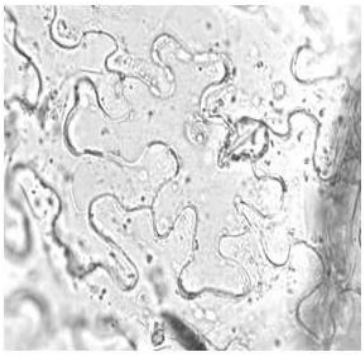
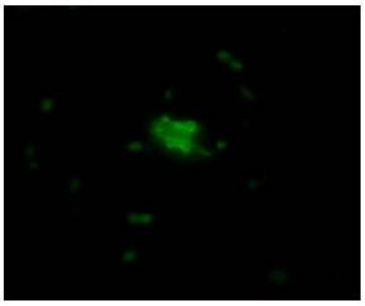
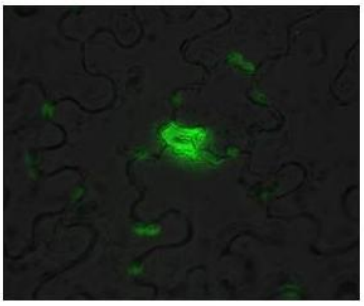
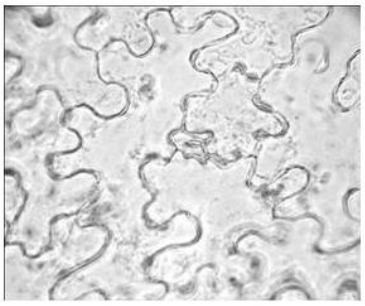
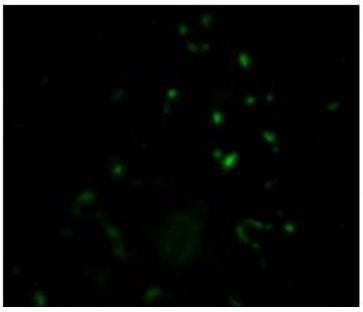
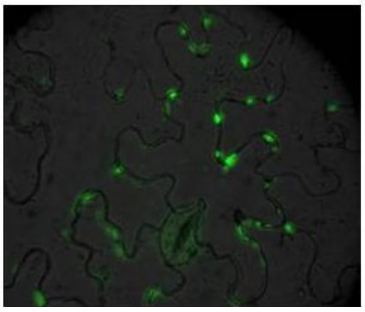
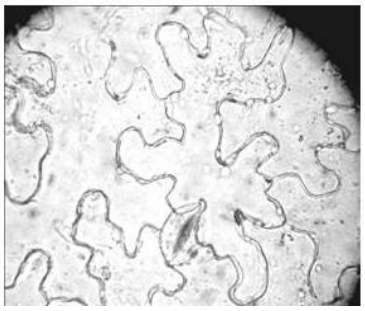
pBin35SmGFP4 N- 30kDa MP (FL)-C expressed in *N.benthamiana* showed significant nummber of flourescent epidermal cells where the fusion protein was localized in the outer cellular membranes and in the plasmodesmata (Fig 32, Slide 3).

The expression of 30kDa MP N- terminus (pBin35SmGFP4 N-MP1-130-C) showed significant amount of flourescent signals under microscope (Fig 32, Slide 4) showing that two ends are close enough to bring split GFP in proximity for the formation of flourescent molecule.

Fusion construct containing C-termius of 30kDa MP (pBin35S mGFP4 N-131-264-C) when expressed in *N. benthamiana* leaves showed flourescene epidermal cells under microscope. The fusion protiens aggregate in the cellular membranes (Fig 32, Slide 5).

When 30kDa MP mutant spliced with mGFP4 (mGFP4 N:C+ MP-1-130-N-131-264-C) was expressed, MP was significantly localized in the membranes (Fig 32, Slide 6) but the fused protein was not detectable on western blot either with or without p19 infiltration (Fig 33).

As a negative control the p19 *Agrobacterium* solution and resuspension buffer was used alone for infiltration, to see any background effect of p19 or tthe buffer.

GFP fluorescence (429nm) after excitation at 365nm Magnification 40x	Overlay of Fluorescence and bright field picture	Bright field picture at a magnification of 40x	Name of Expression Construct
			1:pBin35S mGFP4 N:C
			2:pBin35SmGFP4 N-TGB2-C
			3:pBin35SmGFP4 N-30kDa MP(FL)-C

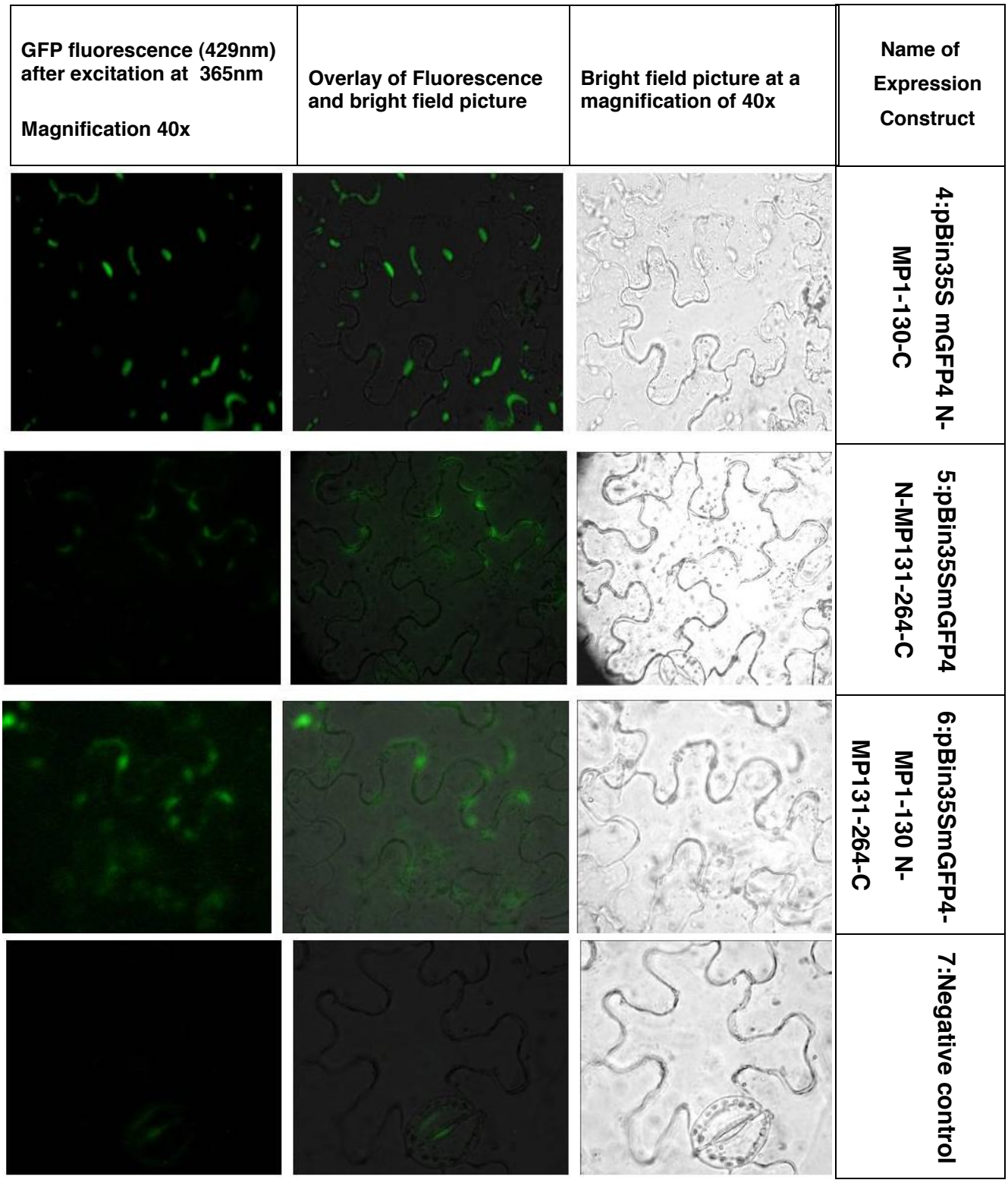


Figure 32. Microscopic analysis of *N. benthamina* epidermal cells infiltrated with 30kDa MP and its deletion mutants. Pictures were taken under the GFP filter and with bright field. The GFP and BF pictures were overlayed to see the exact localization of the GFP fused proteins in the cell.

4.2.5 Isolation of membrane associated mGFP4 N:C fused 30kDa MP and its deletion mutants expressed in *N. benthamiana*

To support the correct localization of MP fusions as detected by flourescent microscopy, a biochemical analysis was conducted. For this purpose leaf samples collected after three days of *Agro-infiltration* were grinded in plant extraction buffer (See 3.4.3). After fine grinding of leaves, the samples were centrifuged at 4 ºC and 4000 x g for 10 minutes to remove cell debris. The supernatant was transfered in a new tube and centrifuged at 30000 x g for 30 minutes at 4 ºC to isolate cytoplasmic and membrane fractions. Supernatant after this centrifugation step contains cytoplasmic protiens and was collected in a new Eppendorf tube. Pellet containing the membrane associated proteins, was resuspended in extraction buffer. Resuspended membrane fractions were then mixed with 5 x SDS loading buffer (See 3.2) and denatured for 5 minutes at 95 ºC. The samples were centrifuged and loaded on polyacrylamide gel for the protien analysis and GFP detection (See 3.2 and 3.2.2).

Immunodetection showed (Fig 33) that mGFP4 protein when expressed alone in *N. benthamiana* cells was present in supernatant fraction containing cytoplasmic protiens. Where as pellet fraction, comprises of ER membranes and other heavier cellular components contains very low or no mGFP4 protein (Fig 33, mGFP4). whereas TGB2 protien, fused with mGFP4, was significantly detectable in the pellet fraction (Fig 33, mGFP4 N-TGB2-C) that also explains membrane localiztion of TGB2 as seen in flourescent microscopy (Fig 32, Slide 2).

Immunodetection of pellet fractions obtained from samples infiltrated with *Agrobacterium* suspension containing expression cassette 30kDa MP N- terminus fusion with mGFP4 showd that the fusion protien was significanlty detectable (Fig 33, pBin35S mGFP4 N-MP1-130-C). This shows the association of deletion mutant with ER and cellular membranes.

Expression of other deletion mutants and the 30kDa MP(FL) fusions were not detected on immunoblot either expressed in the presence of silencing suppresor or

without silencing supressor (Fig 33, pBin35S mGFP4 N-30kDa MP(FL)-C, pBin35S mGFP4 N-MP131-264-C, pBin35S mGFP4 1-130-N-131-264-C).

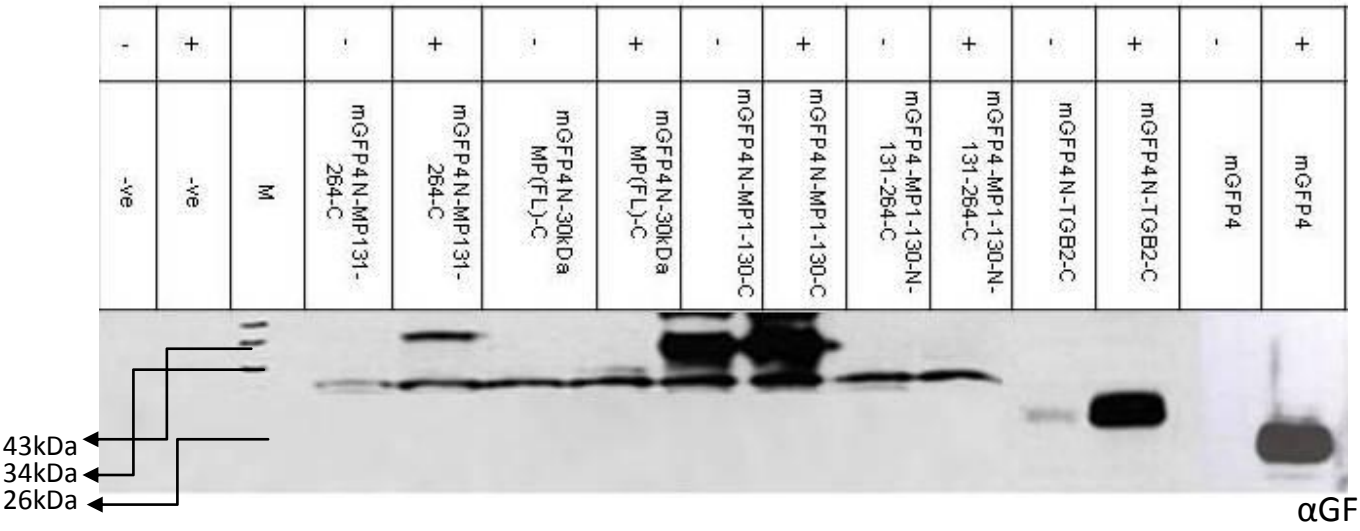


Figure 33. protien analysis of membrane fractions isolated from *N. benthamiana Agro-infiltrated* leaves with 30kDa MP and its deletion mutant. (+) represents co infiltration with p19 and (-) represents without p19. –ve: negrative control, M: Prestained protein marker SM0671.

4.3 Construction of Tomato Mosaic Virus (ToMV) based vectors.

Virus induced gene silencing is an important tool to study gene function. VIGS is based on RNA silencing mechanism where a foreign gene is inserted in the viral genome to study the loss of function in plants, animals or mammals. VIGS has been widely used in plants to study plant virus interactions and the host factors involved in virus replication and spread. Until now most of the studies have been made in *N. benthamiana*, a model plant, for the understanding of host virus dynamics. During the last decade, several viral based silencing constructs have been made to use in crop plants like barley and rice. Tomato is an important crop in the Middle East, Europe and America. One of the major threats for the production of this crop is tomato mosaic virus. Although lots of efforts are being done to get resistance against this virus including the use of resistance genes found in wild relatives. But still the mechanism of resistance is unknown. Host factors involved in the virus replication and the resistance breaking phenotype of the virus are important candidates to understand the strategy of virus resistance. For this purpose we have developed the tomato mosaic virus based silencing vectors to study the function of several candidate genes involved in the resistance mechanism.

4.3.1 Construction of a plant based tomato mosaic virus vector

In the first step we have developed a tomato mosaic virus cDNA clone that can replicate in *N. benthamiana*, *N. tabacum* and *S. lycopersicum.*

4.3.1.1 Cloning of tomato mosaic virus in binary vector.

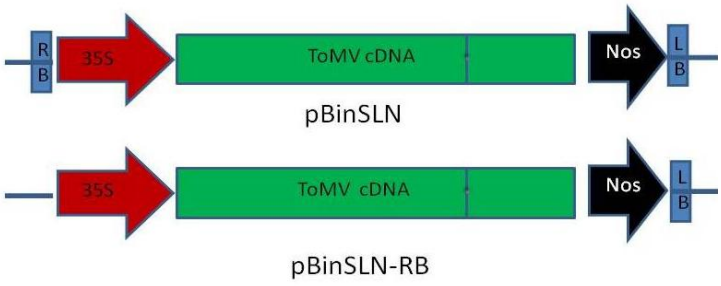
For this purpose cDNA clone of ToMV named as pSLN (35S-ToMV cDNA-nos terminator) was used (Weber *et al.,* 1993). 1 µg DNA of pSLN was digested with two restriction enzymes SacI and SphI. The restriction reaction was incubated at 37 ºC for

two hours and loaded on 1% agarose gel. Desired fragment containing ToMV cDNA along with 35S promoter and nos terminator (~10Kb) was isolated from the gel (See 3.1.10) and cleaned with gel elution kit. pBin19 binary vector was also restricted with SacI and SphI to get linearized vector. Two types of binary vectors containing tomato mosaic virus cDNA were constructed.

1) pBinSLN binary vector contains the left border and right border of T-DNA obtained by partial digestion of pBin19 for 5 minutes.
2) pBinSLN-RB vector contains only the left border of T-DNA, obtained by the complete digestion of pBin19 for 2 hours.

Linearized pBin19 and ToMV cDNA fragment was ligated together in 3:1 ratio (See 3.1.11) and incubated overnight at 4 ºC. Ligation was transformed in DH5α and selection was made against Kanamycin resistance. Five colonies were selected and grown in 5ml of liquid LB medium overnight at 37 ºC. Liquid culture was used to isolate plasmid DNA, that was restricted with SacI and SphI for the confirmation of positive colonies (Fig 34).

A



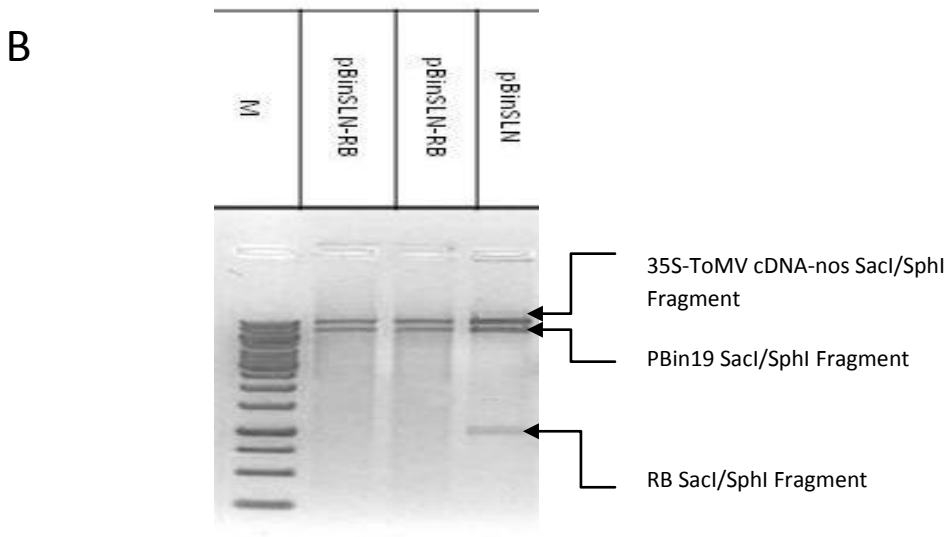


Figure 34. A). Schematic diagram of pBinSLN and pBinSLN-RB, B).Test restriction of pBinSLN and pBinSLN-RB clones with SacI and SphI. M: 1kbp marker.

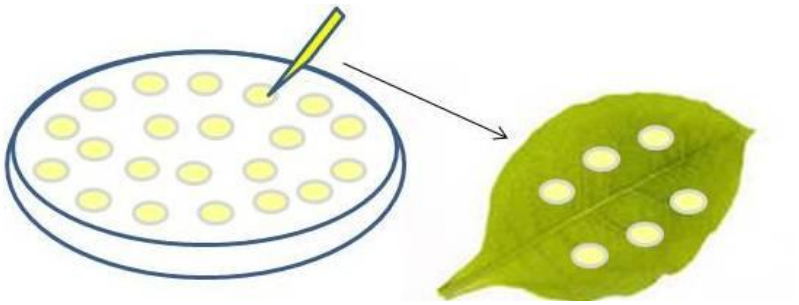
The positive clones were transformed in *Agrobacterium* strain LBA4404 through tri-parental mating (See 3.1.4). Transformed *Agrobacterium* colonies were selected against Kanamycin and Riffampicilin. Positive colonies were confirmed through restriction analysis of isolated plasmid DNA with restriction enzymes SacI and SphI. The confirmed clones were stored as glycerol stocks for further use.

4.3.1.2 Expression of pBinSLN and pBinSLN-RB in plants.

To analyze the replication of ToMV from binary vectorS and effect of T-DNA right border on efficiency of binary vector, *Agrobacterium* containing pBinSLN and pBinSLN-RB were inoculated/infiltrated in *N. benthamiana*, *N. tabacum* and *S. lycopersicum*.

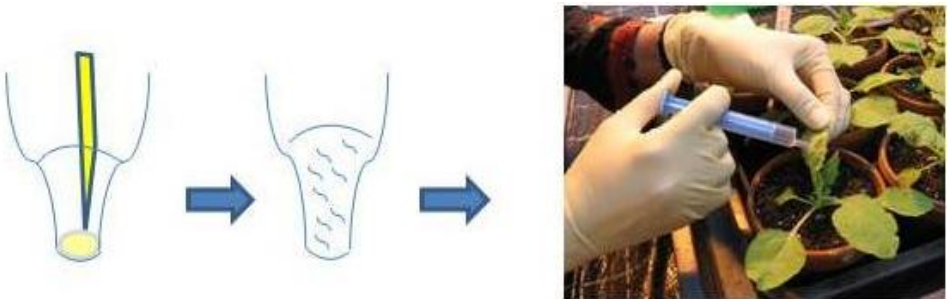
I. Colony inoculation/ *Agro-inoculation* of pBinSLN and pBinSLN-RB in plants

For the colony inoculation, single colonies of both pBinSLN and pBinSLN-RB were streaked on a new LB agar plate and incubated at 30 ºC overnight. The freshly grown colonies were picked up with the help of toothpick and slightly rubbed on upper leaf surface as shown below.



II. *Agro-infiltration* of pBinSLN and pBinSLN-RB in plants.

Single colonies of pBinSLN and pBinSLN-RB were grown in 5 ml of LB medium overnight until the end OD_{600} of 0.5. The cells were centrifuged down and suspended in infiltration buffer. The suspended *Agrobacterium* were infiltrated in the lower leaf surface as shown in the figure below.



Two weeks after pBinSLN and pBinSLN-RB inoculation/infiltration, No virus symptoms were visible on the plants, leaf samples were collected from newly emerging leaves, to analyze the systemic spread of tomato mosaic virus. The virus particles were isolated from the leaf samples as described in 3.4.4.2. 1 µl of the virus extract from each sample was incubated on a nitrocellulose membrane (See 3.2.3) and immune-detected against TMV antibody (Fig 35).

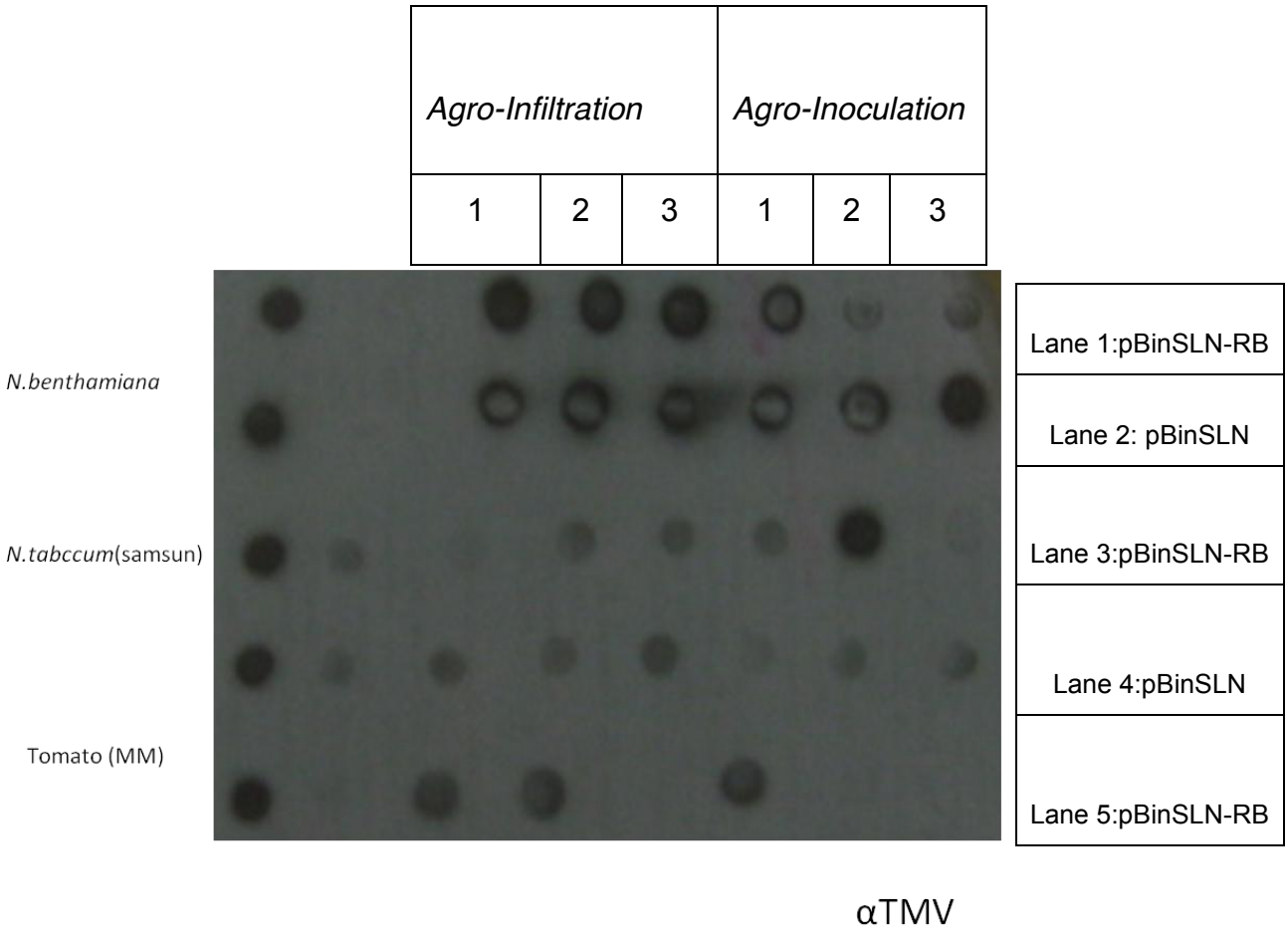


Figure 35. Immuno-detection of leaf samples obtained from *N. benthamiana*, *N. tabacum* and *S. lycopersicum* for tomato mosaic virus replication infiltrated/inoculated with pBinSLN and pBinSLN-RB.

Results (Fig 35) showed ToMV amplification and accumulation in systemic leaves. *N. benthamiana* plants showed equal concentration of virus particles in both *Agro-infiltrated* and *Agro-inoculated* plants (Fig 35, Lane 1 & 2). pBinSLN-RB has also showed no significant difference in virus amplification as compared to pBinSLN.

In *N. tabacum,* no significant virus accumulation was detected both by *Agro-infiltration* or *Agro-inocultaion*. Only one plant, *Agro-inoculated* with pBinSLN-RB showed virus accumulation (Fig 35, Lane 3 &4).

In *S. lycopersicum*, two out of three *Agro-infiltrated* plants showed virus accumulation where as only one out of three *Agro-inoculated* plant showed ToMV accumulation (Fig 35, Lane 5).The results obtained from immune-detection of dot blot showed no significant difference in the replication efficiency of pBinSLN and pBinSLN-RB (Fig 35).

Four weeks after infiltration/inoculation of pBinSLN and pBinSLN-RB, leaves showed curling and yellowing as symptoms of virus infection (Fig. 36). Infected plants were short stature as compared to the mock plant. Sever infection caused plant death on later stages.

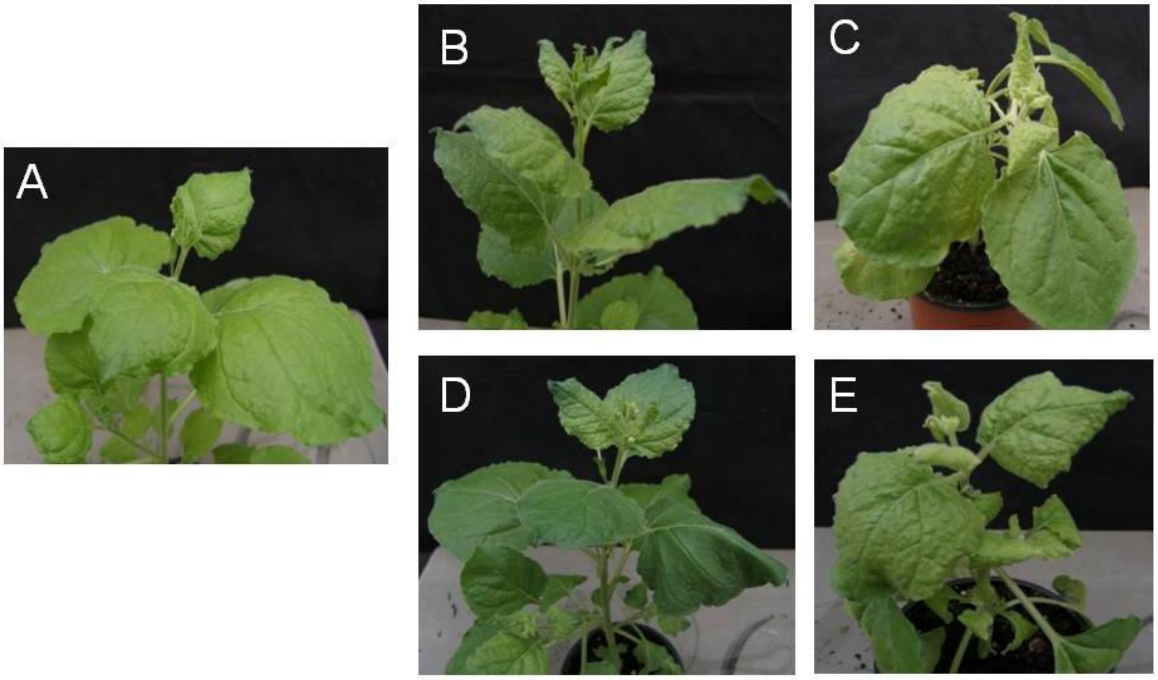


Figure 36. *N. benthamiana* plants *Agro-infiltrated* or *Agro-inoculated* with pBinSLN and pBinSLN-RB. A) Mock plant, B) *Agro-inoculated* with pBinSLN, C) *Agro-infiltrated* with pBinSLN, D) *Agro-inoculated* with pBinSLN-RB, E) *Agro-infiltrated* with pBinSLN-RB.

Virus was isolated from upper systemically infected leaves (See 3.4.4.2). 14 µl of the virus extract was mixed with 4 µl of 5x SDS loading buffer and 2 µl of 2 M DTT (reducing agent). Samples were shortly centrifuged and denatured at 95 ºC for 5 minutes before loading on the SDS gel (See 3.2). 10 µl of prepared sample was loaded on SDS gel and analyzed after staining with coomassie blue (See 3.3.1). Coat protein of

Tomato mosaic virus (~18kDa) was found in leaf samples (Fig 37), thereby confirming the virus infection. Furthermore, no significant difference in virus replication was observed at later infection stage in pBinSLN and pBinSLN-RB infiltrated *N. benthamiana* plants.

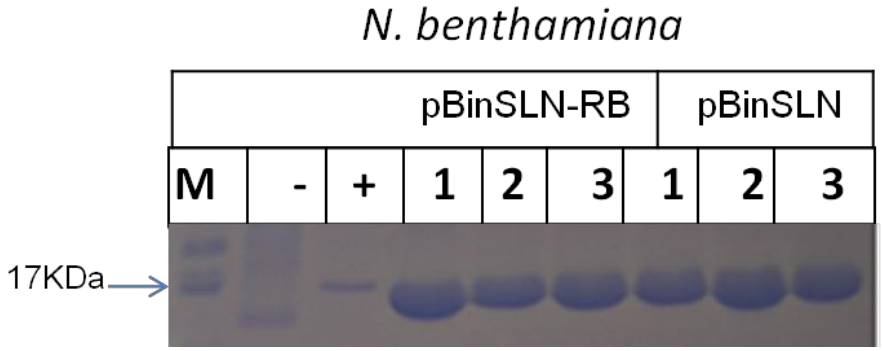


Figure 37. Analysis of tomato mosaic virus infection in *N. benthamiana* on *Agro-infiltration* with pBinSLN or pBinSLN-RB. M. Prestained protein marker, -: Mock plant, +: Plant infected with ToMV virus extract,

Tomato plants, either *Agro-infiltrated* or *Agro-inoculated* with pBinSLN and pBinSLN-RB, also showed virus symptoms four weeks after infection. Infected plants showed yellowing and shortening of leaves as compared to mock plant (Fig. 38A). Plants designated with 1 and 2 were infiltrated with pBinSLN whereas 4 was infiltrated with pBinSLN-RB. Analysis of virus extract, from infected tomato leaves, showed the presences of viral coat protein on SDS polyacrylamide gel, thus confirming the virus replication from pBinSLN and pBinSLN-RB.

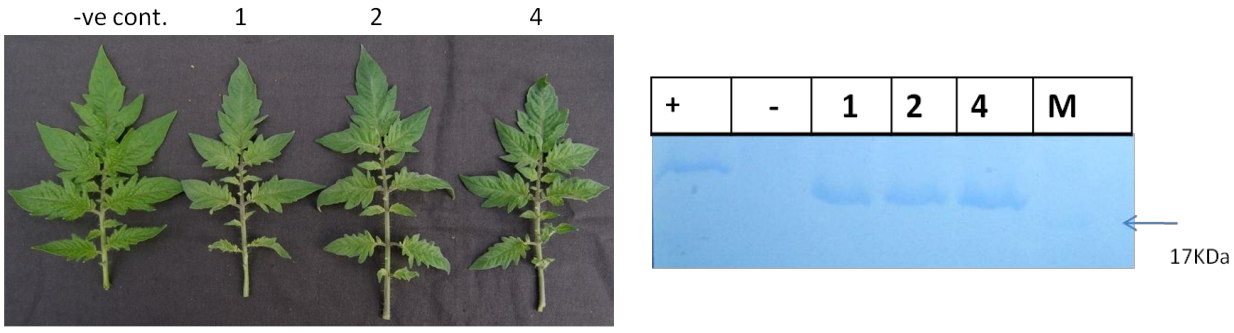


Figure 38. A) Analysis of tomato mosaic virus infection in *S. lycopersicum* , *Agro-infiltrated* with pBinSLN (1 &2) and pBinSLN-RB (4) B) polyacrylamide gel analysis of virus extracts from tomato plants. M. Prestained protein marker, -: Mock plant, +: Plant infected with ToMV virus extract, 1&2 are plants *Agro-infiltrated* with pBinSLN, 4) plant *Agro-infiltrated* with pBinSLN-RB)

4.4. Isolation and characterization of tomato mosaic virus double mutant overcoming both *Tm-2* and *Tm-2²* resistance in tomato

Plant viruses have high mutation rate to overcome resistance barriers for their survival. Therefore, several resistance breaking mutant virus strains have been reported time to time in tomato, cotton and other crops (Pelham, 1972; Mansoor, 2003). Tomato mosaic virus is an important example of such mutations, overcoming resistance caused by the presence of *Tm-2* or *Tm-2²* genes in commercial varieties.

Until now, several *Tm-2* or *Tm-2²* resistance breaking viruses have been isolated. Sequence analysis of these mutant strains showed the mutations in 30kDa MP, hence confirming the interactions of MP with these resistance genes for resistant phenotype (Weber *et al.,* 1993). Emergence of resistance breaking viruses and mutations responsible for overcoming resistance has been discussed in detail in 1.1.1. Till now, no ToMV mutant strain has been isolated that gives resistance to both *Tm-2* and *Tm-2²* together.

In our experiments, we have developed a two step selection scheme for the isolation of a double mutant, overcoming both *Tm-2* and *Tm-2²* resistance. For this purpose, pBinSLN-RB (See 4.2.1.1) was Agro-infiltrated in *S. lycopersicum* GCR 236 (*Tm-2/Tm-2)*. The virus extracts obtained from these plants was used to infect *S. lycopersicum* GCR 267 (*Tm-2²/Tm-2²)* and was analyzed for the emergence of virus symptoms and thus a new mutant strain.

4.4.1 *Agro-infiltration* of pBinSLN-RB in *S. lycopersicum* GCR 236.

Agrobacterium containing pBinSLN-RB was propagated in 50 ml of LB medium under Kanamycin and Riffampicilline selection at 30 ºC with continuous shaking. LB medium was incubated until OD_{600} of 0.5. *Agrobacterium* culture was prepared for infiltration as described in 3.4.5. Four weeks old *S. lycopersicum* GCR 236 plants were infiltrated with 2 ml of *Agrobacterium* suspension and incubated in green house conditions. Three weeks after *Agro-Infiltration*, leaf samples were collected and analyzed for virus replication. Only one out of 25 *Agro-infiltrated* plants showed virus replication and virus symptoms.

4.4.2 Inoculation of *S. lycopersicum* GCR 267 (*Tm-2²/Tm-2²*) and GCR 254(*Tm-1/Tm-2*) plants with the virus extract obtained from *S. lycopersicum* GCR 236.

Virus extract obtained from *S. lycopersicum* GCR 236 infected plant was diluted to 1:50 in Tris-Cl pH 7.5. 50 µl of this diluted virus was inoculated on four weeks old *S. lycopersicum* GCR 267 and GCR 254 plants as described in 3.4.4.1**.** Three weeks after virus inoculation, in case of GCR 267, one out of 25 inoculated plants showed mosaic symptoms as shown in figure 39D, whereas none of the GCR 254 was infected. Leaf samples were collected from inoculated plants and analyzed for virus replication. Virus was isolated from leaf samples and was loaded on 12 % SDS gel. After coomassie staining and de-staining, Results showed the amplification of mutant virus in MM, as well as in GCR 267 plants, where as no virus amplification was visible in GCR 254 plants. In case of wild type ToMV, replication was observed only in MM, but not in GCR 267 and GCR 254 plants (Fig. 40).

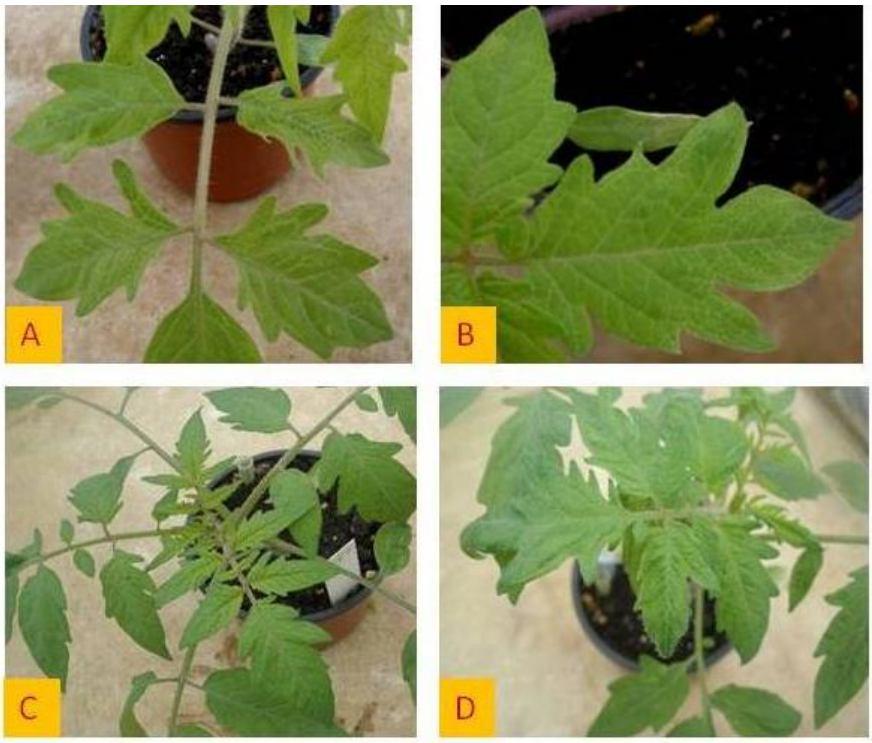


Figure 39. 1b: *Solanum lycopersicum* (MM(A&B) and GCR267(C&D))plants infected with wild type(A &C) and *Tm2²* virus breaking strain (B&D) showing virus symptoms three weeks after infection with 1:50 dilution of virus extracts obtained from MM and GCR236 plants that were Agro-infiltrated with pBinpSLN-RB vector.

Protein analysis of mutant virus revealed a slightly slower migration of virus coat protein as compared to wild type tomato mosaic virus (Fig 39). Due to the instability of *Tm-2²* resistance breaking virus mutants identified so far during sequential propagation, mutant virus was propagated again in two different experiments. The serial propagation of mutant strain has showed that the virus was stable and infectious in the inoculated plants.

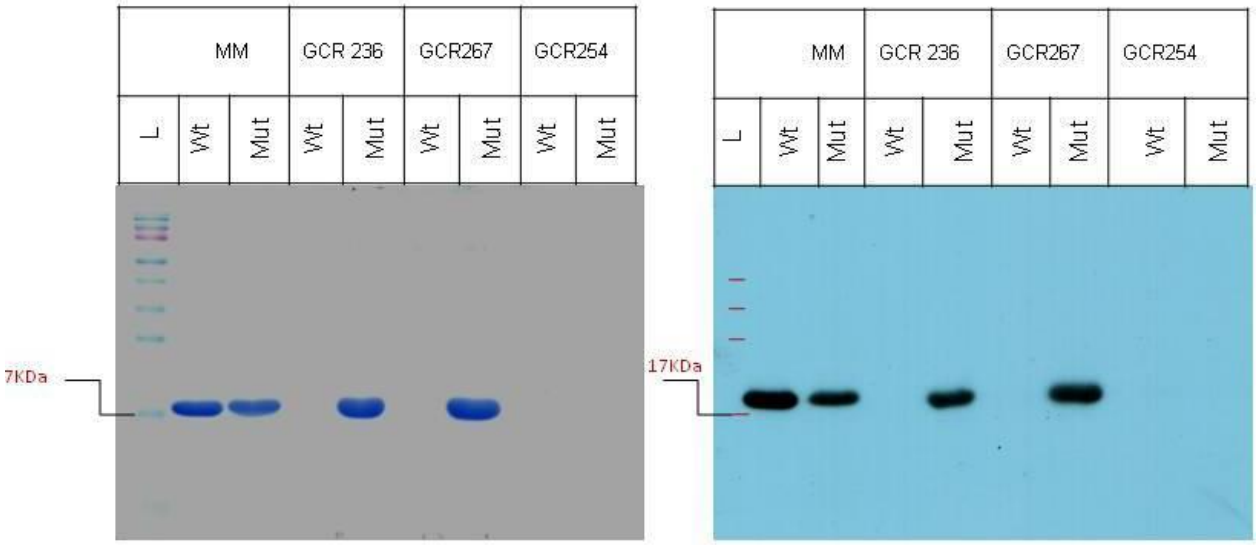


Figure 40. SDS gel analysis of virus extracts isolated from MM, GCR236(*Tm-2/2*),GCR267(*Tm-2²/Tm-2²*),GCR254(*Tm-1/Tm-2*) plants infected with Wild type ToMV(Wt), *Tm-2²* and *Tm-2* resistance breaking strain(Mut). a) Coomassie stained gel showing ToMV coat protein. b) αTMV detections. L: prestained protein Marker (SM0671)

4.4.3 Sequence analysis of mutant strain of tomato mosaic virus (ToMV2-22)

Till now, all mutant tomato mosaic virus strains overcoming *Tm-2* and *Tm-2²* resistance contain aminoacid exchanges in the movement protein responsible for the resistance breaking phenotype as discussed in 1.1.2.

Therefore, Mutant virus isolated from *S. lycopersicum* GCR267 *Tm-2²/Tm-2²* (ToMV2-22) was amplified using the primers based on tomato mosaic virus coat protein

(CP) and movement protein (MP). Amplified fragments were cloned in T-vector and sequenced (See 3.1.8).

Sequence analysis of ToMV2-22 MP (Fig 41) showed that the mutant virus has aminoacid exchanges at aa position 54 and 133 as compared to wild type virus. The aa exchange at position 133 has also been found in other *Tm-2* resistance breaking mutant strains whereas at aa position 54 has not been found before in other resistance breaking strains of tomato mosaic virus as shown in figure 41.

```
                  51                  70
      ToMV   (51) SEVNLLKGVKLIEGGYVCLV
       LII   (51) SEVNLLKGVKLIEGGYVWLV
      LIIa   (51) SEVNLLKGVKLIEGGYVWLV
    ToMV1-2  (51) SKVNLLKGVKLIEGGYVCLV
   ToMV2-22  (51) SKVDLLKGVKLIEGGYVCLV

                 130                 150
      ToMV  (130) KDAEKNIWQVLVNIKNVKMSA
       LII  (130) KDAKKNIWQVLVNIKNVKMSA
      LIIa  (130) EDAEKNIWQVLVNIKNVKMSA
    ToMV1-2 (130) KDAKKNIWQVLVNIKNVKMSA
   ToMV2-22 (130) KDAKKNIWQVLVNIKNVKMSA

            211                                 250
      ToMV  (211) RTKSSKRGPKNNNNLGKGRSGGRPKPKSFDEVEKEFDNLI
       LII  (211) RTKPSKRGPKNNNNLGKGRSGGRPKPKSFDEVEKEFDNLI
      LIIa  (211) RTKSSKRGPKNNNNLGKGRSGGRPKPKSVDEVEEEFDNLI
    ToMV1-2 (211) RTKSSKRGPKNNNNLGKGRSGGRPKPKSFDEVEKEFDNLI
   ToMV2-22 (211) RTKSSKRGPKNNNNLGKGRSGGRPKPKSFDEVEKEFDNLI
```

Figure 41. Comparison of Partial amino acid sequence of the 30KDa protein of wild type ToMV, two *Tm-2* resistance breaking ToMV strains (LII and LIIa), *Tm-1* resistance breaking strain ToMV1-2 and *Tm2* and $Tm2^2$ resistance braking strain ToMV2-22. Amino acid exchanges are depicted In colored letters.

Coat protein analysis of ToMV2-22 showed that mutant strain has 99 % homology with the wild type tomato mosaic virus (Fig 42). Only single amino acid exchange has been found at aa position 155 from Serine (S) to Phenylalanine (F).

```
GENE ID: 920840 ToMVgp4 | 17.5 kDa coat protein [Tomato mosaic virus]
(10 or fewer PubMed links)

 Score =  285 bits (730),  Expect = 7e-75
 Identities = 158/159 (99%), Positives = 158/159 (99%), Gaps = 0/159 (0%)
 Frame = -2

Query  800  MSYSITSPSQFVFLSSVWADPIELLNVCTNSLGNqfqtqqarttvqqQFSEVWKPFPQST  621
            MSYSITSPSQFVFLSSVWADPIELLNVCTNSLGNQFQTQQARTTVQQQFSEVWKPFPQST
Sbjct  1    MSYSITSPSQFVFLSSVWADPIELLNVCTNSLGNQFQTQQARTTVQQQFSEVWKPFPQST  60

Query  620  VRFPGDVYKVYRYNAVLDPLITALLGAFDTRNRIIEVENQQSPTTAETLDATRRVDDATV  441
            VRFPGDVYKVYRYNAVLDPLITALLGAFDTRNRIIEVENQQSPTTAETLDATRRVDDATV
Sbjct  61   VRFPGDVYKVYRYNAVLDPLITALLGAFDTRNRIIEVENQQSPTTAETLDATRRVDDATV  120

Query  440  AIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTFAPAS  324
            AIRSAINNLVNELVRGTGLYNQNTFESMSGLVWT APAS
Sbjct  121  AIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSAPAS  159
```

Figure 42. Alignment of coat protein sequence of ToMV2-22 with that of wild type tomato mosaic virus, obtained from NCBI blast. Amino acid exchange is depicted in blue.

4.5 Tomato mosaic virus as silencing vector

Different viruses have been used for silencing of endogenous genes to study gene function. Among these, Tobacco rattle virus (TRV) and Tobacco mosaic virus (TMV) are important examples of silencing vectors. None of these vectors can be used successfully in tomato to study the resistance mechanism and indentify host factors involved in resistance due to host range and sever symptoms. This has emphasized the need to develop a viral silencing vector that can propagate in tomato and does not damage the plant to higher extent. Tomato mosaic virus is an important pathogen of tomato crop giving mild mosaic symptoms on infection. Therefore, tomato mosaic virus based silencing vector was developed to study the effect of important candidate genes involved in resistance mechanism. Recently, Pflieger and his colleagues have developed a Turnip yellow mosaic virus silencing vector and showed that the insertion of

candidate gene just behind the coat protein sequence results in efficient silencing and does not hinder the virus replication. For this purpose, we have developed a tomato mosaic virus based silencing vector where the candidate gene fragments were inserted behind the coat protein as shown in figure 43A (Pflieger *et al.,* 2008). To analyze the effectiveness of tomato mosaic virus based silencing vector, phytoene desaturase (PDS) was used as a marker gene. The *PDS* gene (Demmig-Adams and Adams, 1992) is ideal to demonstrate the effectiveness of VIGS. This gene participates in carotenoid metabolic pathway, acting on the antenna complex of the thylakoid membranes, and it protects chlorophyll from photooxidation. Silencing of this gene results in a drastic decrease in leaf carotene content, leading to photobleaching symptom (Fig 43B) that is easily detected. It has been frequently used as a positive control of the system (Ruiz *et al.,* 1998; Ratcliff *et al.,* 2001; Holzberg *et al.,* 2002; Liu *et al.,* 2002a; Turnage *et al.,* 2002).

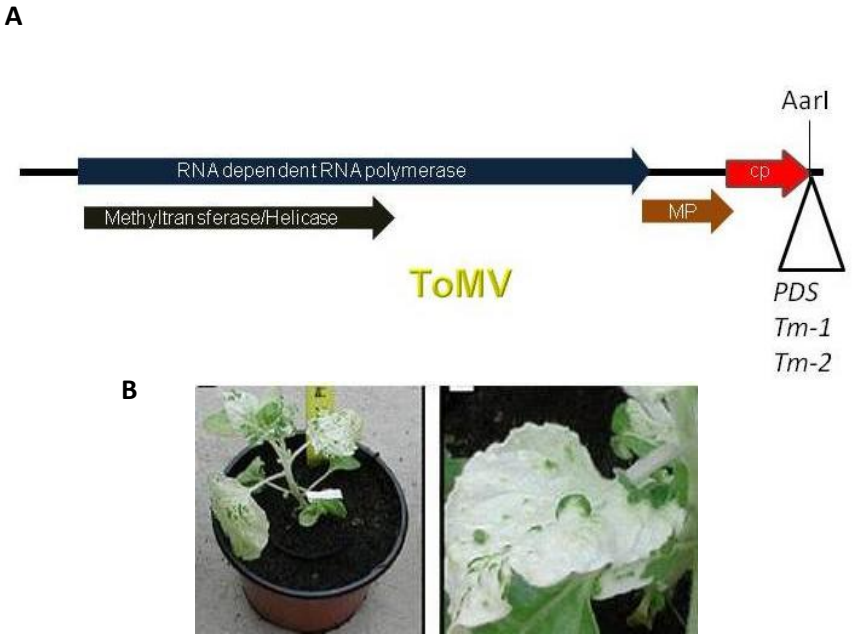


Figure 43. A) Diagrammatic representation of ToMV genome, indicating the point of insertion of target genes. B) *N. benthamiana* plants showing the phenotypic effect of PDS silencing (Ruiz *et al.,* 1998)

4.5.1 Amplification of target gene fragments

Three target genes were selected in the first instance for silencing through tomato mosaic virus based silencing vector, namely phytoene desaturase gene (PDS), *Tm-I*

resistance gene and *Tm-2* resistance gene. PDS gene was used as a marker to test the efficiency of tomato mosaic virus based silencing vector, whereas the silencing of resistance genes was aimed to study the host factors involved in the resistance pathway initiated by the presence of *Tm-I* and *Tm-2* resistance genes.

For the silencing of endogenous gene, 200bp and 500bp fragments of each candidate gene was amplified through standard PCR program (See 3.2.1) with the help of sequence specific primers (See 2.8.1). Amplified fragments were loaded on 1 % agarose gel to analyse the right size amplification (Fig 44).

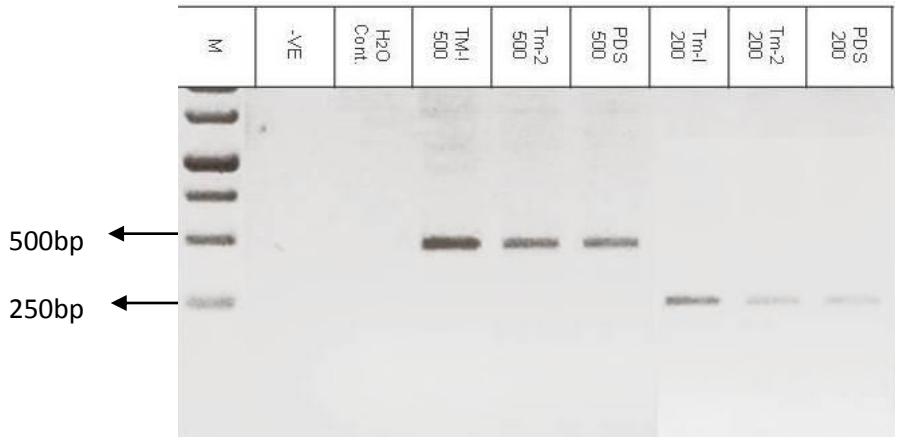


Figure 44. PCR amplification of *Tm-I*, *Tm-2* and *PDS* fragments. M. 1Kb DNA marker, -ve : negative control without *Taq* polymerase, H_2O control : Negative control using H_2O instead of template DNA.

4.5.2 Cloning of amplified fragments in pBinSLN-RB

pBinSLN-RB (4.2.1.1) was used as a silencing vector by cloning the amplified fragments behind coat protein. For this purpose, pBinSLN-RB DNA was cut with AarI enzyme. Restricted DNA was treated with T4 DNA polymerase at 11 ºC for 15 minutes to blunt the restricted ends. This blunt end DNA treated with dideoxynucleotidyl transferase for the incorporation of dt at 3' ends according to supplier's instructions. This vector containing 3' dt ends was used for direct cloning of PCR amplified fragments of PDS, *Tm-I* and *Tm-2* genes. Detailed cloning strategy is explained in figure 45.

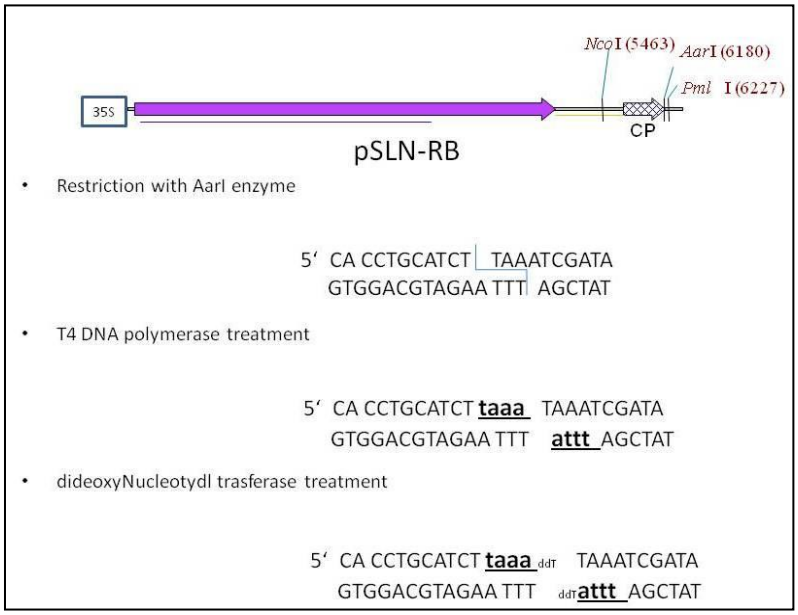


Figure 45. Cloning strategy of amplified gene fragments in pSLN-RB

Positive clones were transformed in LBA4404 through tri-parental mating (See 3.1.4). Transformed colonies were selected against Kanamycin and Riffampicine selection and confirmed through restricting the plasmid DNA with NcoI and PmlI (Fig 46).

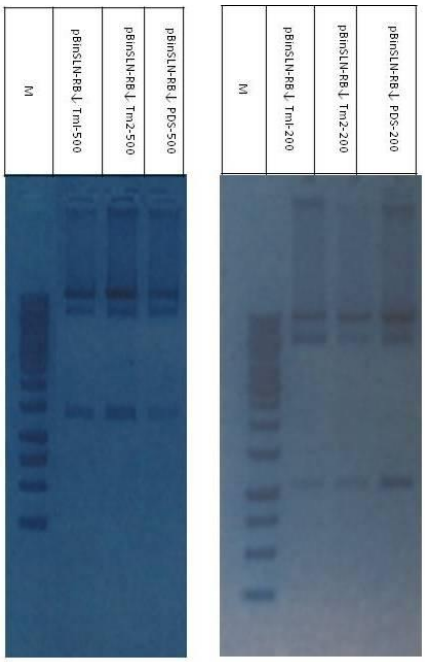


Figure 46. Restriction analysis of pBinSLN-RB↓ Tm-I 200/500, pBinSLN-RB↓ Tm-2 200/500, pBinSLN-RB↓ PDS 200/500 with PmlI and NcoI. M.1Kb Marker.

4.5.3 *Agro-infiltration* of pBinSLN-RB↓ silencing vectors in *N. benthamiana*

pBinSLN-RB based silencing vectors (pBinSLN-RB↓) were agro-infiltrated in *N. benthamiana* (See 3.4.5) to study the virus replication and silencing efficiency. For this purpose, single *Agrobacterium* colonies containing different silencing vectors were grown in 5 ml of LB medium until the end OD_{600} of 0.5. Grown *Agrobacterium* cultures were pelleted and re-suspended in 10 mM $MgCl_2$ + acetosyringone. Four weeks old *N. benthamiana* plants were *Agro-infiltrated* and incubated under green house conditions. Four weeks after infiltration, virus symptoms were observed in *N. benthamiana* plants (Fig 47) but no visible symptom for the silencing of PDS gene was observed in plants infiltrated with pBinSLN-RB↓ PDS200/500 (Fig 47). Leaf samples were collected from systemically infected leaves and analyzed for virus replication (Fig 48).

Figure 47. *N. benthamiana* plants *Agro-infiltrated* with different silencing vectors, showing symptoms four week after infiltration.

Virus particles were isolated from systemically infected *N. benthamiana* leaf samples as described in 3.4.4.2 and analyzed on SDS gel. SDS polyacrylamide gel

analysis of leaf extracts showed virus replication in plant infiltrated with pBinSLN-RB and pBinSLN-RB↓ Tm-I 200/500. Plants infiltrated with other silencing vectors targeting Tm-2 and PDS gene did not show any virus signals on coomassie stained SDS gel (Fig 48).

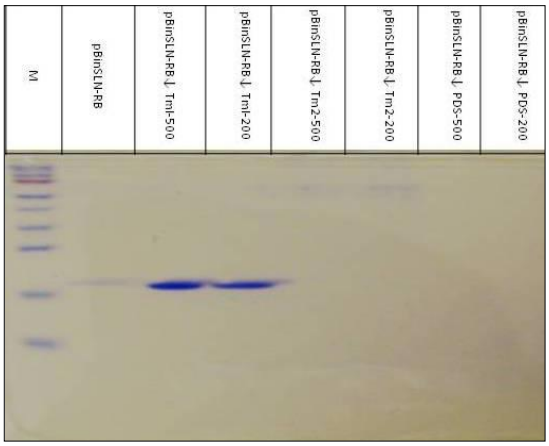


Figure 48. SDS gel analysis of virus extracts obtained from *N. benthamiana* plants, four week after agro-infiltration. M. Prestained protein marker SM0671.

Virus RNA was isolated from the extracts of infected leaves (See 3.4.6.1) and was used for cDNA synthesis. 1 µl of synthesized cDNA was used for the amplification of ToMV coat protein and 3' UTR using sequence specific primer in a standard PCR reaction (See 3.2.1). PCR samples were loaded on 1 % agarose gel for the analysis of amplified products. Results showed that the virus amplification in all plants infiltrated with pBinSLN-RB↓ except for pBinSLN-RB↓ Tm-2-500 and the amplified virus was of similar characteristics as that of positive control (pBinSLN-RB).This shows that the silencing vector was able to amplify upon infiltration in plants but virus, amplified in plants, had excluded the inserted fragment and reverted back to wild type as no change was found in the amplified fragment size of pBinSLN-RB and pBinSLN-RB↓ silencing vectors (Fig 49).

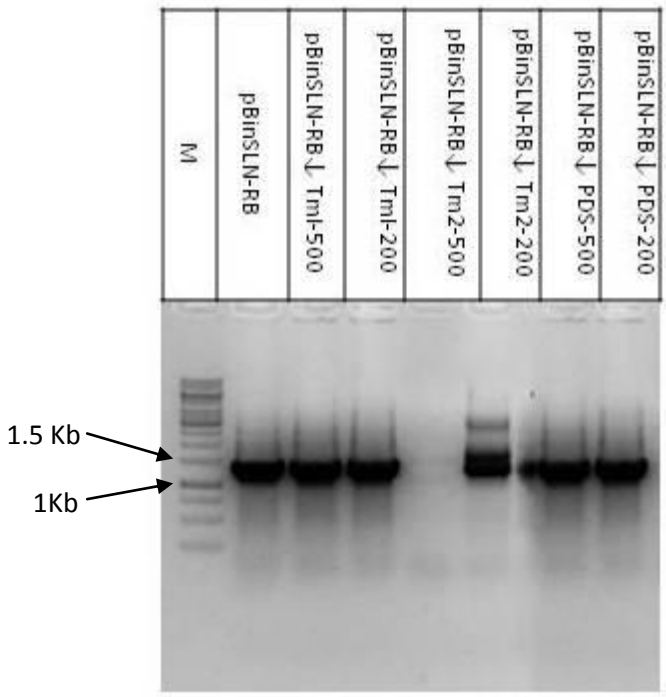


Figure 49. PCR amplification of CP and 3'UTR of tomato mosaic virus from the plant extracts obtained from agro-infiltrated N. benthamiana plants, infiltrated with different silencing vdctors. M. 1Kb DNA marker.

4.5.4 Inoculation of virus extracts from *N. benthamiana* on *S. lycopersicum* for gene silencing

Virus extracts obtained from *N. benthamiana* plants infiltrated with pBinSLN-RB↓ Tm-1/Tm-2/PDS silencing vectors (See 4.4.3) were used to infect *S. lycopersicum* GCR 236, GCR 267, GCR 254 and MM plants. For this purpose, four weeks old tomato plants were infected with 50 µl of virus extract after slightly injuring the plants with Carborandum. Four weeks after infection, samples were collected from tomato plants, to analyze virus multiplication. In case of silencing, virus amplification should be possible in GCR 254 plants, when infected with virus extracts from pBinSLN-RB↓ Tm-I 200/500. Similarly, GCR 267 and GCR 236 plants infected with virus extract from pBinSLN-RB↓ Tm-2 200/500 should also show tomato mosaic virus multiplication upon silencing of *Tm-2* and *Tm-22* genes. Virus was isolated from leaf samples obtained from tomato plants and was loaded on SDS gel to analyze the presence of ToMV (Fig 50).

SDS gel analysis showed that GCR 237, GCR 236, GCR 267 plants infected with corresponding virus extracts from *N. benthamiana* did not show any virus symptoms and virus replication.

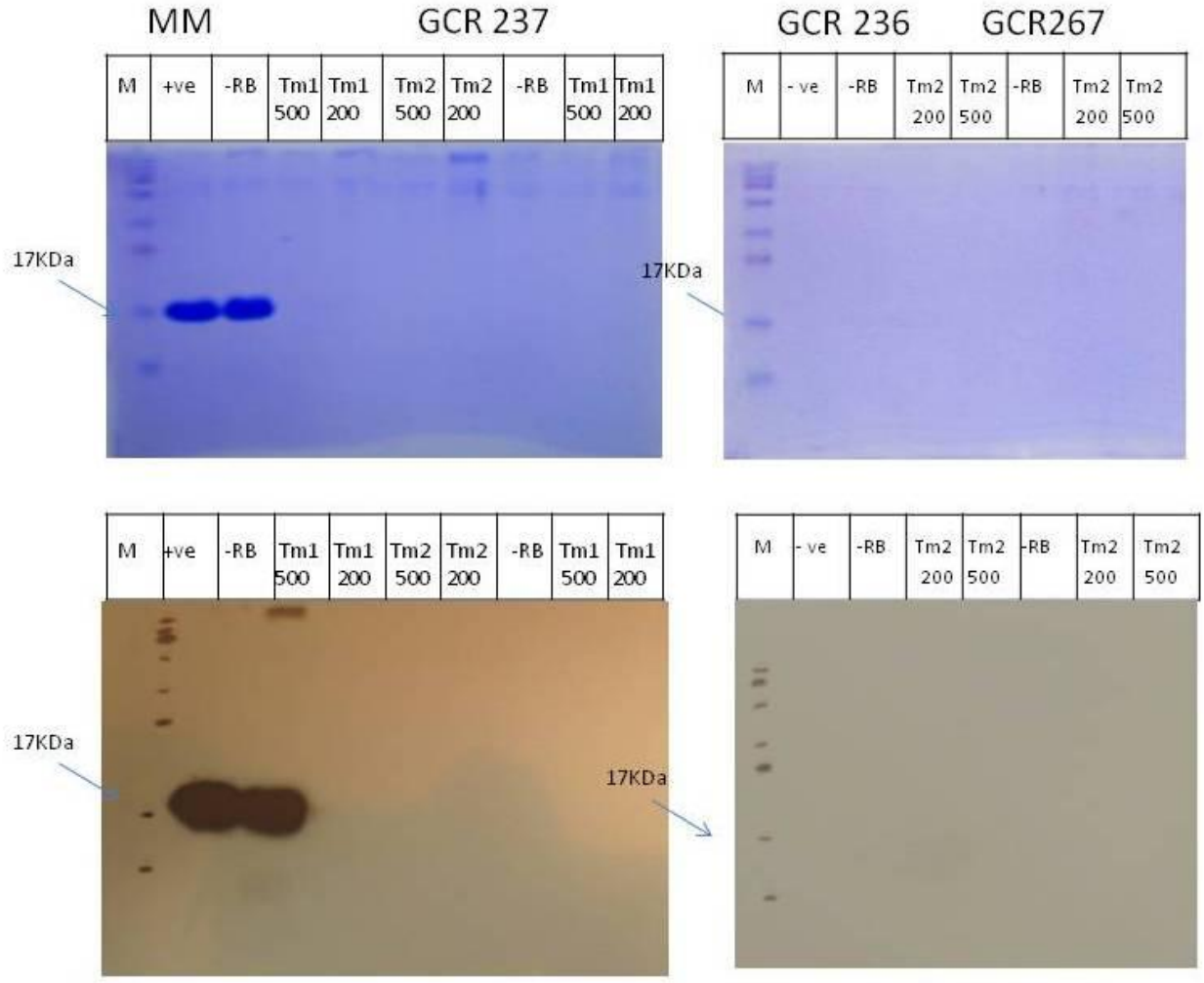


Figure 50. SDS gel analysis of tomato plants infected with virus extracts obtained from 4.4.3. M. Perstained protein marker SM0671, -ve. Mock , +ve: tomato plant infected with wild type ToMV.

5. Discussion

Viruses are important plant pathogens causing sever diseases and loss in crop yield (Kang *et al.,* 2005). Plant viruses, upon infection, replicate at the site of infection and move to the other plant parts making the use of host factors. Therefore, viruses are completely depending upon their host plants to complete their life cycle (Matthews, 1982). Various efforts have been made to investigate the mechanisms of virus infection and their systemic spread in plants.

Several plant species have the ability to arrest virus attack at various stages of infection, a phenomenon named as resistance, i.e. by interfering with the replication or systemic movement. This host resistance can be achieved either by natural resistance sources e.g. resistance genes (*R* genes), that are found in certain plant species, or by genetic engineering e.g. RNA interference (Goldbach *et al.,* 2003). Presence of resistance genes elicit the hypersensitive response (HR) and limit the virus spread to the neighbouring cells, and/or a salicylic acid mediated response is initiated away from the site of infection for systemic resistance (SAR, Soosaar *et al.,* 2005).

Resistance genes (*R* genes) are divided in five different classes based on their structure and function (Bent, 1996; Van Ooijen *et al.,* 2007). CC-NBS-LRR is a major class of *R* genes that has been mostly investigated to understand the mechanism of resistance (Kang *et al.,* 2005). Important members of this group include the Rx gene of potato, as well as *Tm-2* and *Tm-2*^{2} genes in tomato. The mechanism of *R* gene resistance is explained by an elicitor-receptor model (Flor, 1947). One example of this model is resistance obtained in potato plants against PVX virus. The Rx gene of potato acts as receptor and coat protein of PVX as an elicitor. An interaction between receptor and elicitor arrests virus multiplication at a very early stage of infection (Bendahmane *et al.,* 1995; Bendahmane *et al.,* 1997). Similarly, resistance genes *Tm-2* and *Tm-2*^{2} (another example of CC-NBS-LRR resistance genes) confer resistance against tomato mosaic virus in cultivated tomato crops and are introduced from wild relatives by

extensive breeding procedures. Different studies have showed that *Tm-2* and *Tm-2²* recognize tomato mosaic virus 30kDa movement protein as elicitor to initiate the resistance cascade (Pfitzner, 2006). Movement protein, as the name suggests, is involved in inter-cellular virus movement as well as long distance transport (Matthews, 1982).

Various *Tm-2* and *Tm-2²* resistance breaking tomato mosaic virus strains have been isolated during the last decades (Pelham, 1972; Hall, 1980). Sequence analysis of ToMV mutant strains showed that mutations responsible for this resistance breaking phenotype are present in 30kDa movement protein (Calder and Palukaitis, 1992). Molecular analysis of these mutants have shown that exchange at aa 133 (Glu>Lys) or at aa 68 (Cys>Phe) are responsible for partially overcoming the *Tm-2* resistance, where as in the presence of both exchanges, *Tm-2* resistance breaking can be completely achieved (Meshi *et al.,* 1989). Similarly, mutations at aa 130 (Lys>Glu), 238 (Ser>Arg) and 244 (Lys>Glu) were found in *Tm-2²* resistance breaking ToMV strains. Weber and his colleagues in 1998 have also shown that *Tm-2* requires only N-terminus of 30kDa MP, for resistance against ToMV but *Tm-2²* recognizes the C-terminus as well as the N- terminus of 30kDa MP. These results suggest a physical interaction between *R* genes and 30kDa movement protein of tomato mosaic virus.

Previously, Roger Beachy and his group have performed several experiments to shed light on the structure and localization of movement protein. When GFP fused MP was expressed in BY-2 protoplast and *N. benthamiana*, fluorescent signals were observed near the plasma membrane and at the plasmodesmata (Heinlein *et al.,* 1998). TMV MP was also found to be associated with ER. Based on their results, they postulated that 30kDa MP of *tobamoviruses* does not enter in the ER, but is associated with cytoplasmic side of ER membranes. Later on, Brill and his colleagues in 2000, developed a structural model of in-vitro translated 30kDa MP of tobacco mosaic virus (a type member of *tobamoviridae*) based on proteolyses of the microsomal integrated protein followed by an analysis of the resulting peptides by mass spectrometry. They

showed that 30kDa MP is an integral membrane protein with its N and C terminus present in the cytoplasm, whereas the middle part (aa 80-150) in ER lumen (Fig 51A). In the light of previous results, obtained from *Tm-2* and *Tm-2²* resistance genes (Pfitzner, 2006), structural model presented by Brill *et al.,* 2000 is not able to explain the interactions between 30kDa MP and *R* genes (Pfitzner, 2006) and the effect of mutations found in resistance breaking virus strains, hence rising a number of questions to be answered.

Gerhardts (2008) addressed the structural model of 30kDa MP. She pointed out that *Tm-2* and *Tm-2²* are cytoplasmic proteins and mutations responsible for resistance breaking are in the middle part of 30kDa MP. This mutation rich region, however is supposed to be localized in the ER lumen, as described by Brill and his colleagues (2000). Therefore, it is not clear, how two proteins present in different compartments can come together, close enough to make physical interactions. Moreover, the current structural model (Brill *et al.,* 2000) was based on the results obtained from 30kDa MP expressed and integrated in the micosomal membranes under *in-vitro* conditions hich can be unrelated to the *in-vivo* conditions.

In order to answer this question, an alternative structural model of 30kDa MP was proposed by Gerhardts in 2008 (Fig 51B). She used resistance breaking mutants of ToMV to probe the interactions between *Tm-2* and 30kDa MP but also the structure of 30kDa MP for the first time. This alternative model suggests that theToMV 30kDa MP is an integral membrane protein with two trans-membrane domains, but with its N and C terminus as well as the middle part in the cytoplasm.

This model was supported by the results obtained from the mapping of *Tm-2* and *Tm-2²* domains interacting with 30kDa MP (Weber *et al.,* 2004), but additional biochemical data is necessary to confirm the localization of these domains in the cell cytoplasm.

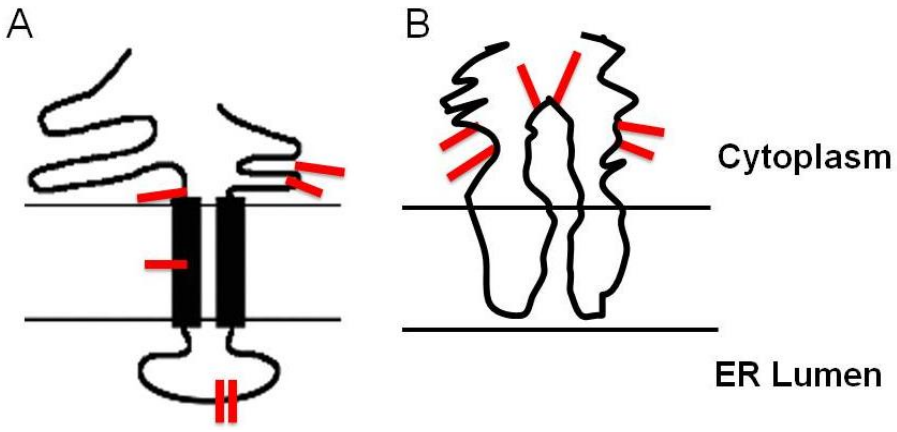


Figure 51. Structural model of 30KDa MP and its membrane association. A) Model Presented by Brill *et al.,* 2000. B) Alternative structural model suggested by Gerhardts 2008. Red lines represent the distribution of resistance breaking mutations found in *Tm-2* and *Tm-2*^{2} resistance breaking ToMV strains.

In this thesis, a novel approach was used, to probe the structure and localization of different domains of the 30kDa MP in yeast and also in plants. A split GFP system, consisting of two non-overlapping halves of the GFP, fused with different deletion mutants of ToMV 30kDa MP. This is different from other split GFP systems, used so far, where one GFP half is fused with one protein and the other is either expressed freely in the cell (Zamyatinin *et al.,* 2006) or fused with different protein to analyse protein-protein interactions (Walter *et al.,* 2004).

5.1 Structural analysis of Tomato mosaic virus movement protein

For the structural analysis of ToMV 30kDa MP, three different deletion mutants were developed to expose the parts of the movement protein containing the resistance breaking mutations. These deletion mutants were fused with split GFP and expressed both in yeast and plants. In contrast to the conventional split GFP approach, both halves of GFP were fused to different domains of same protein in a single construct. On

expression of these fusion constructs, the both halves of GFP depend on protein folding and localization to come close enough for the construction of fluorescent molecule. If the two ends of the protein are localized in different compartments or are away from each other, no fluorescent signals will be observed under the microscope.

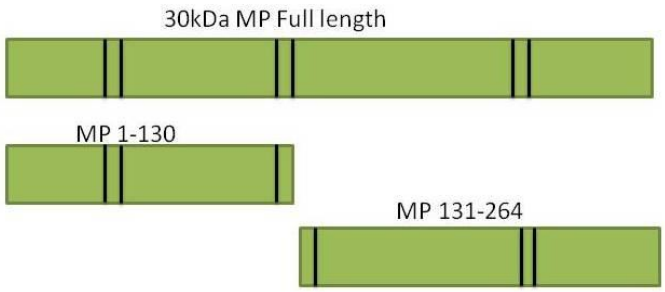


Figure 52. Schematic diagram of ToMV movement protein and its deletion mutants. Black bars represent the amino acid exchanges responsible for resistance breaking phenotype.

In the first construct, complete MP was fused with split GFP (N-MP(FL)-C; Fig 53A), fluorescent signals obtained with this fusion construct showed that both N and C terminus are present in the cytoplasm (Fig 28, slide 5 & Fig 32, slide 3), as hypothesized by both structural models in question (Fig 51). Heinlein and his colleagues in 1998 have shown that the GFP fusion of TMV MP, when expressed in *N. benthamiana*, was localized in the ER membranes and also near the cytoplasmic side of plasmamembrane. Expression of our ToMV MP:GFP fusion constructs showed similar localization patterns (Fig 32, slide 3) as well as aggregation of MP:GFP during later stages. In addition, these results also demonstrate that, this approach to fuse both parts of GFP with different domains of MP is an appropriate method to analyse the *in-vivo* structure of our protein of interest.

The second GFP:MP fusion construct contains the N terminus of ToMV MP (mGFP N-MP 1-130-C) as depicted in figure 53(B). According to the structural model

presented by Brill and his colleagues, where the middle part of ToMV movement protein lies in the ER lumen, there should be no fluorescent signals. Results obtained by the expression of this fusion construct showed significant number of fluorescent cells (Fig 28, slide 6 & Fig 32, slide 4) indicating presence of both ends in the cytoplasm , thus constructing the fluorescent molecule.

The third GFP-ToMV MP fusion construct contains the C terminus of the MP lying between N and C terminus of split GFP (mGFP N-MP131-264-C). Expression of this fusion construct, both in yeast and plants, showed that middle part of ToMV MP protein to be present in the cytoplasm near the C terminus of MP as shown in the figure 53(C). Plasmodesmatal localization of the fusion protein showed that C terminus alone can also be directed to the plasma-membrane (Fig 28, slide 7 & Fig 32 slide 5).

Expression of another fusion construct containing ToMV MP fused between split GFP (mGFP 1-130-N-131-264-C, as depicted diagrammatically in Fig 51(D)) showed fluorescent signals under microscope, where as membrane association of the fusion protein indicates that the functionality of MP was not affected during the cloning of split GFP parts between ToMV MP (Fig 32, slide 6).

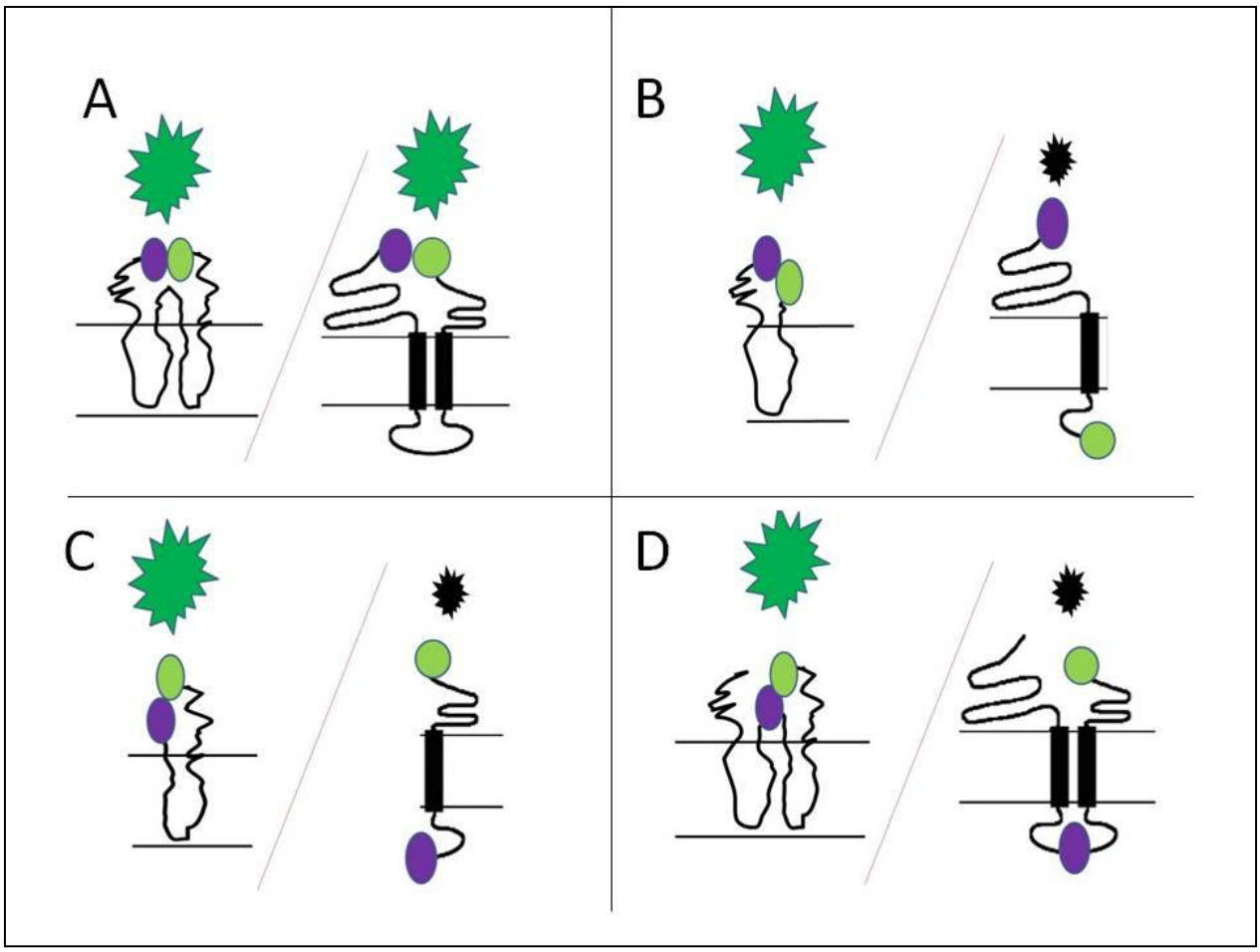


Figure 53. Diagrammatic representation of ToMV MP structure described by Brill (2000) versus Gerhardts (2008). Figures in panel A, B, C and D represents different split GFP fusion constructs and their possible folding when expressed either in yeast or plants. Interaction between both parts of GFP occurs because they are located in close proximity, resulting in the reconstitution of a functional GFP

GFP fluorescence data, obtained during our experiments, strongly support the ToMV MP structural model proposed by Gerhardts in 2008. Immuno detection of expressed proteins (Fig 28 and Fig 33) showed that the GFP protein, when expressed alone, was found in cytoplasm. Whereas, fusion of GFP with different MP deletions has changed the localization pattern from cytoplasm to ER and plasma membrane. Similar results were obtained when Roger Beachy and his group expressed GFP:MP fusions of TMV in *N . benthamiana* and BY2 protoplast (Heinlein *et al.,* 1998). Unfortunately only the N and C terminus deletions of ToMV MP were detected on western blot, whereas the

complete MP was undetectable by immune-detection in several expression experiments. There can be several reasons for this failure as described below.

Virus movement proteins are important substrate for degradation by various cellular proteases and other degradation processes. Tomato mosaic virus MP has also been found to be significantly regulated by RNAi. Expression of our GFP:MP fusions in the presence of silencing suppressors has increased the amount of protein three folds than expressed in the absence of silencing suppressor (Fig 33). Certain fusion proteins could not be detected by western blotting. This suggests that other degradation processes are also involved in the expression of fusion proteins e.g. proteasome degradation etc. Proteasome degradation has previously shown to be an important pathway for the regulation of viral MPs expression *in-vitro* and *in-vivo* (Reichel and Beachy, 2000; Drugeon and Jupin, 2002). A second possible reason for this can be the folding pattern of the fusion proteins. These drawbacks can be avoided either by expressing the fusion proteins in the presence of proteasome inhibitors or detection of proteins with MP specific antibodies. Immuno detection of the fusion proteins was done using MP specific antibodies but this gives huge background and therefore was not able to see the specific bands (data not shown).

5.2 Development of Tomato mosaic virus based vectors

Virus induced gene silencing (VIGS) is a technique, in which the RNA mediated antivirus defence system is used to silence plant genes. Plant viruses, when introduced in plant cells, initiate dsRNA degradation against genomic viral RNA. When viral genome, carrying foreign gene sequence from plant, corresponding genomic mRNAs can be degraded and ultimately results in gene silencing (Lu *et al.,* 2003). Virus induced gene silencing has been widely used for the functional analysis of genes with unknown functions (Baulcombe, 2000). Different viruses have been utilized for the

development of silencing vectors and to understand functions of unknown host factors involved in virus replication and movement in plants (Ruiz *et al.,* 1998). Since the discovery of this technique, various virus vectors have been used in different plant species e.g. Tobacco mosaic virus, Potato virus X and Tobacco rattle virus (Kumagai *et al.,* 1995; Ruiz *et al.,* 1998). Recently, VIGS vectors are extensively used for the understanding of resistance mechanism against plant pathogens. Barley stripe mosaic virus based VIGS vector has been used in wheat to study the resistance mechanism mediated by the *Lr21* gene against leaf rust (Scofield *et al.,* 2005). *Lr21,* belongs to the CC-NBS-LRR class of R genes and has been isolated from cultivated wheat as a resistance source against leaf rust. Similarly, a potato virus X based silencing vector was used in tomato to identify host factors involved in resistance against fungal pathogens (Rowland *et al.,* 2005).

Tomato mosaic virus is an important pathogen and gives mild symptoms when infect tomato plants. Various *R* genes have been isolated, conferring resistance against this virus (*Tm-1*, *Tm-2* and *Tm-2*^{2}). Efforts are made to understand the resistance mechanism and to identify host factors involved in resistance cascade. To contribute in this field, a tomato mosaic virus based silencing vector has been developed for the use in tomato. Tomato mosaic virus was thought to be a suitable virus vector, as it gives milder symptoms in tomato compared to PVX or TMV vectors.

In principle, two types of plant virus vectors have been used so far. One type are *in-vitro* transcription vectors. These vectors, which were first developed by Ahlquest and his co-workers (French *et al.,* 1986), contain a T7 promoter inserted in front of the cDNA sequence of a complete genome of a virus in an *E. coli* plasmid. These can be linearized and *in-vitro* transcribed to generate an infectious copy of the viral genome. The other type of viral vectors are *in-vivo* transcription vectors. These vectors contain a full length cDNA copy of a viral genome behind a promoter active in plant cell i.e. 35S RNA promoter. These vectors generate an infectious viral genome, if they are introduced in plant cells either by DNA infection or by *Agrobacterium tumificiens*.

In this research an "*in-vivo* transcription" tomato mosaic virus vector has been developed which can replicate in plants. For this purpose ToMV cDNA was cloned between the 35S promoter and nos terminator. Two different vectors were developed, one containing both left and right borders of T-DNA and the other containing only the LB to have a minimum possible size of replicating vector (Fig 34). Previously, Horsch and Klee in 1986 showed that deletion of RB from T-DNA allows the transfer of T-DNA genes from Ti plasmid, but the efficiency of stable integration was reduced drastically (Horsch and Klee, 1986). In the present research, it is shown that presence of either one T-DNA border or both does not make any effect on the efficiency of virus replication either by *Agro-infiltration* or in colony inoculation (See 4.3.1, Fig 35). This has been analysed in *N. benthamiana, N. tabacum* and *S. lycopersicum*. Similar results were obtained in different host plants showing that the RB of T-DNA is indispensible for transferring the T-DNA fragment containing virus genomic cDNA in plant genome for transient replication.

Using this plant based tomato mosaic virus vector, a new mutant strains of tomato mosaic virus was selected that can overcome both *Tm-2* and *Tm-2*^{2} resistance. Comparison of ToMV2-22 coat protein ORF (Fig 54) with wild type ToMV CP revealed one amino-acid exchange at position 155 of coat protein from Serine to Phenylalanine which could explain the change in mobility of the protein on SDS gels.

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GENE ID: 920840 ToMVgp4 | 17.5 kDa coat protein [Tomato mosaic virus]
(10 or fewer PubMed links)

 Score =  285 bits (730),  Expect = 7e-75
 Identities = 158/159 (99%), Positives = 158/159 (99%), Gaps = 0/159 (0%)
 Frame = -2

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Sbjct  1    MSYSITSPSQFVFLSSVWADPIELLNVCTNSLGNQFQTQQARTTVQQQFSEVWKPFPQST  60

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Sbjct  121  AIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSAPAS  159
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Figure 54. Alignment of coat protein ORF of wild type ToMV and ToMV2-22 strain. Aminoacid exchange highlighted in blue colour.

The resistance breaking MP of ToMV2-22 contains three aa exchanges in comparison to wt ToMV MP at position 52 (E-K), position 54 (N-D) and position 133 (E-K). So far, one aa exchange at position 133 (E-K) of the 30kDa MP was found in all *Tm-2* resistance breaking viruses, whereas one aa exchange at position 130 (K-E) of the MP is associated with all *Tm-2²* resistance breaking genotypes. These aa exchanges alone are, however, not sufficient for the resistance breaking phenotype of the virus. Additional amino-acid exchanges are needed at position 52 (E-K) or 133 (E-K) or 135 (N-S) for *Tm-2* breaking virus and at position 238 and 244 for a *Tm-2²* resistance breaking virus. Sequence analysis revealed that our virus isolate (ToMV2-22) contains aa exchanges associated with a *Tm-2* resistance breaking genotype (52 E-K, 133 E-K), However, is lacking the substitution, found to be responsible for overcoming *Tm-2²* resistance. Instead, it shows a different exchange at amino acid position 54 (N-D) in the MP which has not been reported before. Deletion analysis of the 30kDa MP in transgenic plant (Weber and Pfitzner, 1998) have already shown, that the C-terminal 30 aa of complete MP are necessary for recognition by the *Tm-2²* gene but region, downstream of aa 230 can also induce a *Tm-2²* dependent necrotic defence reaction.

Therefore because of the high homology of the *Tm-2* and *Tm-2²* proteins, our screening scheme of successive selection for virus mutants breaking *Tm-2* and *Tm-2²* may have led to the isolation of a virus strain that was already adapted to escape the recognition by *Tm-2* type resistance genes. These virus mutants then require only one additional aa exchange to alter the structure of the 30kDa MP to evade *Tm-2²* resistance.

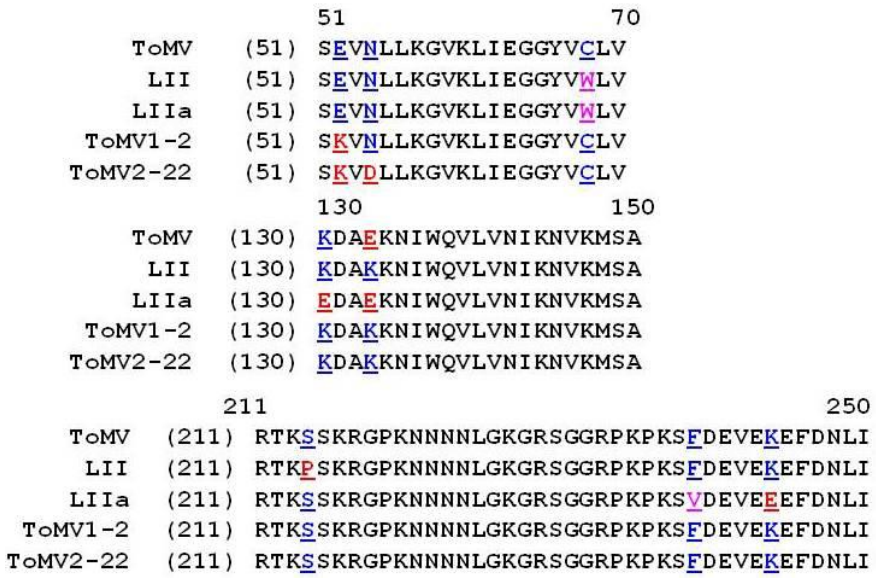

Figure 55. Comparison of Partial amino acid sequence of the 30KDa protein of wild type ToMV, two *Tm-2* resistance breaking ToMV strains (LII and LIIa), *Tm-1* resistance breaking strain ToMV1-2 and *Tm2* and *Tm2²* resistance braking strain ToMV2-22. Amino acid exchanges are depicted in colored letters.

Other *Tm-2²* resistance breaking ToMV strains listed in the literature (Pelham, 1966) are less virulent compared to the wild type ToMV. ToMV2-22 has been tested in three different experiments for growth and further propagation on new host plants. Under green house conditions, it proved to be virulent and the resistance breaking mutations were stable in the viral genome. During infection, it gave symptoms like leaf curling, mosaic development and stunting of tomato plants.

To generate recombinant viral expression vectors, in most cases regulatory elements for the formation of additional sub-genomic mRNA molecules are inserted. Because of the duplication of viral genomic sequences, these vectors are notoriously unstable (Baulcombe, 2000). Therefore, in this thesis, a different approach was taken by using a unique restriction site directly down-stream of the coat protein ORF.

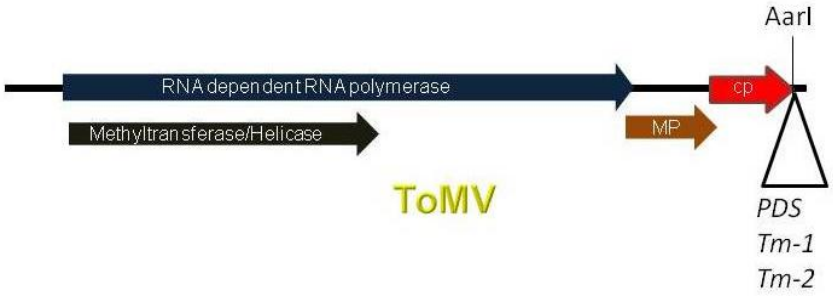

Figure 56. Diagrammatic representation of ToMV genome, indicating the point of insertion of target genes.

Tomato mosaic virus based silencing vectors, were made by inserting different size fragments (250/500bp).Three different candidate genes were used as targets. Phytoene desaturase (PDS) gene was used as a marker of silencing efficiency. PDS gene is involved in the chlorophyll synthesis pathways and has been extensively used to test the silencing efficiency of various VIGS vectors (Ruiz *et al.,*1998).

Similarly *Tm-2* and *Tm-2*^{2} resistance gene fragments were inserted in tomato mosaic virus. Results obtained from our experiments showed however, that the recombinant tomato mosaic virus has the ability of size reversion when it replicates in plant cells. Virus isolated from agro-infiltrated plants always lost the inserted fragments behind the coat protein by recombination and changed to wild type virus. Size reversion is a phenomenon that has been observed extensively in plant viruses especially in ss or ds DNA viruses e.g. Cassava mosaic virus and Cotton leaf curl virus (Etessami *et al.,* 1989; Qian *et al.,* 2008). The reason for this phenomenon in ToMV could be the instability of recombinant viruses during replication because of changes in its genomic structure (Wang *et al.,* 2003). Therefore, further work has to be carried out to understand this mechanism of instability and to develop a strategy to solve this problem.

6. Summary

Viruses are obligatory plant pathogens causing sever diseases, and ultimately great losses in crop yield. Plant viruses, once entered in the cell, make use of host machinery for its own replication and moves from one cell to the other. Natural resistance against virus attack is achieved by the presence of resistance genes (*R* genes). *R* genes recognize viral avirulence (*Avr*) factors in elicitor-receptor manner to initiate resistance cascade. In tomato, the resistance genes *Tm-I*, *Tm-2* and *Tm-2²* are used to protect the plants against infection by tomato mosaic virus. *Tm-2* and *Tm-2²* require recognition of the viral 30kDa movement protein (MP) for triggering resistance response. Sequence analysis of *Tm-2* and *Tm-2²* resistance breaking viruses have shown an amino-acid exchange at position 133 (E>K) is found in all *Tm-2* resistance breaking virus strains, whereas, amino-acid exchange at position 130 (K>E) is associated with *Tm-2²* resistance breaking phenotype (Calder and Palukaitis, 1992). This suggests a physical interaction between resistance genes and 30kDa MP.

In the present study, a unique Split GFP approach is used to analyse the structure and localization of different domains of 30kDa MP in *S. cerviceae* and *N. benthamiana*. Different deletion mutants were fused between two non-overlapping halves of GFP and expressed. Results showed that both N and C terminus as well as the middle part of 30kDa MP (aa 80-150) is present in the cytoplasm with two integral membrane loops. These findings are in contrast with previous *in-vitro* results, which suggest that middle part of 30kDa MP is present in ER lumen, whereas N and C terminus in cytoplasm (Brill *et al.,* 2000). Fluorescence microscopy revealed that GFP fused 30kDa MP deletion mutants were localized on the cytoplasmic side of plasmamembrane and near plasmodesmata. Membrane association of fusion protein confirmed the proper folding and functionality of deletion mutants. Therefore, the structural model of ToMV 30kDa MP has to be revised.

Secondly, to identify the host factors involved in resistance mechanism, initiated by *Tm-2* and *Tm-2²* resistance genes, tomato mosaic virus based vectors were constructed. Two different types of *in-vivo* transcription vectors were constructed, one

containing both right and left border of the T-DNA (pBinSLN) and one without the right border (pBinSLN-RB). Self replication of these vectors were analysed in *N. benthamiana*, *N. tabacum* and *S. lycopersicum*. It was found that the deletion of RB does not affect virus replication, when agro-infiltrated in *N. benthamiana*. pBinSLN-RB was used further for the isolation of a stable and vigorous *Tm-2* and *Tm-2²* resistance breaking ToMV strain through a novel selection scheme. ToMV2-22 contains two amino-acid exchanges at position 54(N>D) and 133(E>K). ToMV2-22 is the first mutant strain of ToMV, which can escape both *Tm-2* and *Tm-2²* resistance simultaneously.

6. Zusammenfassung

Viren sind obligate Parasiten und können bei Kulturpflanzen zu großen Ernteausfällen führen. Wenn Pflanzenviren in die Zelle eingedrungen sind, benutzen sie Wirtsproteine für ihre Replikation und wandern von Zelle zu Zelle. Natürliche Resistenz gegen Virusinfektionen in Pflanzen wird durch Resistenzgene (R-Gene) hervorgerufen. R-Gene erkennen virale Avirulenzfaktoren (Avr) in einer Elicitor-Rezeptorreaktion und lösen eine Abwehrkaskade aus. In Tomatenpflanzen benutzt man die Resistenzgene *Tm-1*, *Tm-2* und *Tm-2²* zum Schutz gegen Tomatenmosaikvirus. *Tm-2* und *Tm-2²* erfordern die Erkennung des viralen 30 kDa „movement protein“ (MP) für die Auslösung der Abwehrreaktion. Sequenzanalyse von *Tm-2* und *Tm-2²* resistenzdurchbrechenden Viren zeigte entweder einen Aminosäureaustausch an Position 133 (E>K) bei allen *Tm-2* durchbrechenden Viren während ein Aminosäureaustausch an Position 130 mit einem *Tm-2²* durchbrechenden Phänotyp assoziiert ist. Diese Befunde weisen auf eine direkte Interaktion zwischen diesen Resistenzgenen und dem 30 kDa MP hin.

In der vorliegenden Arbeit wurde eine besondere Form des „split GFP“ Ansatzes verwendet um die Struktur und Lokalisation verschiedener Domänen des 30 kDa MP in *S. cervisiae* und *N. benthamiana* zu analysieren. Verschiedene Deletionen des MP wurden an zwei nicht überlappende Hälften des grün fluoreszierenden Proteins (GFP) fusioniert und expremiert. Wenn sich diese Domänen im selben Zellkompartiment nahe beieinander befinden, können sich die GFP Teile zusammenlagern und ein fluoreszierendes Protein bilden. Die Ergebnisse dieser Experimente zeigen, dass sich sowohl der N- als auch der C-Terminus und der mittlere Teil des MP (As 80-150) im Cytoplasma befinden und sich zwei integrale Membranbereiche vermuten lassen. Fluoreszenzmikroskopie zeigte eine Lokalisation der 30kDa MP GFP Fusionen an der cytoplasmatischen Seite der Zellmembran in der Nähe der Plasmodesmata. Alle Fusionsproteine waren membranassoziert, was auf eine korrekte Faltung hindeutet.

Diese Befunde stehen im Kontrast zu vorhergehenden *in vitro* Experimenten, die nahelegten, dass sich der mittlere Teil des 30 kDaMP im ER Lumen befindet, während N- und C-Terminus im Cytoplasma sind. Deshalb muß das bisherige Strukturmodel des ToMV 30kDa MP revidiert werden.

Um Wirtsfaktoren zu identifizieren, die in den Resistenzmechanismen involviert sind, welche von den Resistenzgenen *Tm-2* und *Tm-2²* ausgelöst werden, wurden Vektoren auf der Basis des ToMV entwickelt. Zwei unterschiedliche Typen von „*in vivo*“ Transkriptionsvektoren zur Agroinfektion wurden konstruiert. Ein Typus enthält sowohl die rechte als auch die linke „T-border“ Sequenz der T-DNA (pBinSLN) während der andere ohne die rechte „T-border Sequenz konstruiert wurde (pBinSLN-RB). Die Replikation dieser Vektoren wurde *in N. benthamiana, N. tabacum* und *S. lycopersicum* analysiert. Es zeigte sich, das eine Deletion der rechten „T-border“ keinen Einfluß auf die Virusvermehrung nach Agroinfiltration von *N. benthamiana* hatte. pBinSLN-RB wurde verwendet um mit einem neuartigen Selektionssystem eine stabile und schnell wachsende ToMV Mutante isoliert, die sowohl die *Tm-2* als auch die *Tm-2²* Resistenz durchbrechen kann (ToMV2-22). ToMV2-22 besitzt zwei Aminosäureaustausche im 30 kDa MP an Position 54 (N>D) und 133 (E>K). ToMV2-22 ist die erste Mutante von ToMV, die beide Resistenzgene *Tm-2* und *Tm-2²* gleichzeitig überwinden kann.

7. Abbreviations

3' -	carboxyterminus of DNA or RNA
5' -	aminoterminus of DNA or RNA
A	adenine
APS	ammonium persulfate
Avr	Avirulence
bp	base pairs
° C	degrees Celsius
C. elegans	*Caenorhabditis elegans*
c – terminal	on the carboxyterminus
Da	Dalton
DMSO	Dimethyl sulfoxide
DTT	1,4 - Dithiothreitol
dNTP	2’ – deoxynucleotide – 5’ - triphosphate
E. coli	*Escherichia coli*
EDTA	Diaminoethanetetraacetic acid
et *al.*	and others
EtBr	ethidiumbromide
etc.	et cetera
EtOH	ethanol
ER	endoplasmic reticulum
f.c.	final concentration
g	gram
g	gravital acceleration
H	histidine
h	hour(s)
HRP	horse radish peroxidase
IPTG	Isopropyl β – D – 1 - thiogalactopyranoside
kb	kilo base pairs
kDa	kilo Dalton

l	liter
LB	Luria Bertani medium
M	molar
MCS	multiple cloning site
mg	milligram
min	minute(s)
ml	milliliter
mM	mili molar
mol	mol
ng	nanogram
nm	nanometer
n – terminal	at the aminoterminus
OD_x	optical density at x nm
p.a.	for analytical use (pro analysi)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDS	Phytoene desaturase
PEG	polyethyleneglycol
pH	cologarithm of the concentration of hydrogen ions in solution
PMSF	Phenylmethylsulphonyl fluoride
PVX	Potato virus X
R	Resistance
rpm	rounds per minute
SD	synthetic dropout medium
SDS	sodium dodecyl sulfate
s	seconds
T	tryptophan
TAE	Tris – acetate – EDTA buffer
Taq	*Thermophilus aquaticus* DNA polymerase
TB	Tris Borat buffer
TBS	Tris buffered saline
TE	Tris – EDTA buffer

TEMED	Tetramethylethylenediamine
TMV	Tobacco mosaic virus
ToMV	Tomato mosaic virus
Tris	2 – amino – 2 – (hydroxymethyl) propane – 1,3 - diol
TTBS	Tris buffered saline with Tween 20
u	enzyme units
UV	ultraviolet
V	Volt
X – Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
YPAD	yeast extract – peptone – dextrose medium + adenine

8. References

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Affidavit

I hereby declare that this thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no other sources have been used in the preparation of this thesis than those indicated in the thesis itself. This thesis was never submitted in this or any other form to an examination board.

Fariha Tanwir
Stuttgart Hohenheim

Curriculum Vitae

Personal Details

Name	Fariha Tanwir
Date of Birth/Place	18.12.1981/Faisalabad, Pakistan
Nationality	Pakistani
Marital Status	Married

Educational Background

1994-1995 Secondary school certificate/ Matric (Science)
Govt. Girls High School Faisalabad, Pakistan.

1996-1998 Higher secondary school certificate/ F.Sc. (Pre-medical)
Govt. College Madina Town, Faisalabad, Pakistan.

1998-2002 Bechlor of sciences/ B.Sc(Hons) Agriculture
University of Agriculture, Faisalabad, Pakistan.

2003-2005 Master of philosophy (Mphil-Biotechnology)
Quaid-i-Azam University, Islamabad, Pakistan.

2005-2007 Research associate (Wiss. Mitarbeiter)
Dept. of Plant Molecular Biology and Virology
University of Stuttgart, Stuttgart, Germany.

2007-2011 PhD studies under the supervision of Prof. Artur J.P.Pfitzner
University of Hohenheim, Stuttgart, Germany.

List of Publications.

1. Kleinow, T., Nischang, M., Beck, A., Kratzer, U., **Tanwir, F**., Preiss, W., Kepp, G., and Jeske, H. (**2009**a). Three C-terminal phosphorylation sites in the Abutilon mosaic virus movement protein affect symptom development and viral DNA accumulation. **Virology** 390, 89-101.

2. Kleinow, T., **Tanwir, F**., Kocher, C., Krenz, B., Wege, C., and Jeske, H. (**2009**c). Expression dynamics and ultrastructural localization of epitope-tagged Abutilon mosaic virus nuclear shuttle and movement proteins in Nicotiana benthamiana cells. **Virology** 391, 212-220.

Posters & Presentations.

1) Poster presented under the topic of " **Simultaneous silencing of regulatory sequences of DNA A and DNA ß in CLCuV in tobacco**" in the Yearly meeting of the DPG working group "virus diseases of the plants" in 2006 at Black Forest , Stuttgart, Germany.

2) Poster presented under the topic of "**Comparison between different expression systems for AbMV movement proteins in host and non host plants to study protein interactions**" in 2007 at plant biology meeting of American society of plant biologists held in Chicago, America.

3) Presentation delivered under the topic of "**Geminiviruses move through plants using two systems**" in Sept. 2007 at 125th meeting of German Botanical Society in Hamburg, Germany.

4) Poster presentation under the topic of "**Structural analysis of tomato mosaic virus movement protein using split GFP system in yeast**" in Feb. 2010 at 23rd conference of molecular biology of plants in Dabringhausen, Germany.

5) Participated in 55th meeting of Nobel Prize winners in Lindau, Germany in 2005.

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