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Molecular Farming: Production of Antimicrobial Peptides in different *Nicotiana* species

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SUMMARY

As a consequence of their extensive use in the last decades more and more classical antibiotics lose their activity against emerging multi-resistant microorganisms. Hence, the urgent need for new antibiotic compounds is obviously. Antimicrobial peptides (AMPs) are thought to be an attractive alternative to convential antibiotics. Their potential lies not only in their high antimicrobial activity but also in their mode of action, namely targeting microbial membranes. This way of microbe killing hinders bacteria to evolve resistance against AMPs, since this would imply a reorganization of the bacterial membrane. For the application in plant protection and human medicine we developed novel peptides that are much more active against plant and human pathogens than natural occurring antimicrobial peptides. But the synthetic production of these peptides is very expensive. Hence, the economic production in different *Nicotiana* species serving as inexpensive green factories was evaluated during this study. Several options for transfer and heterologous expression of genes encoding for the *de novo* designed peptides in plants were compared, including stable transformation of plants and transient viral expression systems. Next to the use of a commercial transfection method, a *Tobacco mosaic virus* (TMV) derived expression system following the 'full virus vector strategy' with the viral coat protein as fusion partner was generated. The latter was the method of choice for *in planta* production of the short peptides. After infection of different tobacco species as inexpensive and robust green factories, virus-like particles presenting the peptides all over their surface were detected. Following successful production of the desired peptides fused to TMV in *Nicotiana benthamiana*, recombinant virions were purified and the peptides released from their fusion partner by chemical cleavage. An efficient protocol for recombinant peptide purification was elaborated using high resolution chromatographic methods and highest purity was detected. The final yield ranged between 0.2 and 0.5 mg of pure peptide per 20 g of infected fresh leaf biomass and *in vitro* antimicrobial activity under varying assay conditions was similar to synthetic peptides.

Taken together, the developed and subsequently described method is well suited for recombinant peptide production in plants and provides an efficient purification protocol to recover biologically active antimicrobial peptides from plant material.

ABBREVIATIONS

ACN	Acetonitrile
AMP	Antimicrobial peptide
bp	Base pairs
CaMV	*Cauliflower mosaic virus*
cDNA	Complementary DNA
CP	Coat protein
CNBr	Cyanogen bromide
CV	Column volume
DEPC	Diethyl pyrocarbonat
DNA	Deoxyribonucleic acid
dpi	Days post infection
DTT	Dithiothreitol
GFP	Green fluorescent protein
GOI	Gene of interest
GST	Glutathione S-transferase
h	Hour(s)
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HR	Hypersensitive response
kDa	Kilodalton
LB	Luria-Bertani
LMW	Low molecular weight
MALDI	Matrix assisted laser desorption ionisation
MES	2-(*N*-morpholino)ethanesulfonic acid (IUPAC nomenclature)
MIC	Minimal inhibitory concentration
min	Minute(s)
MOPS	3-morpholinopropane-1-sulfonic acid (IUPAC nomenclature)
MS	Murashigge & Skoog
ND	Not determined
Nos	Nopaline synthase
nt(s)	Nucleotide(s)
OD	Optical density
oN	Over night
PCR	Polymerase chain reaction
PMP	Plant-made pharmaceutical
PVDF	Polyvinylidene defluoride
RdRP	RNA-dependent RNA polymerase
RES	Restriction site
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPC	Reversed phase chromatography
rpm	Revolutions per minute

RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide electrophoresis
SEC	Size exclusion chromatography
SEL	Size exclusion limit
SP	Synthetic peptide
SP6	Bacteriophage SP6
Taq	*Thermus aquaticus*
TMV	*Tobacco mosaic virus*
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume

Amino acids and nucleotides are expressed according to the IUPAC code (International Union of Pure and Applied Chemistry). All used base units and derived units are conform to the convention of the SI-system (Système International d'unitès).

LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1 ANTIMICROBIAL PEPTIDES

1.1.1 ANTIMICROBIAL PEPTIDES - ANCIENT MOLECULES IN INNATE HOST DEFENSE

Higher organisms are permanently exposed to a great variety of microorganisms, such as viruses, mycoplasma, bacteria, fungi and nematodes. Mostly, this cohabitate takes place harmoniously and results only seldom in severe infections. The reason for this is not only the more or less strong impermeability of outer barriers of multicellular organisms, but also the development of several defense strategies during evolution. The most sophisticated defense mechanism is the use of antibodies and killer cells to recognize and fight invading pathogens (Thomma et al., 2002). But these adaptive immune responses evolved evolutionary rather late and are elaborated only in a small subset of living species, namely higher vertebrates. Since also insects and plants survive infections, although they are not capable of mounting an antibody mediated defense response, further mechanisms of microbe killing must exist (Strominger, 2009). A large group of low molecular weight natural compounds exhibiting antimicrobial activity has been isolated during the last decades. Many of these defense molecules are antimicrobial peptides (AMPs) and more than 1200 different peptides are described until now displaying antiviral, antifungal, antibacterial and even anticancer activity (Marshall and Arenas, 2003; Wang et al., 2009). AMPs are effective defense weapons distributed throughout the animal and plant kingdom, suggesting that they have played an important role in the successful evolution of complex multicellular organisms (Andreu and Rivas, 1998; Fritig et al., 1998; Garcia-Olmedo et al., 1998; Simmaco et al., 1998; Thomma et al., 2002; Zasloff, 2002; Castro and Fontes, 2005). They are part of the non-specific defense system and include inducible and permanently produced peptides with activities against different types of microorganisms. Because AMPs are thought to target the microbial cellular membrane, a design feature that distinguishes broad species of microbes from multicellular plants and animals, their potential as therapeutics and use as alternative antibiotics is currently under strong investigation (Zasloff, 2002).

1.1.2 CLASSIFICATION AND PHYSICO-CHEMICAL PROPERTIES OF AMPS

Despite their high sequence diversity some characteristics are shared by virtually all known AMPs (van 't Hof et al., 2001). First, short size with a molecular mass of 1-5 kDa, but even di- and tripeptides with antimicrobial activities are known (Chmara et al., 1998; Svenson et al., 2009). Second, a cationic character, achieved by a high number of the basic amino acid residues arginine and lysine distributed all over the peptide. And third the high hydrophobicity. Both latter characteristics result often in amphipathic structures with separate hydrophilic and hydrophobic domains (***Figure 1***). Upon contact with pathogen membranes a helix-formation is often observed, whereas in aqueous solutions many AMPs are disordered or unstructured (Hancock and Diamond, 2000). Ratios of hydrophobic to charged residues can vary from 1:1 to 2:1 (Brogden, 2005).

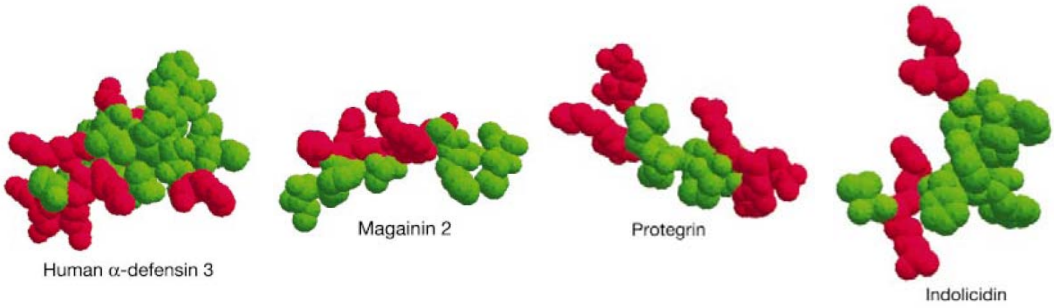

Figure 1 *Clusters of hydrophobic and cationic amino acids in distinct domains of several antimicrobial peptides. Although certain peptide structural groups occur, no positional conservation of even classes of amino acids is known. Red, basic (positively charged) amino acids; green, hydrophobic amino acids (Zasloff, 2002).*

Based on chemical-structural criteria, AMPs are divided into four groups (Garcia-Olmedo et al., 1998; Hancock and Lehrer, 1998; Epand and Vogel, 1999; Bulet et al., 2004; Sitaram, 2006) as shown in ***Figure 2***: (I) Cysteine-containing β–sheet peptides with at least two disulfide bonds, including defensins of several mammals and plants, and protegrins produced in porcine neutrophils.

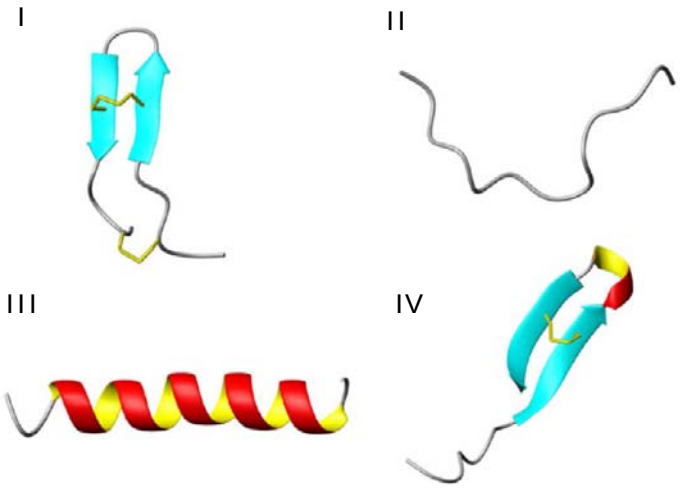

Figure 2 *Grouping of AMPs (Powers and Hancock, 2003). (I) tachyplesin, β–sheet peptide with two disulfide bonds. (II) indolicidin, extended peptide lacking classical secondary structure. (III) magainin, linear α-helical peptide. (IV) thanatin, peptide with loop structure by the presence of a single bond.*

(II) Peptides with over-represented amino acids, e.g. the histidine-rich histatins from human salivary and the tryptophan-rich indolicidin from cattle. (III) Peptides with loops stabilized by one disulfide bridge, e.g. bactenecin from cow neutrophils. (IV) Linear amphipathic α-helical peptides, e.g. magainins whose name giver is found in skin secretions of the African clawed frog *Xenopus laevis* and was one of the first identified and characterized AMP (Chakrabarti et al., 2003). These groups can be subdivided into several families resting on specific characteristics. In addition, there are some peptides harboring antimicrobial activity that cannot be assigned into above mentioned groups. Maximim H5 from amphibians or dermcidin from humans for example, both showing antimicrobial activities, must be classified as anionic peptides. Also fragments of larger proteins are known to exhibit antimicrobial activities, e.g. lactoferricin from lactoferrin or domains of bovine α-lactalbumin, lysozyme and ovalbumin (Brogden, 2005).

1.1.3 ASSUMED MODE OF ACTION

Because of their cationic and hydrophobic features, antimicrobial peptides are thought to interact primarily with negatively charged biomembranes (Epand and Vogel, 1999; Brogden, 2005). Cell membranes of many bacteria are predominantely composed of hydroxylated phospholipids like phosphatidylglycerol and phosphatidylserine and thereby sustain a negative charge (Yeaman and Yount, 2003). This is complemented by lipopolysaccharides of the outer membrane of gram-negative bacteria and teichonic acids of the membrane of gram-positive bacteria. The cationic parts of the peptides interact with these negatively charged structures of biomembranes. Incorporation of the peptides into the membrane is supported by their hydrophobic parts and leads to permeabilization of the membranes, whereas the induction of well-defined pores or the unorganized disintegration is under discussion (Huang, 2006). The insertion of the peptides is explained by several different models, whereas the barrel-stave model, the toroidal-pore model, and the carpet model are the most popular ones (Brogden, 2005).

The barrel-stave model indicates that attached linear α-helical peptides aggregate and insert into the membrane in a way, that the hydrophobic parts of the peptides interact with the lipophilic core of the cell membranes and the hydrophilic parts of the peptides form the interior region of the pore (***Figure 3 A***). Peptides that act via this mechanism must fulfill several criteria and it was shown that only a few AMPs, including e.g. pardaxin or alamethicin, indeed can be considered as channel-forming peptides. First, the AMP's overall hydrophobicity must be high enough to penetrate the lipidic core of the membrane and hence the membrane-peptide interaction is mainly determined by hydrophobic interactions. In addition, AMPs should self-associate in the membrane-bound state to form bundles of transmembrane pores and these pores would increase due to the recruitment of more monomers. To span the lipid bilayer, a minimal length of 22 amino acids for α-helical peptides and 8 amino acids for β-sheeted structures is required. As only a few pores are necessary to disrupt the membrane potential in cells, the MIC of those AMPs would be far below micromolar concentrations (Shai, 2002).

An extension of the barrel-stave model is given by the wormhole- or toroidal pore model where the attached antimicrobial peptides insert into the membrane and induce the lipid monolayers to bend continuously through the pore. In this arrangement the interior part of the pore is formed by both the inserted peptides and the hydrophilic heads of the lipids and was shown to be the mode of action of magainins, protegrins and melittins. In contrast to the barrel-stave model the peptides are always associated with the lipid head groups, even when vertically inserted.

Since the mode of action of only some AMPs matches the barrel-stave or toroidal pore model an alternative mechanism was described that is independent of a specific peptide structure and does not require recognition between membrane-bound monomers and also the insertion of peptides into the hydrophobic core to form transmembrane channels is not necessary. In the so called carpet model the peptides are bound to the surface of the membrane by electrostatic interactions with anionic membrane constituents in a carpet-like manner (***Figure 3 B***).

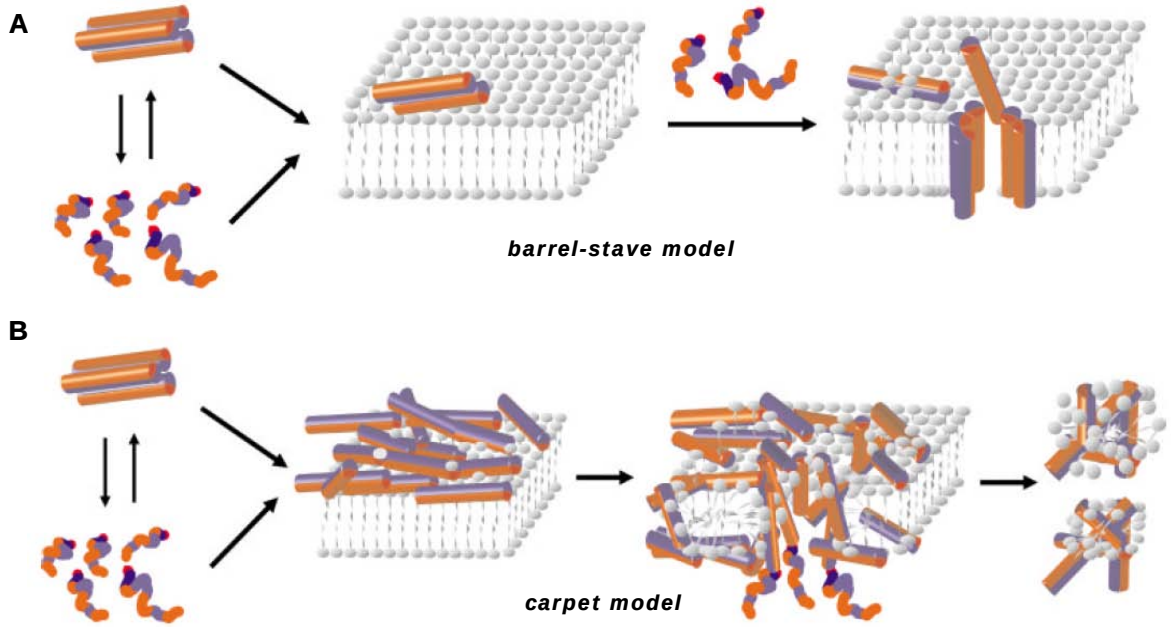

Figure 3 *Proposed modes of action of AMPs (Shai, 2002). (A) The barrel-stave model assumes that peptides first assemble on the surface of the membrane, followed by insertion into the lipid core where more peptide monomers are recruited. (B) The carpet model describes the binding of the peptides with their hydrophobic faces to the membrane. Above a certain threshold the membrane is permeated, transient pores are formed and membrane disintegration is the consequence.*

Above a certain threshold concentration the peptides disrupt the bilayer what may include the formation of holes and allows additional peptides to access the membrane (Shai, 2002; Bechinger and Lohner, 2006).

Next to their antimicrobial activity by cell disruption AMPs may have other targets located intracellulary. There is increasing evidence that some peptides can bind to nucleic acids, thereby disturbing DNA, RNA and protein synthesis. Also the inhibition of enzymatic activity or the activation of autolysin is discussed. One important point necessary to disrupt intracellular key processes is the entry of the AMPs into the cells. Two general mechanisms are proposed, spontaneous lipid-assisted translocation and stereospecific receptor-mediated membrane translocation. However, the precise mechanism for peptide translocation remains largely unknown and may vary from peptide to peptide. But obviously, most AMPs show strong similarities in charge, structure and membrane interactions with cell-penetrating peptides, which are thought to enter mammalian cells by passive transport (Nicolas, 2009).

Bioactivities of AMPs are not restricted to the direct killing of microorganisms. In fact, natural antimicrobial peptides represent lead-molecules that can boost innate immune responses and selectively modulate pathogen-induced inflammatory processes. It is supposed that AMPs can act as immune regulators either by suppression or enhancement of immune responses. So they are involved in many elements of inflammatory processes in mammals, including the recruitment of T-cells by acting as chemotaxins, the suppression of cytokinine production or even the turning on of specific macrophage genes (Hancock and Diamond, 2000; Mookherjee and Hancock, 2007). Because under physiological conditions mammalian AMPs often have weak antimicrobial activities, their

involvement in immunomodulatory processes might be even more important (Hancock and Sahl, 2006).
Also in transgenic plants producing indolicidin and magainin derived AMPs, not only the resistance to pathogens was increased but also the yield and the vegetative growth was enhanced by a still unknown mechanism (Xing et al., 2006). To respect the whole expanse of predicted roles of AMPs in host defense responses and signalling the term 'host defense peptides' is increasingly used (Hancock and Sahl, 2006; Giuliani et al., 2008).

1.1.4 AMPS IN PLANT DEFENSE

Especially plants, which are as consequence of their sessile lifestyle constantly exposed to a large array of pathogenic organisms need a quick and effective defense response. Beside the production of ROS and the alteration of cell-wall constitution upon pathogen interaction huge amounts of preformed or inducible low molecular weight compounds exist in plants to ensure survival and to defend invading pathogens. Among them, antimicrobial peptides play an important role. Because AMPs are single gene products with a small size, they can be produced fast and at variable levels after pathogen contact, but require only minimal amounts of host resources like biomass and energy input (Broekaert et al., 1995). This might explain the success and the evolutionary consistency of AMPs as part of the innate immune system in virtually all multicellular organisms (Giuliani et al., 2008).
A high number of antimicrobial peptides were isolated from different plant species so far (Garcia-Olmedo et al., 1998; Wong and Ng, 2003; De Lucca et al., 2005; Egorov et al., 2005; Wong and Ng, 2005; Ho et al., 2007). Based mainly on the disulfide bond structure the peptides can be divided into different families, including defensins, thionins, lipid transfer proteins, knottins, heveins, and snakins, whereas each family can consist of up to 300 members (Garcia-Olmedo et al., 1998; Berrocal-Lobo et al., 2002; Castro and Fontes, 2005; Silverstein et al., 2005). The majority of the plant antimicrobial peptides shows complex globular structures, with β-sheets stabilized by numerous disulfide bridges (Garcia-Olmedo et al., 1998; Thomma et al., 2002). Furthermore, most of the isolated plant antimicrobial peptides exhibit toxic activities primarily against pathogenic fungi and rarely against pathogenic bacteria (Thomma et al., 2002). This might be an evolutionary consequence, because most plant diseases caused by microorganisms are of fungal origin, thus driving plants to concentrate on the development of effective fungizides (Agrios, 2005). In contrast, most mammalian AMPs show antibacterial activities, because fungi usually seldom cause severe health problems in mammals. Actually, the human ecosystem presents a poor habitat for fungi, due to the neutral pH and the strong bacterial concurrence (Boman, 2003). Enhanced production of several types of antimicrobial peptides has been demonstrated in many different plant species upon pathogen infection (Bohlmann et al., 1988; Molina and Garcia-Olmedo, 1993; Penninckx et al., 1996; Thomma et al., 2001), after treatment with pathogen-derived compounds, such as LPS, Harpin or commercially available plant defense activators (von Rad et al., 2005; Livaja et al., 2008). A lot of effort has been spent on identification of

signalling pathways regulating production of antimicrobial peptides using defensin PDF1.2 of the model plant *Arabidopsis thaliana*. The expression of *PDF1.2* depends on the concomitant activation of ethylene- and jasmonic acid-pathways (Penninckx et al., 1998). Nitric oxide, however, also an important signalling molecule involved in plant defense reactions (Delledonne et al., 1998; Durner et al., 1998), does not induce *PDF1.2* expression. The reason therefore seems to be the NO-induced enhanced biosynthesis of salicylic acid, which is known to inhibit jasmonic acid biosynthesis (Huang et al., 2004). The involvement of different important signalling molecules in activating or inhibiting *PDF1.2* expression demonstrates the possible complex role and regulation of antimicrobial peptides during local and systemic resistance responses in plants (Castro and Fontes, 2005).

1.1.5 THE POTENTIAL OF AMPS AS ALTERNATIVE ANTIBIOTICS OR THERAPEUTIC AGENTS

As a consequence of the extensive use of antibiotics during the last decades more and more resistant microorganisms evolved. Especially in human medicine the resistance against commonly used antibiotics such as penicillin, streptomycin, vancomycin and fluoroquinolones or even worse, the multi-resistance to several antibiotics, might lead in the near future to a loss of effective therapeutics for infections caused by bacteria like *Staphylococcus aureus* (Marshall and Arenas, 2003). In addition, the use of antibiotics against animal pathogens in cattle breeding or in aquaculture contributes to the release of antibiotics into the environment and hence favors the selection of resistant microbes. In plant protection the application of antibiotics is also a common practice, e.g. spraying of streptomycin to control *Erwinia amylovora*, the cause of the fireblight disease mainly on Rosaceae. Particularly in plants bacterial infections are hard to overcome considering that plant disease control is mainly based on the application of chemical pesticides, which are more and more under strong restrictions and regulatory requirements (Montesinos, 2007). Given that of the 36.5% average of total loss of all crops produced worldwide 14.1% are caused by diseases, which was a total annual loss of about 220$ billion in 2002 – still not included the 6-12% losses of crops after harvest (Agrios, 2005) – the urgent need for new antimicrobial agents becomes apparent.

AMPs are considered as natural antibiotics and therefore have attracted the interest of researchers for many years. With the increasing demand for compounds with antimicrobial activity combined with resistance minimizing properties these peptides might serve as templates for novel antibiotics. Especially their mode of action, namely targeting fundamental features of microbial cell membranes, should avoid or at least reduce the risk of resistance development in microbial populations. Indeed, this happened in the past to every new antibiotic within a few years of its utilization (Perron et al., 2006). Resistance formation would imply a reorganisation of microbial membranes or the evolvement of active detoxification mechanisms. Furthermore, the difference in prokaryotic and eukaryotic membrane architecture imparts selectivity of AMPs for microorganisms and reduces toxic side effects against cells of higher organisms. Several attempts are going on to widen the antibiotic spectrum in human medicine with

derivatives of natural occuring AMPs and in 2006 four candidates were successful in phase 3 clinical trials (Hancock and Sahl, 2006).
Also in the field of plant protection and plant breeding AMPs are of great concern. Numerous transgenic plants expressing any kind of AMP have been generated showing enhanced resistance against a diverse array of pathogens. An indolicidin variant expressed in *Nicotiana tabacum* protected not only against *Erwinia carotovora* and *Botrytis cinerea*, but also against TMV (Bhargava et al., 2007). *HBD-2* expressed in Arabidopsis also defended *B. cinerea* successfully (Aerts et al., 2007) and potato plants transformed with an alfalfa defensin were more resistant against *Verticillium dahliae* as wildtyp plants (Gao et al., 2000).
Beside natural occuring AMPs several *de novo* designed peptides were produced in plants. The 17 amino acids counting peptide D4E1 showed high and broad activity against several fungal pathogens, when expressed in transgenic tobacco or cotton (Rajasekaran et al., 2005). The advantage of synthetic peptides is obvious. Several examples show, that they are more target specific (Powell et al., 2000), possess increased efficacy at lower concentrations and a broader spectrum of activity. In addition, they are less susceptible to proteolytic degradation and are less toxic to plant cells or mammalian cells, due to a low hemolytic activity (Ali and Reddy, 2000). Since the cationic and hydrophobic characteristics of the antimicrobial peptides determine their mode of action, direct modification of these features allows the rational design and targeted construction of new AMPs.

1.2 MOLECULAR PHARMING

1.2.1 PLANT BASED PRODUCTION OF BIOPHARMACEUTICALS – PROMISES

For centuries, plants have served as a natural source of treatments and therapies. During the last decade, also interest in plants as heterologous expression system has significantly increased, especially for the production of biomedically important proteins (Koprowski and Yusibov, 2001). Indeed, it has become obvious over the last couple of years that current fermentation capacities will not be sufficient to manufacture all biopharmaceuticals because the market and demand for biologics is continuously and very rapidly growing (Knäblein, 2005). New advances in biotechnology make it possible to turn plants into ‘factories’ that produce therapeutic proteins (***Figure 4***). Developing therapeutic proteins in plants can be a safer, more efficient and cost-effective method of protein production (http://www.bio.org/healthcare/pmp/keypoints.asp) for several reasons. (I) Plants are the most efficient producers of biomass and proteins on the planet and can do so without propagation of human pathogens or other mammalian contaminants. Compared to existing production methods, plants have a superior ability to assimilate genetic information and produce complex proteins that can be used to make more effective therapeutics. All they require is sunlight, water, carbon dioxide, and minerals but they provide an optimal system for recombinant protein expression (Wagner et al., 2004).

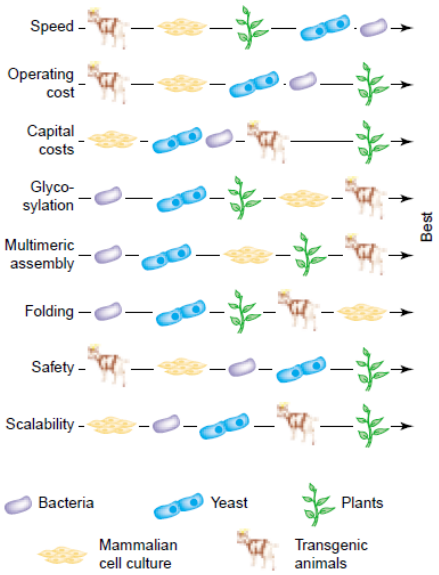

Figure 4 *The use of plants for commercial protein production. Recombinant protein expression in plants is safe, up-scalable and requires low operating and capital costs. Even the multimeric assembly of several protein subunits performs better in plants than in mammalian cell culture (Raskin et al., 2002).*

(II) Traditional cell culture methods require significant capital and labor investment (Lico et al., 2008). There are significantly lower facility and production costs associated with PMPs because the technology is being developed in natural, renewable resources. The estimated costs of producing recombinant proteins in plants could be 10- to 50-fold lower than producing the same protein by *E. coli* fermentation (Kusnadi et al., 1997). And because plant growth is not limited to special manufacturing facilities, it will be relatively easy to scale up production to meet increased and varied demand. (III) Traditional protein-manufacturing practices are facing a major global capacity shortage for the production of biotechnology medicines. Worldwide, there are less than two dozen facilities capable of large-scale biotechnological manufacturing. Thus, the biotechnology industry faces a worldwide factory shortage. Traditional methods cannot produce sufficient quantities of therapeutic proteins to meet patient population needs. The addition of plants, improved through biotechnology to become protein-producing facilities, can help alleviate this shortage. (IV) Also the infrastructure and expertise for planting, harvesting and processing of plant material already exists. Production and cost advantages of plant-made pharmaceuticals can allow more capital to be invested in research and development of new therapeutics, and expanded manufacturing capacity of high-quality proteins will stimulate development of more medicines by removing a key hurdle to mass production. (V) Higher plants generally synthesize proteins from eukaryotes with correct folding and activity and plant cells harbor the capability to direct proteins to environments that reduce degradation (Horn et al., 2004).

1.2.2 PLANT BASED PRODUCTION OF BIOPHARMACEUTICALS – PITFALLS

Although plants have many advantages over other production systems, notably in terms of practicality, economy and safety there are several constraints hindering the widespread use of plants as bioreactors that have to be adressed (Twyman et al., 2003).

Important factors are quality and homogeneity of the final product. Especially in the case of producing pharmaceuticals in nonedible plants like tobacco, precise purification procedures have to be established to avoid copurification of toxic or antigenic plant metabolites (Giddings et al., 2000). Also differences in glycosylation have to be considered, as the glycosylation pattern of transproteins in plants differs from those produced in animals or animal cells *in vitro* (Gomord et al., 2010). But also recombinant protein production in animals or animal cells holds risks like transmission of zoonotic diseases caused by bacteria, viruses or prions, contamination with animal proteins and DNA or alterations in the structure of the recombinant protein. Even protein glycosylation is of concern as not only between mammalian species, but actually between cell types or metabolic states of the same cell the pattern of N-linked glycosylation varies (Kind and Schnieke, 2008).

To date, only few recombinant plant products have reached the marketplace, but some plant based biopharmaceuticals are already in phase III clinical trials (Horn et al., 2004; Knäblein, 2005; Coku, 2007). Beside these biological aspects also regulatory criteria must be taken into account. Despite encouraging evidence concerning the positive socioeconomic and environmental benefits linked to 'genetically modified plant biotechnology' further development is often constrained by a lack of harmonization among national regulatory frameworks. Benefits could be lost, if the regulations in different parts of the world are not brought into line or are at least made mutually compatible (Ramessar et al., 2008).

1.2.3 STRATEGIES FOR HETEROLOGOUS PROTEIN PRODUCTION IN PLANTS

There are different methods to produce recombinant proteins in plants whereas stable nuclear transformation is the most common. To transfer foreign genes into plants and incorporate them stable into the host nuclear genome *Agrobacterium tumefaciens* or particle bombardment is usually used (Tzfira and Citovsky, 2006). In many cases, the transgene of interest is controlled by a constitutive promoter as the CaMV 35S promoter for strong expression or the Nos promoter for moderate expression. But also tissue-specific promoters are used and notably seed-specific promoters cause the accumulation of the desired proteins in mature seeds (Boothe et al., 2010). Beneficial of latter approach is that proteins of interest are stable in seeds for a prolonged period and harvested seeds can easily be stored. Thereby, upstream and downstream processing operations can be disconnected to simplify purification procedures. But also on the protein level the proteins of interest can be targeted to subcellular compartments by including signal peptides to make use of the plants' secretory pathways (Gleba et al., 2005; Vitale and Pedrazzini, 2005). Although recombinant protein production following this strategy is well established, the yields are quite low, mainly in the range of 0.1-0.5% of total soluble protein and the R&D duration time usually takes more than 18 months (***Table 1***). In addition, the eventuality to cross with native species or food crops has to be kept in mind even though meanwhile technologies exist to prevent or at least minimize outcrossing events (Horn et al., 2004).

Table 1 *Proteins of commercial interest produced in plants (Kusnadi et al., 1997).*

Recombinant protein	Molecular weight (kD/subunit)	Origin	Host	Production level
α-Amylase	55.2	*Bacillus licheniformis*	Tobacco	0.3% of soluble leaf protein
Aprotinin	6	Bovine	Corn	0.07% of soluble seed protein
Avidin	15	Chicken	Corn	6.0% of soluble seed protein
Chymosin	30	Calf	Tobacco, potato	0.1–0.5% of soluble protein
Cyclodextrin glucanotransferase	76	*Klebsiella pneumoniae*	Potato	<0.01% of soluble tuber protein
Enkephalin	0.5	Human	Arabidopsis	0.1% of soluble seed protein
Erythropoietin	37	Human	Tobacco	0.0026% of soluble protein
Glucoamylase	74	*Aspergillus niger*	Potato	Not available
β-Glucuronidase	68	*Escherichia coli*	Corn	0.7% of soluble seed protein
Growth hormone	21	Trout	Tobacco	0.1% of soluble leaf protein
Heat-labile enterotoxin B	39	*Escherichia coli*	Tobacco, potato	0.001% of soluble leaf protein, 0.01% of soluble tuber protein, resp.
Hepatitis B surface antigen	24	Hepatitis B virus	Tobacco	0.0066% of soluble leaf protein
Hirudin	11	*Hirudo medicinalis*	Canola	1% of seed weight
γ- and κ-chains hybridoma	45 and 27	Mouse	Tobacco	1.3% of soluble leaf protein
β-Interferon	20	Human	Tobacco	0.000017% of fresh weight
Levansucrase	49.9	*B. subtilis, Streptococcus mutans*	Tobacco, potato	Not detectable
Lysozyme	14.4	Chicken	Tobacco	0.003% of fresh leaf tissue

Next to the nuclear genome, transgenes can be stable integrated into the plastid chromosome by biolistic transformation, but tobacco appears to be the only species where this method has been established as routine. As mature leaf cells contain about 100 chloroplasts each containing about 100 copies of plastid circular DNA, expression driven by a plastid gene can be as high as 30% or even more of total soluble protein making this protein production technology very attractive (Oey et al., 2009a; Oey et al., 2009b). In terms of post-translational modifications the recombinant protein is confined to the plastids' modifying capabilities. Similar to nuclear transformation, plastid transformation is a slow process, requiring about 12-18 months for the generation of transgenic lines. Regarding the undesired spreading of the transgene, this technique can be considered as safe as plastid genes are usually not pollen transmitted.

In contrast to above mentioned techniques where the gene of interest is stable integrated into either the nuclear or chloroplast genome transient expression systems can be used for recombinant protein production in plants. In principle, three approaches exist that often fall back on *Agrobacteria's* property of gene delivery into plant cells and utilize plant viral genetic elements to force transgene expression and protein production in plants (Pogue et al., 2010). The simplest and probably eldest method is named agroinfiltration and is related to the procedure deployed for generating stable transgenic plants and even uses the same vector plasmids (D'Aoust et al., 2009). In brief, a high concentrated solution of *Agrobacteria* carrying the gene-of-interest (GOI) on a binary vector plasmid is infiltrated into the leaves of the selected host plant either by syringae or vacuum. After transfer of the T-DNA, composed of the GOI under the control of a

strong plant-specific promoter into the cells, transgene expression and protein synthesis is initiated in every infected cell. As this technique is simple and fast performed, it is well suited for initial studies of transgene expression and *in planta* protein production. However, protein yields are often lower than that found in stable trangenic plants, likely due to the low number of infected cells. Furthermore, it also has to be considered that only infiltrated leaves are able to produce the transprotein (Gleba et al., 2005). In addition, large bacterial cultures are necessary to infect high amounts of plants and the optimal bacterial density of the infiltration suspension has to be determined for every host plant experimentally. In general, suspensions with densities below OD_{600nm} of 0.1 result in weak transgene expression whereas densities above OD_{600nm} 1.0 are often detrimental for plants as tissue yellowing or wilting can be observed (Wroblewski et al., 2005). Virus-based technologies depict further transient expression approaches with mainly high expression levels and the capability for use on an industrial scale (***Table 2***). At first, there are two strategies: *(i)* the full virus vector strategy and *(ii)* the deconstructed virus vector strategy (Gleba et al., 2007). Both exploit plant viral attributes like speed of expression and high yield of transprotein synthesis, and harbor the advantages of reduced costs and R&D duration.

Table 2 *Comparison of plant expression systems (Koprowski and Yusibov, 2001)*

Transgenic plants	Plant virus vectors
Method of direct expression of foreign genes in plant	Method for transient expression of foreign genes in plant
Foreign gene is	
Stably integrated into plant genome	Stably integrated into viral but not plant genome
Located in and expressed through nuclear apparatus	Located and expressed in cytoplasm and amplified by virus replicase
Inherited	Not inherited

	Transgenic plants	Plant virus vectors
Expression	Limited to single transgenic plant species	Applicable to all plant species susceptible to a given plant virus
Cycle time[a]	1–3 years	1–3 months
Scale-up	1 year	1–3 months
Cost of final product[b]	High	Low

[a] Time required for cloning and expression of foreign genes in plants.
[b] Includes product development, scale-up and manufacturing costs.

Transient expression systems following the full virus vector strategy make use of functional plant viruses, with mainly RNA viruses like *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX) and *Alfalfa mosaic virus* serving as 'templates' for efficient vector development (Gleba et al., 2007; Lico et al., 2008). Usually, a cDNA clone of the appropriate virus is easily modified by introducing an additional ORF encoding the GOI under the control of a viral subgenomic promoter. Alternatively, GOIs can be fused to viral proteins like the movement protein or the coat protein (D'Aoust et al., 2010). After inoculating plants with infectious transcripts of the recombinant virus a fully functional virus should form *in planta* showing the ability for systemic infection (Lindbo, 2007a, b). Depending on the vector and its ability to move systemically, 2-5 weeks will be necessary to infect as many organs and cells of the host plant as possible. The protein of interest will then accumulate in all infected plant tissues (***Figure 5***).

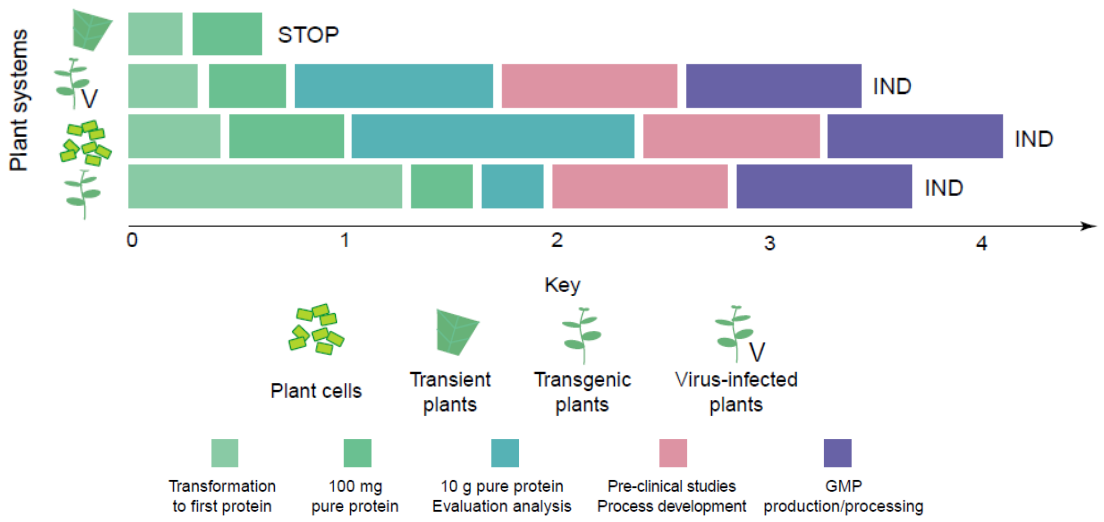

Figure 5 *Performance of virus-based expression system in comparison to other strategies for protein production in plants (Twyman et al., 2003). The axis indicates time span in years necessary for pre-commercial development. Plants infected with modified viruses provide in short time high amounts of recombinant proteins. This process is up-scalable and already lead to investigational new drugs (IND).*

In the best case, a viral vector might achieve a level of heterologous gene expression comparable to that of the wildtyp viral coat protein, an amount regarded as a biological limit by many virologist (Turpen, 1999). As these expression systems are based on wildtyp plant viruses possible plant resistance mechanisms have to be considered and only susceptible host plants with high virus multiplication rates should be chosen. In most cases the only host with satisfying amplification of viral vectors is *Nicotiana benthamiana*, a hyper-susceptible Australian *Nicotiana* species (Goodin et al., 2008). Unfortunately, this is a plant with high susceptibility to many diseases that should be replaced by virus-susceptible tobacco cultivars whenever possible (Gleba et al., 2007).

There are several limitations of transient expression systems based on the full virus vector strategy like low infectivity of viral vectors or the inability to carry average-size or large transgene inserts. To overcome these restrictions, a viral expression system following the deconstructed viral vector strategy 'magnICON®' was developed (Marillonnet et al., 2004; Marillonnet et al., 2005). This system is based on *in planta* assembly of functional viral vectors from separate pro-vector modules. Instead of supplying a plant cell with a complete viral vector as a mature viral particle, or in form of a RNA or DNA molecule, *Agrobacteria* are used to deliver various modules that are assembled inside the cell with the help of a site-specific recombinase (Marillonnet et al., 2004). Even vectors derived from two different plant viruses were found to coreplicate in a non-competing way in the same cell. Using this system GFP, GUS, assembled antibodies and further proteins could be successfully produced with yields as high as 0.5-1.0 g/kg of fresh leaf biomass (Gils et al., 2005; Giritch et al., 2006). Another advantage of this system is the possible transferability from *Nicotiana benthamiana* to other host plants that might be better suited, although reduced protein yields have to be accepted. A further improvement was achieved by making the viral vectors more mRNA-like which favors the use of *Agrobacteria* for delivering the viral sequences into the plant cells (Gelvin, 2005). As *Agrobacteria* transfer the T-DNA into the nucleus the viral amplicon

undergoes a nuclear phase – usually nonexistent in the life cycle of most plant viruses. This new phase might cause problems for the viral vector as it is exposed to the host cells pre-mRNA processing machinery and undesired splicing could occur which might result in a replication-deficient virus. By removing cryptic splicing sites and introducing several plant introns the nuclear RNA processing as well as the RNA export is favored and directed towards the release of functional viral replicons into the cytosol. There, the natural life cycle of the recombinant virus will start without restrictions (Marillonnet et al., 2005).

1.3 *TOBACCO MOSAIC VIRUS* AS BIOTECHNOLOGICAL TOOL

1.3.1 TMV – A PLANT PATHOGEN

Viruses are the cause of many plant diseases, generally characterized by the induction of developmental abnormalities, chlorosis, necrosis and in some cases the death of the host plant (Culver and Padmanabhan, 2007). They lead to significant agricultural losses worldwide and have been studied intensively with regard to improve the resistance of plants against viruses (Agrios, 2005). Probably, the best studied plant virus is *Tobacco mosaic virus* where intensive research already started at the end of the 19th century when M. Beijerinck elucidated that TMV was a *contagium vivum fluidum* or in modern parlance, a substance that was filterable and could move through an agar medium (Scholthof, 2004). Especially at the beginning, the driving force in TMV research was the strong dependence of many farmers on the tobacco crop in the USA as well as in Europe. So, the tobacco growers were in need of practical solutions to control TMV, that caused in the late 1940s an annual average loss of 20,000 tons of tobacco, accounting for 2-3% of the crop (Scholthof, 2004).

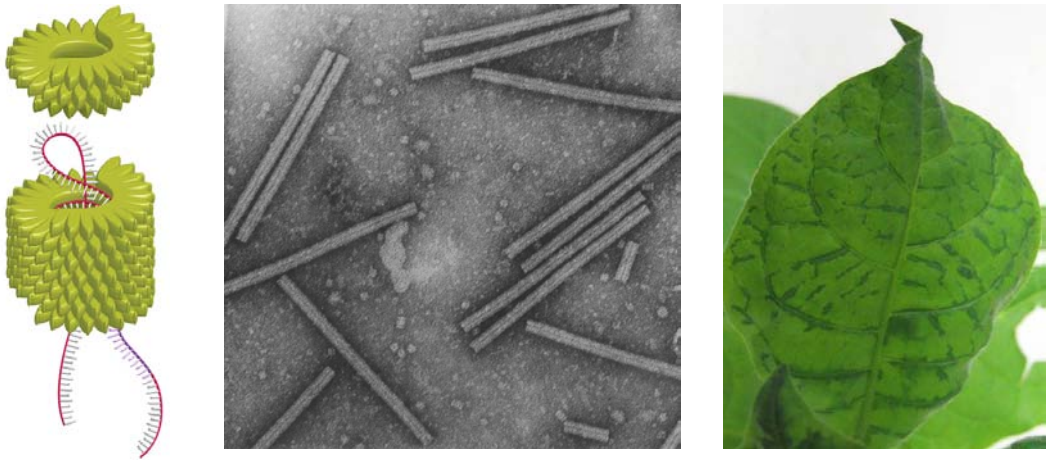

Figure 6 *Schematic model (left) and electron micrography of TMV virion (middle) (Knapp and Lewandowski, 2001). The typical mosaic symptoms induced on susceptible tobacco plants by TMV are shown on the right picture.*

The increasing knowledge about TMV and its simplicity made TMV later to a model in molecular biology and plant pathology, shortly displaced by *E. coli* as molecular biological

model, but with a second advent as reverse genetics made it possible to modify RNA easily on cDNA level.

TMV is one of the simplest viruses known and represents the classical example of a rod-shaped virus with a size of approximately 300 x 18 nm (***Figure 6***). The shape is a result of its basic design, namely a regular, right-handed helix of 2130 identical coat protein subunits with a single 6395 nt RNA molecule embedded, wound as a helix and three nucleotides bound per capsid protein (Klug, 1999). The RNA genome and coat protein subunits are able to 'self-assemble' upon contact under certain conditions *in vivo* as well as *in vitro* preceding the formation of a stable 'disc' composed of 17 coat protein subunits. Assembly starts at an internal site on the TMV RNA, the so called 'origin of assembly' about 1 kb upstream from its 3'-terminus. Nucleation is believed to occur by the insertion of a stem-loop hairpin structure of RNA into the central hole of the protein disc and between the two layers of subunits (Butler, 1999; Klug, 1999). The sequence of the 6395 nt containing TMV RNA genome was revealed completely in 1982 by Goelet and colleagues (Goelet et al., 1982) and a lot of information is available about its primary structure and genome organization. The TMV genome contains only four ORFs that are translated into proteins and one additional ORF coding for a putative 54 kDa protein, that was not detected till now. The 69 nucleotides at the 5'-terminus – also called Ω-sequence – contain a m^7GpppG cap structure and act as translational enhancer. Following the Ω-sequence the ORFs encoding for the replicase proteins are located. The TMV replicase complex is composed of a 126 kDa protein harboring a methyltransferase domain implicated in viral RNA capping, and a helicase domain involved in double-stranded RNA unwinding. The 'leaky' amber stop codon allows the 5%-read through of the 126 kDa ORF resulting in the 183 kDa protein with a clear RNA-dependent RNA polymerase domain (Padmanabhan et al., 2005). Overlapping with the five 3'-terminal codons of the 183 kDa ORF the movement protein (MP), also named 30 kDa protein of TMV is located that is produced from subgenomic mRNAs and mediates the local cell-to-cell spread of TMV. The MP accumulates in plasmodesmata to increase their permeability by rising the size exclusion limit (SEL) from 0.5 kDa to over 10 kDa (Fujiki et al., 2006) or even induces the formation of new 'secondary' plasmodesmata. Furthermore, it co-aligns with microtubules and is tightly associated with ER-derived membranes of infected cells. Notably, the 30 kDa protein is believed to occur as a phosphoprotein or might be at least transiently phosphorylated at C-proximal sites and can be a substrate of cell wall-associated protein kinases (Karger et al., 2003). Free TMV RNA moves through the plasmodesmata by forming complexes with the MP to build elongated structures that are below the modified plasmodesmatal SEL. Hence, the MP is believed to have at least two functions: binding of the TMV RNA genome to form movable complexes and to widen the plasmodesmata allowing the MP-RNA complex to pass. In addition, possible roles in the regulation of TMV genome expression are discussed (Okada, 1999; Karger et al., 2003). The 3'-proximal located ORF encodes for the coat protein (CP) that provides protective function via encapsidation of the viral RNA genome by forming virus particles. For virus replication and cell-to-cell spread the CP is not required, but it is essential for systemic long-distance movement (Bendahmane et al., 2002). The CP of wildtyp TMV is composed

of 158 amino acids after cleavage of the initial methionine and can accumulate in infected plant tissue up to 7-10% of the total host protein in quantity (Li et al., 2006) which is actually the highest amount of foreign protein that can be produced in plants.
In the natural environment TMV infection begins with the delivery of virus particles to a plant. As TMV is not transmitted by vectors like insects, nematodes or fungi contact between healthy and infected plants or contaminated surfaces is necessary for virus-transfer. Virus enters into the cells most likely through wounds, but also passage through the cell wall via ectodesma or by formation of endocytic vesicles is discussed (Shaw, 1999). The virus moves to other cells and tissues via the symplast and virus multiplication is launched after uncoating in a cotranslational manner. Replication of the TMV genome involves synthesis of negative-strand RNA using the genomic positive-strand RNA as template followed by positive-strand RNA synthesis on negative-strand RNA templates (Buck, 1999). Already during uncoating translation of the viral RNA-dependent RNA polymerase occurs on the genomic RNA and continues on the new synthesized full-length positive-strand RNA. The MP and CP, in contrast, are translated from subgenomic mRNAs (Okada, 1999). By spreading from cell-to-cell a local infection is established as first phase of the disease and virions accumulate after assembly of genomic RNA with viral coat proteins. During this stage leaves usually display no visible symptoms (Dawson, 1999). The next phase is characterized by the entry of virions into the phloem and its movement into young leaves, that are still expanding and not yet photosynthetically independent. These areas exhibit vein clearing and, depending on the strain, a yellow to light-green pattern. In the third phase of symptomology also the smaller leaves, still undergoing cell division, become infected and develop uneven patterns of dark-green zones on light-green background often along with leaf deformity (Dawson, 1999).
For plant pathologists the interaction between TMV and tobacco became a classical model system for the study of gene-for-gene disease resistance in resistant plants as well as for the investigation of disease development in susceptible plants (Erickson et al., 1999a). The dominant *N* gene from *Nicotiana glutinosa* confers resistance to all known tobamoviruses exept the Ob strain (Padgett et al., 1997; Dawson, 1999; Erickson et al., 1999a) by restricting the virus to its entry site by rapid induction of HR. And by now, not only the product of *N* was determined as an intracellular receptor of the NBS-LRR class of *R* genes but also the corresponding avirulence gene product of TMV. Namley, a part of the helicase domain of the 126 kDa protein was identified to elicit the HR in resistant plants (Bao et al., 1996; Erickson et al., 1999b). Beside *N* also the semi-dominant *N'* occurs in *Nicotiana sylvestris* and other tobacco varieties. *N'* provides resistance to several TMV strains by using the CP as trigger for defense responses (Dawson, 1999).

1.3.2 BIOTECHNOLOGICAL APPLICATIONS OF TMV

The immense knowledge about TMV and its hosts as well as the ease of manipulation of the virus on cDNA level made TMV well suited for use in modern plant biotechnology. Despite the use as expression vectors for recombinant protein production in plants TMV found its way into a lot of further applications in different fields of biotechnology (Pogue

et al., 2002). As TMV tolerates additional peptides fused to the N- or C-terminus or an internal loop region of its CP, it is possible to display epitops in an ordered, repetitive array all over the surface of a virus particle. In vaccine applications such virus-like particles induce an enhanced immune response in comparison to vaccination with the free antigen (Bendahmane et al., 1999; Jiang et al., 2006; Palmer et al., 2006; Smith et al., 2006; Smith et al., 2009). By adding a linker it was even possible to display a fragment of protein A on TMV's surface to provide a matrix as immunoadsorbent for antibody purification (Werner et al., 2006). Next to the use of the complete virus in biotechnological applications also parts either on protein or DNA level are employed. Transgenically expressed CP was shown to interfere with disassembly of TMV particles and thereby protects plants from virus infections. This coat protein mediated resistance is similar to cross-protection and by the development of coat protein variants high level of resistance against a wide range of tobamoviruses could be achieved (Beachy, 1999; Asurmendi et al., 2007; Bendahmane et al., 2007). Since the 5'-UTR of TMV, oΩ - sequence, acts as translational enhancer it is often found in binary vector plasmids used for plant transformation to increase transprotein synthesis either in stable or transient expression approaches (Buck, 1999). Beside the direct utilization, essential features of plant viruses like TMV, especially the precise and ordered structure of the capsid surface or the interior and exterior surface can serve as biotemplates for nanomaterials with high potential for applications in nanotechnology (Young et al., 2008).

1.4 PRELIMINARY WORK AND GOALS OF THE STUDY AND STRATEGY

Most of the natural antimicrobial peptides are 10 to 50 amino acids in length, positively charged and show a high portion of hydrophobic amino acids. This simple composition facilitates the design of synthetic peptides with improved antimicrobial activities. For the application in plant protection and human medicine novel peptides were developed at the Institute of Biochemical Plant Pathology (Zeitler et al., *2010*) that are much more active against pathogens than natural occurring antimicrobial peptides and which are not toxic for plant or animal cells. Peptide activity was proven in *in vitro* assays with bacterial plant and human pathogens as well as with the *Human immunodeficiency virus* (HIV). Additionally, the peptides reduced the proliferation of bacterial plant pathogens on leaf surfaces demonstrating their possible use as plant protecting agent. In total, more than 60 peptides were designed and screened for their activity against plant and human pathogens. Interestingly, minor modifications in the peptide sequences resulted partly in a dramatic increase in activity. The broad spectrum activity covered not only important plant pathogenic bacteria like *Pseudomonas syringae pv. syringae* but also clinically important human pathogens like *Staphylococcus aureus* or *Pseudomonas aeruginosa* and the *Human immunodeficiency virus*. We conclude that the rational design of AMPs represents a potent weapon in the battle against plant and human pathogens.

As the synthetic production of these peptides is very expensive, the production in plants with *Nicotiana* species serving as inexpensive green factory is aspired as economic alternative. For transfer and heterologous expression of genes encoding for the *de novo*

designed peptides in plants two options are available: stable and transient transformation whereas the latter often uses viral expression vectors.

The first aim of this study was to determine the most suitable production system with highest expression levels of the designed peptides *in planta*. Therefore, stable transgenic plants should be developed that express genes encoding for the antimicrobial peptides. Furthermore, the applicability of the *Magnifection* technology magnICON® (Bayer – formerly Icon Genetics) for the desired purpose had to be investigated. Finally, an own *Tobacco Mosaic Virus* (TMV) based transient expression system should be generated following the "full virus strategy" with the genes coding for the peptides fused to the viral coat protein (***Figure 7***).

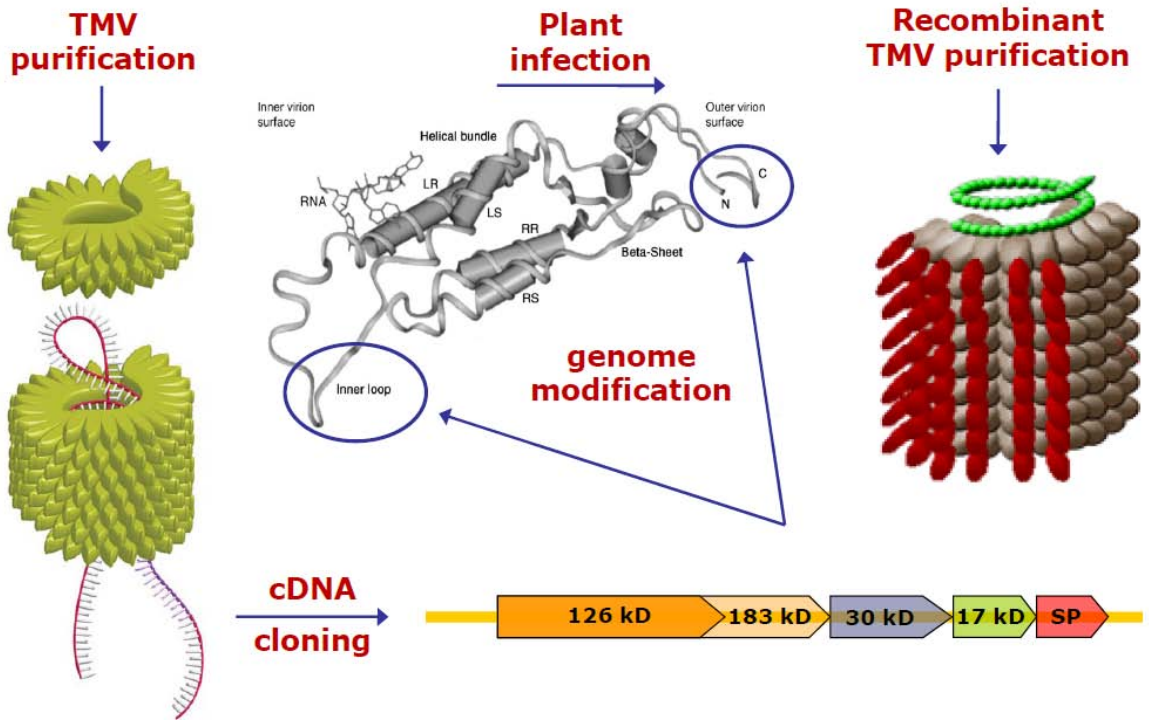

Figure 7 *Strategy for the generation of a TMV-based full virus expression system. Functional virions carrying fused peptide sequences on their surface enable local cell-to-cell movement and systemic movement in plants and act in addition as stable fusion partner to favor protein accumulation in the cell and reduce proteolytic degradation.*

After successful peptide production in plants, an efficient purification protocol had to be established to isolate the recombinant AMPs from plant material. The obtained peptides had to be analyzed for purity and their antimicrobial activities *in vitro*.

2. MATERIAL

2.1 PLANT MATERIAL

The plants used in this study were the *Nicotiana tabacum* L. cultivars Xanthi nn, Xanthi NN, Samsun, Samsun NN and *Nicotiana sylvestris* as well as *Nicotiana benthamiana.* Plants were loosely sown in Einheitserde Typ T (NPK 310, 300, 420 mg/l) mixed with perlite in a ratio of 1:5 to improve root aeration and water supply. Three weeks after germination when the plantlets reached a height of about three cm they were piqued in 11x11x12 cm pots (Teku Tainer, Pöppelmann) and further cultivated for three to four weeks till use.
Controlled climate conditions were assured by cultivation in a growth chamber with 14 h light (approx. 200 $\mu E * m^{-2} * s^{-1}$) at 25 °C and 10 h dark at 20 °C. The relative humidity was set constantly to 70%.

2.2 MICROORGANISMS

2.2.1 BACTERIA (LABORATORY)

Escherichia coli DH5a F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-

Agrobacterium tumefaciens GV3101 pMP90
Rifampicin resistance, chromosomal encoded
Gentamycin resistance, pMP90 encoded

Both laboratory strains were available at the institute.

2.2.2 BACTERIA (PHYTOPATHOGENIC)

Pseudomonas syringae pv. syringae	(DSM 10604)
Pseudomonas syringae pv. tomato	(DSM 50315)
Pseudomonas corrugata	(DSM 7228)
Pectobacterium carotovorum ssp. carotovorum	(DSM 30168)
Clavibacter michiganensis ssp. michiganensis	(DSM 46294)
Xanthomonas vesicatoria	(DSM 50861)

All plant pathogenic bacteria were obtained from the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

E. coli were cultivated at 37 °C, all other bacteria at 28 °C on LB agar plates or in liquid cultures supplemented with the appropriate antibiotics for selection according to standard methods (Sambrook and Russell, 2001). For long-term storage bacterial cultures were mixed with sterile glycerol (final conc. 20% v/v), frozen in liquid nitrogen and stored at -80 °C.

2.2.3 VIRUSES

The plant virus *Tobacco mosaic virus* (TMV) was available at the institute and stored in infected leaves of *Nicotiana tabacum* cv. Xanthi nn at -80 °C.

2.3 KITS AND REACTION SYSTEMS

Name / Description	Company
QIAquick® PCR Purification Kit, No. 28104	Qiagen GmbH, Hilden, Germany
MinElute® PCR Purification Kit, No. 28004	Qiagen GmbH, Hilden, Germany
QIAprep® Spin Miniprep Kit, No. 27104	Qiagen GmbH, Hilden, Germany
QIAquick® Gel Extraction Kit, No. 28704	Qiagen GmbH, Hilden, Germany
TOPO® XL PCR Cloning Kit, No. 45-0008	Invitrogen, Karlsruhe, Germany
Gateway® BP Clonase™ Enzyme Mix, No.11789-013	Invitrogen, Karlsruhe, Germany
Gateway® LR Clonase™ Enzyme Mix, No.11791-019	Invitrogen, Karlsruhe, Germany
RiboMAX™ Large Scale RNA Production Systems – SP6 and T7, No. P1280	Promega, Mannheim, Germany
Ribo m^7G Cap Analog, No. P1711	Promega, Mannheim, Germany
Absolute™ QPCR SYBR® Green Low ROX Mix, No. AB-1322/A	Thermo Fisher Scientific, Munich, Germany
PCR DIG Probe Synthesis Kit, No. 11636090910	Roche Diagnostics, Mannheim, Germany
DIG Easy Hyb Granules, No.1796895	Roche Diagnostics, Mannheim, Germany
DIG Wash and Block Buffer Set, No. 11585762001	Roche Diagnostics, Mannheim, Germany
DNA Molecular Weight Marker II, Digoxigenin-labeled, No. 11218590910	Roche Diagnostics, Mannheim, Germany

2.4 BUFFERS AND SOLUTIONS

All buffers and solutions were prepared in double distilled H_2O and if required autoclaved for 10 min at 121 °C on liquid cycle or filter sterilized (0.22 µm).

For extraction, gel electrophoretic separation, transfer and detection of nucleic acids	
TE buffer	10 mM Tris/HCl, pH 8.0 1 mM EDTA, pH 8.0

DNA extraction buffer	100 mM Tris/HCl, pH 8.0 50 mM EDTA, pH 8.0 500 mM NaCl 2% (w/v) SDS 2% (v/v) ß-mercaptoethanol *After autoclaving and just before use complement* 1% (w/v, final conc.) PVP
P:C:I	phenol:chloroform:isoamyl alcohol in a ratio of 25:24:1
DNA depurination buffer	250 mM HCl
DNA denaturation buffer	1.5 M NaCl 0.5 M NaOH
DNA neutralization buffer	1.5 M NaCl 0.5 M Tris/HCl, pH 7.5
20x SSC transfer buffer	3.0 M NaCl 0.3 M sodium citrate *Adjust to pH 7.5*
50x TAE running buffer	2.0 M Tris base 5.71 % (v/v) glacial acetic acid 50 mM EDTA
RNA extraction buffer (Tri-Reagent)	400 mM ammonium thiocyanate 800 mM guanidine thiocyanate 100 mM sodium acetate 5% (v/v) glycerol *Adjust to pH 5.0 before adding* 38% (v/v, final conc.) water saturated phenol, pH 4.5-5.0
10x MOPS buffer	200 mM MOPS 20 mM sodium acetate 10 mM EDTA *Adjust to pH 7.0*
10x RNA loading buffer	50% (w/v) glycerol 10 mM EDTA, pH 8.0 0.25% (w/v) bromphenol blue 0.25% (w/v) xylene cyanol FF

For Glycine-SDS polyacrylamide gel electrophoresis according to Lämmli

Coomassie R-250 staining solution	0.25% (w/v) Commassie Brilliant Blue R-250 50% (v/v) methanol 10% (v/v) glacial acetic acid

Coomassie R-250 destaining solution	30% (v/v) methanol 10% (v/v) glacial acetic acid
10x SDS running buffer	250 mM Tris base 2 M glycine 1% (w/v) SDS
Resolving gel buffer	1.5 M Tris/HCl, pH 8.8 0.4% (w/v) SDS
Stacking gel buffer	0.5 M Tris/HCl, pH 6.8 0.4% (w/v) SDS
2x reducing sample buffer	100 mM Tris/HCl, pH 6.8 4% (w/v) SDS 0.2% (w/v) bromphenol blue 20% (w/v) glycerol 200 mM DTT

For Tricine-SDS polyacrylamide gel electrophoresis according to Schägger

Coomassie G-250 staining solution	0.025% Coomassie Blue G-250 10% (v/v) glacial acetic acid
Coomassie G-250 destaining solution	10% (v/v) glacial acetic acid
Fixing solution	50% (v/v) methanol 10% (v/v) glacial acetic acid 100 mM ammonium acetate (*added just before use*)
10x anode buffer	1.0 M Tris base 225 mM HCl
10x cathode buffer	1.0 M Tris base 1.0 M tricine 1.0% (w/v) SDS
3x gel buffer	3.0 M Tris base 1.0 M HCl 0.3% (v/v) SDS
4x reducing sample buffer	150 mM Tris/HCl, pH 7.0 12% (w/v) SDS 0.05% (w/v) Coomassie blue G-250 30% (w/v) glycerol 6% (v/v) ß-mercaptoethanol

For transfer and immunodetection of proteins and peptides

TBS-T buffer	0.5% (w/v) Tween 20 in TBS buffer

TBS buffer	10 mM Tris/HCl, pH 7.4 150 mM NaCl 1 mM $MgCl_2$
Blocking buffer	4% (w/v) dry milk 1% (w/v) BSA in TBS-T buffer
Alkaline phosphatase buffer	0.1 M Tris/HCl, pH 9.5 0.1 M NaCl
Electrode buffer (Schägger, 2006)	0.3 M Tris base 0.1 M glacial acetic acid
Transfer buffer (Duchesne and Fernig, 2007)	40 mM Tris base 40 mM Tricine 0.04% (w/v) SDS 20% (v/v) methanol
BCIP solution	5% (w/v) BCIP in 100% DMF
NBT solution	5% (w/v) NBT in 70% DMF

Further solutions

Infiltration buffer	10 mM $MgSO_4$ 10 mM MES, pH 5.7
RNA infection buffer (Hariharasubramanian et al., 1970)	50 mM $Na_4P_2O_7$ 50 mM Tris/HCl, pH 8.6
Peptide dilution buffer	0.01% (v/v) glacial acetic acid 0.2% (w/v) BSA *Sterile filtrated (0.22 µm) before use*

Phytohormones (stock solutions)

2-iP	6-y-y-(Dimethylallylamino)-Purine 75 mg/ml in 1 M NaOH
p-CPA	para-Chlorophenoxyacetic acid 10 mg/ml in 100% (v/v) ethanol

Antibiotics (stock solutions)

Carbenicillin (Carb)	100 mg/ml in 20% (v/v) EtOH
Rifampicin (Rif)	50 mg/ml in DMF
Gentamycin (Gent)	25 mg/ml in ddH_2O

Kanamycin (Kan)	50 mg/ml in ddH_2O
Spectinomycin (Spec)	100 mg/ml in ddH_2O
Cefotaxim (Cef)	100 mg/ml in 20% (v/v) EtOH

2.5 MEDIA

All media were prepared in double distilled H_2O and autoclaved for 10 min at 121 °C on liquid cycle.

Name	Content
LB medium	1% (w/v) tryptone 0.5% (w/v) yeast extract 0.5% (w/v) NaCl *Adjust to pH 7.0* 1.5% (w/v) agar for solid media
SOC medium	2% (w/v) tryptone 0.5% (w/v) yeast extract 0.05% (w/v) NaCl 2.5 mM KCl *Adjust to pH 7.0 and complement before use* 10 mM (final conc.) $MgCl_2$ (filter sterilized, 0.22 µm) 20 mM (final conc.) glucose (filter sterilized, 0.22 µm)
MS medium (Duchefa)	0.44% (w/v) MS salts incl. MS vitamins 3% (w/v) sucrose *Adjust to pH 5.7* 0.8% (w/v) gelrite for solid media
MS pre/cocultivation medium	MS medium containing 0.1 mg/l p-CPA 7.5 mg/l 2-iP
MS selection medium	MS medium containing 0.1 mg/l p-CPA 7.5 mg/l 2-iP 300 mg/l kanamycin 250 mg/l cefotaxime
MS regeneration medium	MS medium containing 300 mg/l kanamycin
B5 medium (Duchefa)	0.44% (w/v) MS salts incl. Gamborg B5 vitamins 3% (w/v) sucrose *Adjust to pH 5.7* 0.8% (w/v) gelrite for solid media

2.6 CHEMICALS AND CONSUMED MATERIAL

All chemicals used were of high purity grade and mainly obtained from Sigma Aldrich GmbH (Taufkirchen, Germany). Handling was performed following standard protocols and the manufacturer's instructions. If any application required chemicals from specific suppliers this is mentioned in the text, otherwise replacement with any chemical of equal purity of any supplier should be possible. Standard laboratory material was used for all experiments and was not restricted to a specific provider.

2.7 ENZYMES

If not stated elsewise all restriction endonucleases were purchased from New England Biolabs (Frankfurt, Germany) or MBI Fermentas (St. Leon-Rot, Germany).

Further enzymes used were:

Name / Description	Company
Antarctic Phosphatase	New England Biolabs, Frankfurt, Germany
Phusion High Fidelity DNA Polymerase	New England Biolabs, Frankfurt, Germany
RQ1 RNase-free DNase	Promega, Mannheim, Germany
Superscript II Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
T4 DNA Ligase	New England Biolabs, Frankfurt, Germany
Taq DNA Polymerase	Agrobiogen, Hilgertshausen, Germany
RNase A	Qiagen GmbH, Hilden, Germany

2.8 ANTIBODIES

Name / Description	Company
Primary antibodies	
Anti-TMV-IgG (rabbit), polyclonal, No. AS-0041	DSMZ, Braunschweig, Germany
Anti-Digoxigenin-AP Fab fragments, No. 11093274910	Roche Diagnostics, Mannheim, Germany

Anti-SP1-IgG (rat), monoclonal	Serviceeinheit Monoklonale Antikörper, Dr. Kremmer, Institut für Molekulare Immunologie, HMGU
Secondary antibodies	
Anti-Rat IgG-AP, A-6066	Sigma-Aldrich GmbH, Taufkirchen, Germany
Anti-Rabbit IgG (Fc)-AP, S3731	Promega, Mannheim, Germany

2.9 PEPTIDES

Synthetic peptides (> 80% purity, lyophilized) were purchased from metabion (Munich, Germany) or emc microcollections (Tübingen, Germany). Peptides were dissolved in dilution buffer and aliquots were stored at -80 °C. The following peptides were used in this study:

Name	Sequence
SP1	RKKRLKLLKRLV-NH_2
SP1-1	RKKRLKLLKRLL-NH_2
SP6	ALAHFLKKAIKK-NH_2
SP10-2	LRFLKKALKKLF-NH_2
SP13	KRKLIFLAAFLAALALFKKR-NH_2
SP15	KRKLIFLAAFLAALALFKKR-NH_2

2.10 OLIGONUCLEOTIDES

Lyophilized oligonucleotides were purchased from either Sigma-Aldrich GmbH (Taufkirchen, Germany) or Eurofins MWG Operon (Martinsried, Germany). 100 µM stock solutions of deprotected and salt-free oligonucleotides were prepared in sterile ddH_2O and stored at -20 °C.

Oligonucleotides for DNA-sequencing	
SeqTMV4241	5'-AAGAACACGAACTGAGATGG-3'
SeqTMV5092	5'-GACAAATACTTCACCAG-3'
SeqTMV5943	5'-TCCGTTTGGATTGAAGTG-3'

SeqTMV6794	5'-ACCCTCTTGAAAGCAGAGC-3'
SeqTMV7645	5'-CAACATCCAAGACTCCAAAC-3'
SeqTMV8496	5'-GGCAAGCTTAGTATGCAGG-3'
M13F_MediGX	5'-TGTAAAACGACGGCCAGT-3'
M13R2_MediGX	5'-GGAAACAGCTATGACCATG-3'
SeqNosT	5'-ATAATTGCGGGACTCTAATC-3'

Oligonucleotides for generating 3'-provector modules

attB1_Sp13_for	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGAGAAGATTG ATTGCTAGAATTTTG-3'
attB2_SP13_rev	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATCATCTCTTCTTAACCA AAGCTCTAGCA-3'
GST-SP13_for	5'-TTTGGTCTCAAGGTATGTCCCCTATACTAGGTTATTGG-3'
GST-SP13_rev	5'-ATATCTAGATCATCTCTTCTTAACCAAAGCTC-3'
GFP_for	5'-ATATCCATGGTGAGCAAGGGCGAGGAGC-3'
GFP_rev	5'-ATATGTGCACCTTGTACAGCTCGTCCATGC-3'
GFP4_rev	5'-CAAACCAATCTCTTCAACAACTTCAATCTCTTCTTTC-3'
GFPfusSP1_rev	5'-CGACTCTAGATCAAACCAATCTCTTCAAC-3'

Oligonucleotides for cloning TMV and generating the RNA infection vector pT2SB

TMV_for	5'-GATCAAGCTTGTATTTTTACAACAATTACC-3' (HindIII)
TMV_rev	5'-TACGCCCGGGTGGGCCCCTACCGGGGGTAACG-3' (XmaI/SmaI)
TMV_SP6	5'-G*ATTTAGGTGACACTATAGA*ATTTTTACAACAATTACCAAC-3' (*trunc. SP 6 promoter*)
TMV_BseYI	5'-GCTGGGCCCCTACCGGGGGTAACGG-3' (BseYI)

Oligonucleotides for generating the agroinfection vector pAGRO:T2SB

11599agro_for	5'-CTATCCTAGGGTCAAGCAGATCGTTCAAACATTTG-3' (AvrII)
11599agro_rev	5'-GCTACTCGAGAGTACATTAAAAACGTCCGCAA-3' (XhoI)
35sagro_for	5'-CTAGCTCGAGACTAGAGCCAAGCTGATCTCCTTTGC-3' (XhoI)

35sagro_rev	5’-GCATACCGGTTCGACTAGAATAGTAAATTGTAATG-3’ (AgeI)
BR322_for	5’-GACTGGTACCCCCGTAGAAAAGATCAAAGGA-3’ (Acc65I)
BR322_rev	5’-GACTGGTACCATGTGAGCAAAAGGCCA-3’ (Acc65I)
AGRO322_for	5’-GACTGGTACCGGGGTCTGACGCTCAGT-3’ (Acc65I)
AGRO322_rev	5’-GACTGGTACCGGCAGATCCTAGATGTG-3’ (Acc65I)
T2SBagro_for	5’-CTATCCTAGGGCTGGGCCCCTACCGGGGGTAAC-3’ (AvrII)
T2SBagro_rev	5’-GTCAACCGGTATCTGCAGAATTCGCCCTTGAT-3’ (AgeI)

Oligonucleotides for mutagenesis PCR

SP1.1cc_for	5’-GCAATCTCTTAAGAAGCTTAAGTCTCTTCTTTCTCATTTCCTCATCATCTTCATCATCAGTTGCAGGACCAGAGGTCCAAACCAAACCAGAAGAGC-3’
SP1.1cc_rev	5’-GCTCTTCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTGATGATGAAGATGATGAGGAAATGAGAAAGAAGAGACTTAAGCTTCTTAAGAGATTGC-3’
SP10.2cc_for	5’-GCTTCTTAAGAGCCTTCTTAAGGAACCTCAACATATCATCTTCATCATCAGTTGCAGGACCAGAGGTCCAAACCAAACCAGAAGAGC-3’
SP10.2cc_rev	5’-GCTCTTCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTGATGATGAAGATGATATGTTGAGGTTCCTTAAGAAGGCTCTTAAGAAGC-3’
SP6cc_for	5’-CATCTTGACTACCTCAAGTTGCAGGACCAGAGGTTCACTTCTTAATAGCCTTCTTAAGAAAATGAGCAAGAGCCTTGTCGTCGTCGTCTTCCCAAACCAAACCAGAAGAGCTCTCGAAAG-3’
SP6cc_rev	5’-CTTTCGAGAGCTCTTCTGGTTTGGTTTGGGAAGACGACGACGACAAGGCTCTTGCTCATTTTCTTAAGAAGGCTATTAAGAAGTGAACCTCTGGTCCTGCAACTTGAGGTAGTCAAGATG-3’
SP6met_for	5’-GGTTTGGGAAGACGACGACATGGCTCTTGCTCATTTTC-3’
SP6met_rev	5’-GAAAATGAGCAAGAGCCATGTCGTCGTCTTCCCAAACC-3’
SP13-2_for	5’-GACTACCTCAAGTTGCAGGACCAGAGGTTCATCTCTTCTTAACCAAAGCTCTAGCAGCCAATCTCAAAATTCTAGCAATCAATCTTCTCTTCATCCAAACCAAACCAGAAGAGCTCTCG-3’
SP13-2_rev	5’-CGAGAGCTCTTCTGGTTTGGTTTGGATGAAGAGAAGATTGATTGCTAGAATTTTGAGATTGGCTGCTAGAGCTTTGGTTAAGAAGAGATGAACCTCTGGTCCTGCAACTTGAGGTAGTC-3’

CtermCC_for	5'-GACAAGAACACGAACTGGGATGGAGTAGTGATTTCTTCATCTTCATC ATCTTCACTGTAAGACATATTTAAAACGAATCC-3'
CtermCC_rev	5'-GGATTCGTTTTAAATATGTCTTACAGTGAAGATGATGAAGATGAAGA AATCACTACTCCATCCCAGTTCGTGTTCTTGTC-3'
SP1.1loop_for	5'-GGTCTAATACCGCATTGTACCTGTACACCTTAAAGTCCATAAGCAAT CTCTTAAGAAGCTTAAGTCTCTTCTTTCTCATAGGGAACCTAACAGTTAC TTGTGGTGAAGGTTTCC-3'
SP1.1loop_rev	5'-GGAAACCTTCACCACAAGTAACTGTTAGGTTCCCTATGAGAAAGAA GAGACTTAAGCTTCTTAAGAGATTGCTTATGGACTTTAAGGTGTACAGGT ACAATGCGGTATTAGACC-3'
SP1.1_for	5'-GTACGCACCACGTGTGATTACGGACACAATCCGTTATTTATTATGCA TCTTGACTACCTCAAAGCAATCTCTTAAGAAGCTTAAGTCTCTTCTTTCTC ATAGTTGCAGGACCAGAGGTCCAAACCAAACCAGAAGAGCT-3'
SP1.1_rev	5'-AGCTCTTCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTATGAGAA AGAAGAGACTTAAGCTTCTTAAGAGATTGCTTTGAGGTAGTCAAGATGC ATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCGTAC-3'
T2SBSP10_for	5'-GGACACAATCCGTTATTTATTATGCATCTTGACTACCTCAGAAAAGC TTCTTAAGAGCCTTCTTAAGGAACCTCAACATAGTTGCAGGACCAGAGG TCCAAACCAAACCAGAAGAGC-3'
T2SBSP10_rev	5'-GCTCTTCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTATGTTGAGG TTCCTTAAGAAGGCTCTTAAGAAGCTTTTCTGAGGTAGTCAAGATGCATA ATAAATAACGGATTGTGTCC-3'

Oligonucleotides for screening for transgenic plants

NtSP1-1_rev	5'-TCAAAGCAATCTCTTAAGAAGC-3'
NtSP10-2_rev	5'-GAAAAGCTTCTTAAGAGCCTTC-3'
NtSP13_for	5'-AAGTTCATTTCATTTGGAGAGGACTCC-3'
NtSP13_rev	5'-TCATCTCTTCTTAACCAAAGCTCTAGCAG-3'
35S-pA5-fw	5'-GACACTCTCGTCTACTCCAAG-3'

2.11 PLASMIDS

Plasmid DNA was extracted from *E. coli* cells as described in chapter 3.3 and stored in ddH_2O at -20 °C.

Name	Source	Selection	Description / Use
pICH17388	Icon Genetics (Halle, Germany)	Amp/Carb	Plant Transfection magnICON® 5'-module

pICH14011	Icon Genetics (Halle, Germany)	Amp/Carb	Plant Transfection magnICON® integrase
pICH7410	Icon Genetics (Halle, Germany)	Amp/Carb	Plant Transfection magnICON® 3'-module:GFP
pICH11599	Icon Genetics (Halle, Germany)	Amp/Carb	Plant Transfection magnICON® 3'-module
pICH10990	Icon Genetics (Halle, Germany)	Amp/Carb	Plant Transfection magnICON® 3'-module
p11599:SP1	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:SP1
p11599:SP13	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:SP13
p11599:SP15	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:SP13
p11599:His6-SP1	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:His6-SP1
p11599:His6-SP13	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:His6-SP13
p11599:His6-SP15	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:His6-SP15
P10990:GST-SP13	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:GST-SP13
p10990:GHS	this work	Amp/Carb	Plant Transfection magnICON® 3'-module:GFP-His6-SP1
pCR-XL-TOPO	Invitrogen, Karlsruhe, Germany	Kan	TA-cloning vector
pCR-XL-TOPO:TMV	this work	Kan	TMV cDNA clone in PCR-XL-TOPO
pK2GW7	Department of Plant Systems Biology, Ghent University (Belgium)	Spec	Gateway compatible vector for stable transformation of plants; Kanamycin resistance gene for selection of transgenic plants
pK2GW7:SP1-1	this work	Spec	Stable transformation of SP1-1
pK2GW7:SP10-2	this work	Spec	Stable transformation of SP10-2
pK2GW7:SP13	this work	Spec	Stable transformation of SP13

pDest15	Invitrogen (Karlsruhe, Germany)	Amp/Carb	Gateway expression vector
pT2SB	this work	Kan	RNA infection of TMV
pT2SB:SP1-1	this work	Kan	RNA infection of TMV:SP1-1
pT2SB:ccSP1-1	this work	Kan	RNA infection of TMV:SP1-1, N-terminal charge compensation
pT2SB:SP1-1cc	this work	Kan	RNA infection of TMV:SP1-1, C-terminal charge compensation
pT2SB:SP1-1 loop	this work	Kan	RNA infection of TMV:SP1-1, inserted in loop region
pT2SB:SP10-2	this work	Kan	RNA infection of TMV:SP10-2
pT2SB:SP10-2cc	this work	Kan	RNA infection of TMV:SP10-2, C-terminal charge compensation
pT2SB:SP6	this work	Kan	RNA infection of TMV:SP6
pT2SB:SP6cc	this work	Kan	RNA infection of TMV:SP6, C-terminal charge compensation
pT2SB:SP13	this work	Kan	RNA infection of TMV:SP13
pAGRO	this work	Amp/Carb	Binary vector plasmid, pUC ORI
pAGRO322	this work	Amp/Carb	Binary vector plasmid, pBR322 ORI
pAGRO:T2SB	this work	Amp/Carb	Agroinfection of TMV
pAGRO:T2SB-SP1-1	this work	Amp/Carb	Agroinfection of TMV:SP1-1
pAGRO:T2SB-SP1-1cc	this work	Amp/Carb	Agroinfection of TMV:SP1-1, C-terminal charge compensation
pAGRO:T2SB-ccSP1-1	this work	Amp/Carb	Agroinfection of TMV:SP1-1, N-terminal charge compensation
pAGRO:T2SB-SP10-2	this work	Amp/Carb	Agroinfection of TMV:SP10-2
pAGRO:T2SB-SP10-2cc	this work	Amp/Carb	Agroinfection of TMV:SP10-2, C-terminal charge compensation
pAGRO:T2SB-SP6cc	this work	Amp/Carb	Agroinfection of TMV:SP6, C-terminal charge compensation
pAGRO:T2SB-SP13	this work	Amp/Carb	Agroinfection of TMV:SP13

2.12 INSTRUMENTS AND ACCESSORIES

Name / Description	Type	Company
Autoclave	D-150	Systec
Balance	LC 620 S	Sartorius
	A 210 P	Sartorius
	L 2200 P	Sartorius
Camera	Powershot G2	Canon
Centrifuge	Beckman J2-21	Beckmann Coulter
	Beckman L7-65	Beckmann Coulter
	Centrifuge 5415 D	Eppendorf
	Centrifuge 5810 R	Eppendorf
	Biofuge 28 RS	Heraeus
DNA Transfer Unit	LKB VacuGene XL Vacuum Blotter	Pharmacia
Electrophoresis systems	Mini-vertical electrophoresis unit SE250	Hoefer
	Electrophoresis chamber B2	Owl electrophoresis systems
Electroporator	Gene Pulser Electroporation system	Bio-Rad
Flow Hood	Gelaire BSB 4A	Flow Laboratories
FPLC system	ÄKTA Explorer 10S	GE Healthcare
Freeze-Dryer	Alpha 1/5	Christ
Fume Hood	CAPTAIR Filtersystem 804N	Captair
Gel Caster	Multiple gel caster SE215	Hoefer
Gel Documentation	Benchtop 2UV Transilluminator & PhotoDocIt Imaging System	UVP
Magnetic stirrer	IKA-Combimag Ret	Jahnke & Kunke
pH measurement	pH Meter pH 523	WTW
	pH electrode SenTix 21	
Power supply	Electrophoresis power supply EPS 601	GE Healthcare
	Electrophoresis power supply E 802	Consort
Protein Transfer Unit	SemiPhor semidry transfer unit	Hoefer

Reversed phase medium	LiChrosorb RP-8	Merck
Rotors	JA-10, JA-20 F45-24-11	Beckman Coulter Eppendorf
Scanner	Image Scanner II	GE Healthcare
Shaker	UniTherm HB Reax 2	UniEquip Heidolph
Size exclusion column	Superdex Peptide 10/300 GL	GE Healthcare
Spectrophotometer	NanoDrop ND-1000 Ultrospec 3100 pro	NanoDrop Technologies Amersham
Temperature thermostat	RW 6	Lauda
Thermal Cycler	Hybaid PCR express	Thermo Life Sciences
Thermoblock	Thermomix Comfort	Eppendorf
Ultra-pure water system	Ultra Clear Direct	SG
Ultra sonic bath	Sonorex Super 10 P	Bandelin
UV lamp	B-100 AP	UVP
Vacuum concentrator	Univapo 150W	UniEquip
Vortexer	Vortex-Genie 2	Scientific Industries

2.13 SOFTWARE AND WEB SITES / WEB TOOLS

Vector NTI 9.1.0© 2004 Invitrogen Corporation
Unicorn 5.11© 2006 General Electric Company
ImageJ 1.41 of the National Institutes of Health, USA (http://rsb.info.nih.gov/ij/)
Primer express 2.0 Applied Bioscience
http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp
http://www.expasy.ch/
http://fermentas.kd-services.de/?c=DE
http://www.neb.com/nebecomm/products/category1.asp?#2
http://multalin.toulouse.inra.fr/multalin/help.html
http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed
http://www.kazusa.or.jp/java/codon_table_java/
http://www.psb.ugent.be/gateway/index.php
http://www.scripps.edu/~cdputnam/protcalc.html
http://isoelectric.ovh.org/files/calculate.php
http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp
http://www.currentprotocols.com/categories
http://www.cbs.dtu.dk/services/NetGene2/

3. METHODS

3.1 PREPARATION OF ELECTROCOMPETENT BACTERIAL CELLS

3.1.1 *ESCHERICHIA COLI* DH5A

Electrocompetent *E. coli* cells were prepared as described in Sambrook and Russell (2001) with some modifications. A single colony from a fresh LB agar plate was transferred into a flask containing 30 ml LB medium and incubated over night (oN) at 37 °C with shaking (300 rpm). The following day, 300 ml LB medium in a 1000 ml flask were inoculated with 10 ml of the oN bacterial culture and incubated at 37 °C with shaking (300 rpm). When the OD_{600nm} of the culture reached 0.4 - 0.5 the flask was chilled for 20 min in an ice-water bath with occasionaly swirling. Afterwards, the culture was transferred to a prechilled centrifuge bottle and pelleted at 3,000 g for 20 min at 4 °C (4200 rpm in a Beckman JA-10 rotor). The pellet was washed twice in 50 ml ice-cold, sterile ddH_2O and twice in 50 ml ice-cold, sterile 10% (w/v) glycerol. Finally, the pellet was resuspended in 0.5 ml ice-cold, sterile 10% (w/v) glycerol and the OD_{600nm} of a 1:100 dilution was measured. The cell suspension was adjusted to a concentration of approx. $2 * 10^{10}$ cells/ml (1.0 OD_{600nm} = ~ $2.5 * 10^8$ cells/ml) with ice-cold, sterile 10% (w/v) glycerol and aliquots of 40 µl were frozen in liquid nitrogen and stored at -80 °C until required.

3.1.2 *AGROBACTERIUM TUMEFACIENS* GV3101 PMP90

A single colony from a fresh LB agar plate containing the antibiotics rifampicin (final conc. 50 µg/ml) and gentamycin (final conc. 25 µg/ml) was transferred to 5 ml LB medium supplemented with above mentioned antibiotics and grown oN at 28 °C with shaking (300 rpm). The following day, 200 ml LB medium containing rifampicin (final conc. 50 µg/ml) and gentamycin (final conc. 25 µg/ml) in a 1000 ml flask were inoculated with the oN bacterial culture and incubated for 24 h at 28 °C with shaking (300 rpm). Without measuring the OD_{600nm} the culture was divided in four prechilled 50 ml tubes and pelleted at 3,000 g for 20 min at 4 °C. Each pellet was washed twice in 50 ml ice-cold, sterile 10% (w/v) glycerol, afterwards resuspended in 10 ml ice-cold, sterile 10% (w/v) glycerol, mixed together and again centrifuged. Finally, the pellet was resuspended in 0.25 ml ice-cold, sterile 10% (w/v) glycerol and aliquots of 40 µl were frozen in liquid nitrogen and stored at -80 °C until required.

3.2 TRANSFORMATION OF BACTERIA

3.2.1 ELECTROPORATION OF *E. COLI*

Bacterial transformation was carried out by electroporation using a Gene Pulser Electroporation System (Bio-Rad, Munich, Germany). For electroporation, *E. coli* DH5*α* electrocompetent cells were thawed on ice, mixed with either plasmid DNA (1-10 ng dissolved in ddH_2O) or with maximal 1 µl of an enzymatic reaction and transferred to a prechilled 1 mm Gene Pulser cuvette (Bio-Rad, Munich, Germany). An electric pulse of 25

µF capacitance, 1.7 kV and 200 Ω resistance was applied. Afterwards, cells were immediately suspended in 1 mL SOC medium and incubated at 37 °C for 1 h with shaking (300 rpm). 20 – 200 µl were plated on LB agar plates containing the appropriate antibiotics and incubated oN at 37 °C.

3.2.2 ELECTROPORATION OF *A. TUMEFACIENS*

Transformation of *A. tumefaciens* GV3101 pMP90 was performed as described above considering following differences. An electric pulse of 25 µF capacitance, 1.25 kV and 400 Ω resistance was applied. Afterwards, cells were immediately suspended in 1 mL SOC medium and incubated at 28 °C for 1 h with shaking (300 rpm). 20 – 200 µl were plated on LB agar plates containing the appropriate antibiotics and incubated 2 – 3 days at 28 °C.

3.3 PLASMID DNA EXTRACTION FROM *E. COLI* CELLS

For the purification of plasmid DNA a single *E. coli* colony from a selective LB agar plate was transferred into 5 ml LB medium containing the appropriate antibiotic in a 15 ml Falcon tube and grown oN at 37 °C with shaking (300 rpm). Bacterial cells were harvested by centrifugation at 3,000 g for 20 min at 4 °C and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To elute the DNA from the column sterile ddH_2O was used and plasmid DNA was quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

3.4 DNA EXTRACTION FROM PLANT MATERIAL

Total DNA was isolated from plant leaves as described in Lin et al. (2001). 100 mg of leaves were homogenized in a 1.5 ml Eppendorf tube with a pipette tip, mixed with 600 µl DNA extraction buffer and incubated at 65 °C for 15 min in a thermoblock. After centrifugation at 12,000 g for 10 min at 4 °C the supernatant was mixed with 5 µl RNAse A (4 µg/ml) and incubated at 37 °C for 10 min. An equal volume of P:C:I was added and after inverting several times, the phases were separated by centrifugation at 12,000 g for 3 min at 4 °C. The aqueous phase was transferred into a new 1.5 ml Eppendorf tube and the DNA was precipitated by adding 0.6 volumes of ice-cold isopropanol and incubation at -20 °C for 10 min. DNA was pelleted by centrifugation at 12,000 g for 10 min at 4 °C and washed twice in 70% ice-cold ethanol. After air drying the DNA was dissolved in 50-100 µl steril ddH_2O and quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Isolated DNA was stored at -20 °C until use.

3.5 RNA EXTRACTION FROM PLANT MATERIAL

Total RNA was isolated from leaves using TRIZOL® (Invitrogen, Hilden, Germany) according to the manufacturer's manual. 100 mg leaves were homogenized in liquid N_2 using a pestle and mortar, mixed with 1 ml TRIZOL® reagent and incubated for 5 min at RT. After the addition of 200 µl Chloroform, the tubes were shaken vigorously by hand and incubated for 2 to 3 min at RT. Following centrifugation at 18,000 g for 15 min at 4°C the aqueous phase was transferred into a fresh tube and mixed with 500 µl isopropanol for RNA precipitation. Samples were incubated at RT for 10 min, RNA pelleted by centrifugation at 18,000 g for 10 min at 4°C and washed twice in 70% ice-cold ethanol. After air drying the RNA was dissolved in 50-100 µl steril ddH_2O and quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Isolated RNA was stored at -80 °C until use.

3.6 DNA SEQUENCING

Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany) using the Value Read service. Purified plasmid DNA or linear DNA fragments and oligonucleotides for sequencing were premixed in tubes as recommended. Sequencing results were analyzed using the Vector NTIi Advance 10 (Invitrogen) tools AlignX and ContigExpress.

3.7 RESTRICTION ENZYME CLEAVAGE

Restriction digests were performed with restriction enzymes from New England Biolabs (Frankfurt am Main, Germany) or MBI Fermentas (St Leon-Rot, Germany) with the appropriate 1x buffer at a temperature that was recommended for a particular enzyme by the manufacturer. Incubations were carried out in an incubator or thermoblock, and bovine serum albumin (BSA) was added if recommended by the manufacturer. Enzyme concentration was chosen in agreement with the manufacturer's instructions. For double digestions reaction conditions were chosen as suggested by the manufacturers (http://www.fermentas.com/doubledigest/index.html, http://www.neb.com/nebecomm/DoubleDigestCalculator.asp?).

3.8 EXTRACTION AND PURIFICATION OF DNA FRAGMENTS FROM AGAROSE GELS

DNA bands were cut out of the gel with a scalpel and transferred into a Eppendorf reaction tube. The isolation procedure was performed either with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) or the TOPO® XL Gel Purification Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's manual. DNA was quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

3.9 ENZYME REMOVAL FROM DNA SAMPLES

If necessary, DNA was purified after enzymatic reactions using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany). DNA was quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

3.10 DNA GEL ELECTROPHORESIS

The separation of DNA fragments according to length was done in agarose gels using TAE buffer. Samples were mixed with 6x loading dye solution (MBI Fermentas, St Leon-Rot, Germany) before loading and gels were run at voltage of 100 V. For the detection of DNA fragments, 0.05 µg/mL ethidium bromide was added to the liquid agarose. After separation, the fragments were visualised by UV light illumination (302 nm).

3.11 CDNA SYNTHESIS AND QRT-PCR

First-strand cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) following the supplied protocol and as described in chapter 3.17.2.

For qRT-PCR the Absolute™ qPCR Sybr® Green Low ROX Mix (Thermo Fisher Scientific, Munich, Germany) was used. Specific oligonucleotide sequences were determined using the Primer Express 2.0 software (Applied Biosystems, Darmstadt, Germany). The oligonucleotides were designed to have a length of maximum 30 bases and to generate an amplificate of maximum 100 bp. The 25 µl reaction mixture was composed of 12.5 µl Absolute™ qPCR Sybr® Green Low ROX Mix, 10.5 µl cDNA (1:20 dilution) and 1 µl of each gene-specific forward and reverse primer (70 nM final concentration). Analysis were performed in the ABI Prism 7500 with following conditions:

Step	Temperature	Duration	Cycles
Enzyme activation	95 °C	15 min	1
Denaturation	95 °C	15 sec	
Annealing	60 °C	30 sec	40
Extension	72 °C	30 sec	

A melting curve was measured after every run to monitor possible formation of primer dimers.

3.12 PROTEIN EXTRACTION FROM PLANT MATERIAL

For isolation of proteins, harvested leaf material was immediately frozen and subsequently homogenized using a pestle and mortar in liquid N_2. Proteins were

extracted routinely by incubation on ice with vortexing in at least 2 volumes of extraction buffer composed of: 50 mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 5 mM ß-ME, 0.02% Triton-X-100. If necessary, the buffer was supplemented with 8 M urea (final conc.) or further additives as indicated in the text. Cell debris and insoluble material was pellet by centrifugation at 18,000 g for 30 min at 4 °C. Supernatant was recovered and protein content determined by the Bradford assay (Bradford, 1976). If necessary, defined amounts of proteins were precipitated by adding ice-cold acetone to a final concentration of 80%. After incubation for 1 h at -20 °C the precipitate was collected by centrifugation at 18,000 g for 30 min at 4 °C. Protein samples were stored at -80 °C.

3.13 PROTEIN ELECTROPHORESIS

Electrophoretic separation of proteins on denaturating mini-gels was performed in a Hoefer SE 250 Mini-Vertical Gel Electrophoresis unit (GE Healthcare, Freiburg, Germany) with the temperature controlled by a Lauda RW 6T compact low temperature thermostat (Lauda-Königshofen, Germany) with water as coolant, if necessary. All gels, 8 x 7 cm in size and 0.75 cm thick, were prepared in a Hoefer Mighty Small dual gel caster (GE Healthcare, Freiburg, Germany) according to the manual and an Electrophoresis Power Supply EPS 601 (GE Healthcare, Freiburg, Germany) was used as power source.

3.13.1 GLYCINE-SDS-PAGE (LÄMMLI)

For separation of proteins in the mass range > 10 kDa Glycine-SDS-PAGE was used. The preparation of buffers and solutions as well as the 4% stacking gels and 12% or 15% resolving gels for Glycine-SDS-PAGE was performed as described in Sambrook and Russell (2001). Protein samples in solution were mixed with 6x sample buffer (final conc. 1x) or lyophilized protein extracts were resuspended in 1x sample buffer, boiled for 5 min in a thermomixer and after a quick centrifugation step not more than 25 µl of protein sample was loaded per well. Gels were run at constant 25 mA per gel till the bromphenol blue front reached the bottom of the gel. Afterwards, gels were stained for at least 1 h in Coomassie R-250 staining solution followed by destaining till the background became clear again.

3.13.2 TRICINE-SDS-PAGE (SCHÄGGER)

To separate proteins in the mass range < 10 kDa Tricine-SDS-PAGE was used that offers high resolution separation of low molecular weight proteins and peptides. The preparation of buffers and solutions as well as the 4% stacking gels and 10%, 12% or 14% resolving gels for Tricine-SDS-PAGE was performed as described by Schägger (2006). Protein samples in solution were mixed with 4x sample buffer (final conc. 1x) or lyophilized protein extracts were resuspended in 1x sample buffer, incubated for 30 min at 37 °C in a thermomixer and after a quick centrifugation step not more than 20 µl of protein sample was loaded per well. Gels were run at an initial voltage of 30 V till the

samples entered the resolving gel. Next voltage steps were 120 V till the middle of the resolving gel and 190 V at the end of the run. To avoid heating of the gel a temperature thermostat was connected to the electrophoresis unit and the coolant temperature was set to 15 °C. When the run was finished proteins were fixed in the gel by incubation in fixing solution for 30 min and the gel was stained in Coomassie G-250 staining solution for 1 h followed by destaining till the background became clear again.

3.14 PROTEIN TRANSFER AND IMMUNOBLOTTING

For immunochemical detection of proteins on membranes (Western Blot) the semi-dry blotting method was used. Proteins were transferred either on Hybond-P PVDF membranes with 0.45 µm pore size or Hybond-LFP PVDF membranes with 0.2 µm pore size (GE Healthcare, Freiburg, Germany) depending on the molecular weight of the protein or peptide to detect on a Hoefer SemiPhor semi-dry transfer unit (GE Healthcare, Freiburg, Germany).

3.14.1 TRANSFER AND DETECTION OF PROTEINS ON MEMBRANES

After completion of Glycine-SDS-PAGE, the resolving gel was removed from the electrophoresis unit, separated from the stacking gel and incubated in transfer buffer (Duchesne and Fernig, 2007) for 10 min to dilute free SDS in the gel. At the same time, the Hybond-P PVDF membrane was prepared by activating it in 100% MeOH for 5 sec, followed by a short wash step in ddH_2O and subsequent equilibration in transfer buffer for 10 min. A blotting sandwich was assembled from equilibrated gel and membrane and buffer soaked blotting paper according to the transfer unit's manual and protein transfer was performed by applying a current of 1.5 mA per cm^2 of gel area for 1 h. After transfer, free binding sites on the membrane were blocked by incubation in 30 ml blocking buffer for 1 h at RT with slight shaking, followed by three wash steps each in 30 ml TBS-T for 10 min. The primary antibody raised against TMV (Anti-TMV-IgG (rabbit), polyclonal, No. AS-0041) was diluted 1:1,000 in 10 ml TBS-T and the membrane was incubated in the primary antibody solution again for 1 h at RT with shaking. Following a wash step as described above the membrane was incubated in 10 ml secondary antibody solution, 1:10,000 dilution of Anti-Rabbit IgG (Fc)-AP (S3731) in TBS-T, for 1 h at RT with shaking. Subsequently, the membrane was washed twice in 30 ml TBS-T and once in 30 ml TBS as described above. Colorimetric detection of transferred proteins was performed by incubating the membrane in 10 ml AP-buffer supplemented with 66 µl NBT solution and 33 µl BCIP solution. When signal intensity was satisfactory, the membrane was washed in ddH_2O and air-dried.

3.14.2 TRANSFER AND DETECTION OF PEPTIDES ON MEMBRANES

After completion of Tricine-SDS-PAGE, the resolving gel was removed from the electrophoresis unit, separated from the stacking gel and incubated in electrode buffer

(Schägger, 2006) for 10 min to dilute free SDS in the gel. At the same time, the Hybond-LFP PVDF membrane was prepared by activating it in 100% MeOH for 5 sec, followed by a short wash step in ddH_2O and subsequent equilibration in electrode buffer for 10 min. A blotting sandwich was assembled from equilibrated gel and membrane and buffer soaked blotting paper according to the transfer unit's manual and protein transfer was performed oN by applying a current of 0.4 mA per cm^2 of gel area. After transfer, free binding sites on the membrane were blocked by incubation in 30 ml blocking buffer for 1 h at RT with slight shaking, followed by three wash steps each in 30 ml TBS-T for 10 min. The primary antibody raised against SP1-1 (Anti-SP1-IgG (rat), monoclonal) was diluted 1:50 in 10 ml TBS-T and the membrane was incubated in the primary antibody solution for 1 h at 37 °C with shaking. Following a wash step as described above the membrane was incubated in 10 ml secondary antibody solution, 1:10,000 dilution of Anti-Rat IgG-AP (A-6066) in TBS-T, for 1 h at RT with shaking. Subsequently, the membrane was washed twice in 30 ml TBS-T and once in 30 ml TBS as described above. Colorimetric detection of transferred proteins was performed by incubating the membrane in 10 ml AP-buffer supplemented with 66 µl NBT solution and 33 µl BCIP solution. After 10 min the membrane was transferred into ddH_2O and incubated therein oN to enhance signal intensity before the membrane was air-dried.

3.15 STABLE TRANSFORMATION OF *NICOTIANA TABACUM*

3.15.1 INITIATION OF STERILE SHOOT CULTURE

To obtain sterile starting material, seeds were surface sterilized by submerging first in water containing 0.2% (w/v) Tween 20 for 3 min on a shaker (1,000 rpm). Then the water was removed, the seeds covered with 70% (v/v) ethanol and shaked for additional 2 min. After removal of the ethanol, seeds were treated with 1% (v/v) sodium hypochlorite in 0.2% (w/v) Tween 20 for 15 min with shaking. In a sterile flow hood the sodium hypochlorite solution was decanted, seeds washed 4 times in sterile ddH_2O and allowed to dry on sterile filter paper. To initiate sterile shoot cultures, seeds were sown on MS medium with 10 – 12 seeds per plate and germinated under light in a plant growth chamber with conditions as described in 2.1. Shoots were subcultured by cutting of the tops and placing them on fresh medium in Steri Vent containers (Duchefa, Haarlem, Netherlands) every three weeks.

3.15.2 *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION OF LEAF EXPLANTS

Stable transformation of *Nicotiana tabacum* cv. Xanthi NN was performed as described in Firoozabady and Kuehnle (1995). Leaf sections of 1 cm x 1 cm were prepared from the top three fully expanded leaves of sterile shoot cultures, placed an MS pre/cocultivation medium and precultured for three days in the dark. For transformation, *Agrobacterium tumefaciens* pMP90 containing the desired binary vector plasmid were grown to an OD_{600nm} of 0.8 – 1.0 oN in liquid LB medium supplemented with the appropriate antibiotics. Bacteria were harvested by centrifugation at 3,000 g for 20 min at 4 °C and

resuspended in liquid MS medium to an OD_{600nm} of 0.6. Acetosyringone (final conc. 200 µM) was added to the bacterial solution and bacteria were incubated for 30 min at 28 °C with shaking. Subsequently, leaf sections were submerged for 3 – 5 min in the solution, blotted on sterile filter paper and cocultivated on fresh MS pre/cocultivation medium in the dark to allow T-DNA transfer. After three days the explants were washed three times in liquid MS medium supplemented with 250 mg/l cefotaxime, transferred to MS selection medium plates and incubated in the light. About four weeks after transformation tiny shoots appeared around the leaf sections and explants were transferred on fresh plates to ensure nutrient supply and antibiotics exposure. When shoots were 10 – 15 mm in length they were removed from the leaf sections and transferred on MS regeneration medium where real transformants rooted within a few days but escapees were not able to develop roots under selective conditions. Only one to two shoots per explant were selected for further cultivation to avoid undesired accumulation of clones. Shoot cultures were subcultivated as described above, but under constant selection pressure on MS regeneration medium and kept *in vitro* till screening was finished.

Positive screened transformants with normal phenotyp and well developed roots were removed from MS regeneration medium and remaining medium was washed from roots under warm water. Shoots were potted in soil as described above, well watered and transferred to a plant growth chamber with above mentioned conditions. To facilitate *extra vitrum* adaption plantlets were covered with a plastic bag for several days to ensure high humidity.

3.15.3 SCREENING FOR TRANSGENIC TOBACCO PLANTS FOR EXISTENCE OF INSERT BY PCR

To confirm the introduction of the peptide sequences into the genome of positive transformants a PCR screening was performed with oligonucleotides annealing in the CaMV 35S promoter region and in the peptide sequences or solely in the peptide sequences. As neither the CaMV 35S promoter nor the sequences encoding for the peptides appear in untransformed plants, only real transgenic plants should give a positive signal. According to this, wildtyp *Nicotiana tabacum* cv. Xanthi NN served as negative control whereas the plasmids carrying the constructs used to transform plants served as positive controls. To ensure correctness of the PCR analysis and to exclude contamination of the reaction mixture a water sample was always included as additional negative control.

The reaction was set up as follows:

10x reaction buffer	2.0 µl
25 mM $MgCl_2$	1.2 µl
20 mM dNTPs	0.2 µl
10 mM 35s-pA5-fw	1.0 µl
or 10 mM NtSPxx_for*	1.0 µl
10 mM NtSPxx_rev*	1.0 µl
DMSO	0.5 µl
Taq DNA Polymerase (5 U/µl)	0.1 µl

DNA		50.0	ng
ddH_2O	ad	20.0	µl

PCR amplification was performed in a Hybaid PCR-Express thermocycler with following cycling conditions for 35s-pA5-fw/NtSPxx_rev*:

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	
Annealing	53 °C	30 sec	30
Extension	72 °C	30 sec	
Final extension	72 °C	5 min	1
Storage	4 °C	forever	1

Or following cycling conditions for NtSPxx_for*/NtSPxx_rev*:

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	
Annealing	63 °C	20 sec	30
Extension	72 °C	15 sec	
Final extension	72 °C	5 min	1
Storage	4 °C	forever	1

(*) indicates the different forward primer NtSP1-1_for, NtSP10-2_for, NtSP13_for and the accordant reverse primer NtSP1-1_rev, NtSP10-2_rev, NtSP13_rev.

3.15.4 SOUTHERN BLOT ANALYSIS

Copy number of transgenes in stable transformed plants was determined using the 'DIG-System for nonradioactive detection of nucleic acids on membranes' (Roche Diagnostics, Mannheim, Germany) exactly as recommended by the manufacturer. 20 µg of extracted genomic DNA was Xba I digested and separated according to the size on a 0.8% agarose gel, including the DIG-DNA MW II marker (Roche Diagnostics, Mannheim, Germany). DNA was depurinated, denaturated and blotted on a nylon membrane by vacuum exactly as described in the 'LKB VacuGene XL Vacuum – DNA transfer unit' instructions. The 834 bp probe for detection was amplified following the 'PCR DIG Probe synthesis Kit' manual (Roche Diagnostics, Mannheim, Germany) with following cycling conditions for p35s_for/NtSP1.1_rev or p35s_for/NtSP10.2_rev or p35s_for/NtSP13_rev:

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	3 min	1

Denaturation	95 °C	30 sec	
Annealing	53 °C	30 sec	35
Extension	72 °C	48 sec	
Final extension	72 °C	5 min	1
Storage	4 °C	forever	1

Hybridization was performed oN, followed by UV cross-linking and washing of the membrane as described in the instructions of 'DIG Wash and Block buffer set' and 'DIG Easy Hyb granules' (Roche Diagnostics, Mannheim, Germany). Luminescence detection was conducted exactly as described in the 'DIG Luminescent Detection Kit' (Roche Diagnostics, Mannheim, Germany). Finally, membrane was exposed to luminescence detection film for 1 to 3 hr, which was developed using an automatic film processor (Curix 60, Agfa).

3.16 TRANSIENT EXPRESSION SYSTEM MAGNICON®

The modular transfection system magnICON® (Bayer CropScience GmbH, formerly Icon Genetics) was analyzed for its suitability for peptide production in tobacco. As this expression system is not self-made and in addition patent protected it was not the method of choice and thus just a brief description of performed experiments is given. Detailled information can be found in Marillonnet et al. (2004; 2005).

3.16.1 PREPARATION OF BACTERIA AND INFILTRATION OF PLANTS WITH GFP-ENCODING 3'-PROVECTOR MODULE TO EVALUATE TRANSGENE EXPRESSION

First studies aimed to determine the proper host plants and bacterial density in the infiltration solution for expression experiments and were done by analyzing recombinant GFP production in transfected plants. The vectors used were: pICH14011 carrying the phage C31 integrase, pICH17388 carrying the 5'-provector module and pICH7410 carrying the 3'-provector module with GFP as GOI. Cultures of *A. tumefaciens* pMP90 carrying the different plasmids were grown for 24 h at 28 °C with shaking (300 rpm) and harvested by centrifugation at 3,000 g for 20 min at 4 °C. Bacteria were carefully resuspended in infiltration buffer to remove cell fragments and remaining medium and again pelleted as described above. The washed bacteria were again resuspended in infiltration buffer and the OD_{600nm} of the solution was adjusted to 0.001 and 0.01 and 0.1 for each culture. The different cultures were mixed in equal ratio to yield the final infiltration solutions with OD_{600nm} of 0.003, 0.03 and 0.3. About five to six week old plants of the *Nicotiana tabacum* cultivars Xanthi NN, Xanthi nn, Samsun NN, Samsun nn and Petit Havana as well as *Nicotiana benthamiana* plants were investigated for expression performance. Single leaves were infiltrated with a syringae without a needle from the abaxial side that was scratched slightly afore with a scalpel to enhance penetration of the infiltration suspension. To infect whole plants vacuum infiltration was used. Therefore a beaker with one to five liter, depending on the size of the plants, of infiltration solution was placed in a vacuum chamber and the aerial parts of the plant

were submerged head first in the solution. A slight vacuum of 0.5 – 1.0 bar was applied and kept for 1 min before air was drawn in quickly. If necessary, this procedure was repeated once and afterwards most submerged parts of the plants were sucked with the bacteria solution. The presence of detergents like Silwet-L77 to enhance infiltration or acetosyringone to improve T-DNA transfer in the infiltration solution had no visible effect on transient expression and thus was not necessary (Wroblewski et al., 2005). After infiltration plants were kept under standard growth conditions and 5 dpi the GFP expression was monitored under fluorescent light and verified by SDS-PAGE.

3.16.2 TRANSIENT EXPRESSION EXPERIMENTS WITH PEPTIDE-ENCODING 3'-PROVECTOR MODULES

For analyzing the expression of peptides SP1, SP13 and SP15 the sequences optimized for plant codon usage were introduced into the empty 3'-provector module pICH11599 between the restriction sites NcoI and XbaI. Therefore, single stranded oligonucleotides encoding the direct and complementary peptide sequence including start and stop codons and Nco I and Xba I overhanging nucleotides were ligated into double digested pICH11599 following standard protocols with the start codon in frame with the Nco I ATG (as recommended in the Icon Genetics instructions). The resulting plamids were named p11599:SP1, p11599:SP13 and p11599:SP15. To facilitate possible purification plasmids with an additional His-Tag (6x His) directly downstream the start ATG were created in the same way and named p11599:His6-SP1, p11599:His6-SP13 and p11599:His6-SP15. Infiltration of *Nicotiana benthamiana* was performed as described above and the GFP-containing 3'-provector module served as infiltration and expression control.

3.16.3 TRANSIENT EXPRESSION EXPERIMENTS WITH PEPTIDE-FUSION PROTEIN-ENCODING 3'-PROVECTOR MODULES

As the small, cationic peptides might be instable in plant cells due to proteolytic degradation and to reduce loss during purification by possible aggregation with cell fragments or LMW-compounds, vectors with peptide sequences fused to GFP and GST were generated. SP13 was fused to GST by using the Gateway® Cloning Technology (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. First, the peptide was amplified by PCR from p11599:SP13 with the oligonucleotides attB1_SP13_for/attB2_SP13_rev and transferred into the donor vector pDONR221. Recombination with the expression vector pDEST15 produced a plasmid where SP13 is located downstream the GST fusion partner. The GST-SP13 containing expression cassette was amplified by PCR with the oligonucleotides GST-SP13_for/GST-SP13_rev to yield a fragment with a 5'-attached Bsa I restriction site and a 3'-attached Xba I restriction site. The purified and double digested PCR product was ligated into the Bsa I/Xba I double digested vector pICH10990 and the obtained plasmid named p10990:GST-SP13.

For fusion of SP1 to green fluorescent protein, GFP was first PCR amplified from pICH7410 with oligonucleotides GFP_for/GFP_rev. SP1 including preceding His-Tag was

attached to the purified GFP fragment in two successive PCR reactions with oligonucleotides GFP_for/GFP4_rev and GFP_for/GFPfusSP1_rev. The final PCR product contained in this order GFP, a His-Tag and SP1 and was flanked by a NcoI restrictions site at the 5'-end and a Xba I restriction site at the 3'-end. The purified and double digested PCR product was ligated into the Nco I/Xba I double digested pICH11599 and the obtained plasmid named p11599:GHS. Infiltration of *Nicotiana benthamiana* was performed as described above and the GFP-containing 3'-provector module served again as infiltration and expression control.

3.17 PURIFICATION AND CDNA CLONING OF TMV

3.17.1 TMV ISOLATION AND RNA EXTRACTION

The wildtyp virus was propagated on the susceptible host *Nicotiana tabacum* L. cv. Xanthi nn where viral particles accumulated to high amounts and could be easily purified by virion precipitation in the presence of PEG as described in Chapman (1998). For initial infection freeze-dried leaves of TMV infected plants were homogenized in 10 mM phosphate buffer using a pestle and mortar till the tissue was well macerated. Two lower leaves of six to seven weeks old tobacco plants were dusted with silicon carbide as abbrasive and infected by gently rubbing the plant sap on the surface (rub inoculation). Afterwards, leaves were washed with ddH_2O to remove remaining sap and plants continued to grow till most of the systemic leaves showed typical mosaic symptoms (approx. 4 weeks after infection). For virus purification 20 g of systemically infected leaves were homogenized using a pestle and mortar in 60 ml virion extraction buffer with little acid-washed sand to aid homogenization and the homogenate was filtered through two layers of Miracloth into 30 ml Corex centrifuge tubes. 1-Butanol (0.8 ml/10 ml of filtrate) was added dropwise to the filtrate, while swirling the tube contents. After 15 min of incubation at RT with inversion every few minutes chlorophyll and coagulated material collected in the upper organic phase. The tube contents were centrifuged at 10,000 g for 30 min at 12 °C and the lightly pigmented aqueous phase was recovered. The clarified extract was filtrated again through two layers of Miracloth into fresh centrifuge tubes and 20% (w/v) PEG 8000 was added to give a final concentration of 4% (v/v) followed by a 15 min incubation step on ice with periodical mixing. As the solution turns cloudy the virus precipitates and can be pelleted by centrifugation at 10,000 g for 15 min at 4 °C. The supernatant is completely removed and the whitish pellet dissolved in 8 ml of 10 mM phosphate buffer and centrifuged at 10,000 g for 15 min at 4 °C to remove traces of pigmented material. After transferring the supernatant to a fresh tube 5 M NaCl (1.7 ml per 8 ml of supernatant) and 20% (w/v) PEG 8000 (2.42 ml per 8 ml of supernatant) was added and the mixture incubated on ice for 15 min. Centrifugation at 10,000 g for 15 min at 4 °C yields a white viral pellet that was dissolved in 2 ml of 10 mM phosphate buffer after complete removal of the superntant. The purity with regard to contaminating plant proteins was determined by SDS-PAGE where the 17.5 kDa coat protein should

appear as the only visible protein band after Coomassie staining and aliquots of the viral preparation were frozen in liquid nitrogen and stored at -80 °C.
To extract the viral RNA 0.8 ml of the virion preparation was mixed with 0.2 ml of 5x RNA extraction buffer and transferred to a 2 ml microcentrifuge tube. 1 ml of phenol:chloroform was added and the mixture was vortexed briefly until an emulsion formed and centrifuged at 13,000 g for 5 min at RT. The upper aqueous phase was transferred to a fresh microcentrifuge tube and the phenol:chloroform extraction step was repeated twice. To the aqueous phase of the third step an equal volume of chloroform was added, the mixture vortexed briefly to form an emulsion and phases were separated by centrifugation at 13,000 g for 5 min at RT. Again, the upper aqueous phase was transferred to a fresh microcentrifuge tube and the RNA was precipitated for 15 min at -20 °C after adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Centrifugation at 13,000 g for 15 min at 4 °C yields a visible white pellet that was dissolved in 0.2 ml of DEPC-treated ddH_2O after air-drying. RNA was quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and RNA integrity was determined by gel electrophoresis in agarose gels containing formaldehyd with subsequent visualisation by UV light illumination (302 nm). Aliquots of the viral RNA were frozen in liquid nitrogen and stored at -80 °C.

3.17.2 cDNA CLONING OF TMV

First-strand cDNA synthesis of the viral genomic RNA was performed using SuperScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) following the supplied protocol. The TMV specific oligonucleotide TMV_rev was derived from the complete genome of TMV variant 1, GenBank V01408 (Goelet et al., 1982) and was aimed to anneal to the last 22 nucleotides in the 3'-UTR. For optional subcloning steps a XmaI/SmaI restriction site was attached to the 5'-end of the oligonucleotide as overhanging sequence preceded by four randomly chosen nucleotides to enhance restriction enzyme cleavage.
The reaction was set up as follows:

TMV-RNA (full length)		2.5 µg
10 µM TMV_rev		1.0 µl
20 mM dNTPs		1.0 µl
ddH_2O	ad	11.0 µl

After heating to 72 °C for 10 min the reaction was quick chilled on ice and following components were added:

5x first-strand buffer	4.0 µl
0.1 M DTT	2.0 µl
RNaseOUT (40 U/µl)	1.0 µl
Superscript II RT (200 U/µl)	2.0 µl

The reaction was incubated oN at 42 °C in a thermocycler and afterwards inactivated by heating to 70 °C for 15 min.
The TMV cDNA was used without prior purification as template for second-strand synthesis that was performed using the Phusion High-Fidelity DNA Polymerase (NEB, Frankfurt, Germany) according to the manufacturer's instructions. The removal of RNA complementary to the cDNA by *E. coli* Rnase H was not necessary. The TMV specific oligonucleotide TMV_for was derived from the complete genome of TMV variant 1, GenBank V01408 (Goelet et al., 1982) and was aimed to anneal to the first 20 nucleotides in the 5'-UTR. For optional subcloning steps a Hind III restriction site was attached to the 5'-end of the oligonucleotide as overhanging sequence preceded by four randomly chosen nucleotides to enhance restriction enzyme cleavage.
The reaction was composed of:

5x Phusion HF buffer		4.0 µl
20 mM dNTPs		0.2 µl
10 µM TMV_for		1.0 µl
10 µM TMV_rev		1.0 µl
DMSO		0.5 µl
HF DNA Polymerase (2 U/µl)		0.4 µl
cDNA reaction		2.0 µl
ddH_2O	ad	20.0 µl

PCR amplification was performed in a Hybaid PCR-Express thermocycler with following cycling conditions:

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98 °C	3 min	1
Denaturation	98 °C	10 sec	
Annealing	56 °C	20 sec	35
Extension	72 °C	7 min	
Final extension	72 °C	10 min	1
Storage	4 °C	forever	1

Before further processing the result of the reaction was analyzed on a 0.7% agarose gel. To remove excess oligonucleotides and remaining components of the PCR reaction the 6415 bp PCR product was gel-purified using the TOPO® XL Gel Purification Kit supplied with the TOPO® XL PCR Cloning Kit (Invitrogen, Karlsruhe, Germany) as described in the manual but eluted from the column with ddH_2O. The purified PCR product was quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) as well as by agarose gel electrophoresis.
As the TMV cDNA clone was intended to be used in TA-Cloning into pCR®-XL-TOPO (Invitrogen, Karlsruhe, Germany) and the Phusion DNA polymerase lacks the nontemplate-dependent terminal deoxyadenosine transferase activity an additional A-

tailing step had to be performed. Therefore, Taq DNA polymerase (Agrobiogen, Hilgertshausen, Germany) was used to ensure the addition of 3' A-overhangs.
The A-tailing reaction was set up as follows:

Gel-purified PCR product (57 ng/µl)	6.0 µl
2 mM dATP	1.0 µl
25 mM $MgCl_2$	1.0 µl
10x reaction buffer	1.0 µl
Taq DNA Polymerase (5 U/µl)	1.0 µl

A-tailing was achieved by incubation at 72 °C for 20 min. The reaction mixture was directly used in the TOPO Cloning reaction that was performed exactly as described in the manual.

A-tailing reaction mixture	4.0 µl
pCR-XL-TOPO vector	1.0 µl

After 5 min of incubation at RT, 1 µl of the 6x TOPO® Cloning stop solution was added and finally 1 µl of this reaction mixture was used to transform electrocompetent *E. coli* cells as described above.
To analyze transformants several colonies were picked and cultured oN in LB medium supplemented with the appropriate antibiotic for subsequent plasmid DNA isolation. Plasmids were analyzed by restriction digest and those showing the right pattern of restriction fragments were sequenced using the following oligonucleotides: SeqTMV4241, SeqTMV5092, SeqTMV5943, SeqTMV6794, SeqTMV7645, SeqTMV8496, M13F_MediGX, M13R2_MediGX. In sum, the single sequencing reactions covered the whole genome of the cloned TMV and provided sufficient amounts of overlapping nucleotides to reconstruct the contiguous sequence using the Vector NTI tool ContigExpress. The obtained sequences were aligned with published sequences of TMV to verify accuracy or identify mutations using the Vector NTI tool AlignX and a positive clone pCR-XL-TOPO:TMV could be detected.

3.18 GENERATION OF A TRANSIENT EXPRESSION SYSTEM BASED ON TMV

3.18.1 RNA INFECTION VECTOR PT2SB

Plasmid pCR-XL-TOPO:TMV harboring a putative functional TMV cDNA clone served as template for the subsequent cloning steps to creat the final RNA infection vector pT2SB. The TMV sequence was amplified out of the plasmid using the oligonucleotides TMV_for/TMV_rev under above mentioned reaction conditions with 25 ng of the plasmid as template. The PCR product was gel-purified and served itself as template for PCR amplification with the oligonucleotides TMV_SP6, carrying a truncated SP 6 promoter as overhanging sequence at its 5'-end and TMV_BseYI, carrying a BseYI restriction site as overhanging sequence at its 5'-end.

The PCR reaction was set up as follows:

5x Phusion HF buffer		4.0 µl
20 mM dNTPs		0.2 µl
10 µM TMV_SP6		1.0 µl
10 µM TMV_BseYI		1.0 µl
DMSO		0.5 µl
HF DNA Polymerase (2 U/µl)		0.2 µl
Purified PCR product (89 ng/µl)		0.2 µl
ddH_2O	ad	20.0 µl

PCR amplification was performed in a Hybaid PCR-Express thermocycler with following cycling conditions:

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98 °C	3 min	1
Denaturation	98 °C	10 sec	
Annealing	52 °C	20 sec	35
Extension	72 °C	7 min	
Final extension	72 °C	10 min	1
Storage	4 °C	forever	1

Before further processing the result of the reaction was analyzed on a 0.7% agarose gel, again gel-purified using the TOPO® XL Gel Purification Kit supplied with the TOPO® XL PCR Cloning Kit (Invitrogen, Karlsruhe, Germany) and A-tailed for TA cloning.
The A-tailing reaction was set up as follows:

Gel-purified PCR product (21 ng/µl)	15.3 µl
2 mM dATP	1.0 µl
25 mM $MgCl_2$	1.2 µl
10x reaction buffer	2.0 µl
Taq DNA Polymerase (5 U/µl)	0.5 µl

A-tailing was achieved by incubation at 72 °C for 20 min. The reaction mixture was directly used in the TOPO Cloning reaction that was performed exactly as described in the manual.

A-tailing reaction mixture	4.0 µl
pCR-XL-TOPO vector	1.0 µl

Transformation of *E. coli* cells and screening for positive transformants was performed as described above. After sequencing, the putative functional clone named pT2SB could be identified.

3.18.2 LARGE INSERTION MUTAGENESIS PCR

The nucleotide sequences for designed peptides were introduced into pT2SB by mutagenesis PCR using long complementary oligonucleotides allowing direct fusion to the coat protein.
The reaction was set up as follows:

5x Phusion HF buffer		4.0 µl
20 mM dNTPs		0.2 µl
1 µM forward oligonucletide		1.0 µl
1 µM reverse oligonucleotide		1.0 µl
DMSO		0.5 µl
HF DNA Polymerase (2 U/µl)		0.2 µl
pT2SB in ddH_2O		25.0 µg
ddH_2O	ad	20.0 µl

PCR amplification was performed in a Hybaid PCR-Express thermocycler with following cycling conditions:

Step	Temperature	Duration	Cycles
Initial denaturation	98 °C	3 min	1
Denaturation	98 °C	15 sec	
Annealing	52 °C	20 sec	25
Extension	72 °C	7 min	
Final extension	72 °C	10 min	1
Storage	4 °C	forever	1

Before further processing the result of the reaction was analyzed on a 0.7% agarose gel. Afterwards, the PCR reaction was diluted 1:3 with sterile ddH_2O and a DpnI restriction digest was performed to remove the template plasmid before transforming *E. coli* cells as described above.

3.18.3 AGROINFECTION VECTOR PAGRO:T2SB

To simplify infection of plants, the vector pAGRO:T2SB was generated allowing the transfer of TMV into the plant cells by agroinfiltration. The backbone was derived from pICH11599 (Icon Genetics, Halle) providing the genetic elements for replication in bacteria, a selection marker as well as the T-DNA left and right border and the Nos terminator. The pUC ori was replaced by the pBR322 ori taken from pDest15 (Invitrogen, Germany) to reduce plasmid copy number in *E. coli* cells. The CaMV 35S promoter was amplified from the binary vector plasmid pK2GW7 (Uni Ghent, Belgium) and introduced downstream of the T-DNA left border. pT2SB served as template for amplification of the TMV cDNA.

First, the backbone was amplified from pICH11599 using the oligonucleotides 11599agro_for, harboring an Avr II restriction site and 11599agro_rev, harboring a Xho I restriction site. The nucleotide sequence of the CaMV 35S promoter was amplified from pK2GW7 using the oligonucleotides 35sagro_for, containing a Xho I restriction site and 35sagro_rev, containing an Age I restriction site. Both fragments were gel purified and Xho I restriction digested following standard conditions. After fragment clean up using the MinElute® PCR Purification Kit (Qiagen, Hilden, Germany) they were ligated to yield the plasmid pAGRO. This plasmid in turn was amplified using the oligonucleotides pAGRO322_for and pAGRO322_rev both having an Acc 65I restriction site attached to their 5'-ends to yield a linear fragment of pAGRO lacking the pUC ori sequence. To replace the primary origin of replication the pBR322 ori was amplified from pDest15 (Invitrogen, Karlsruhe, Germany) using the oligonucleotides pBR322_for and pBR322_rev both also harboring an Acc 65I restriction site. The fragments were gel purified, Acc 65I restriction digested, column purified as described above and ligated to yield the empty binary vector plasmid pAGRO322. The final agroinfiltration vector was created by amplifying the TMV cDNA from pT2SB using the oligonucleotides T2SBagro_for harboring an Avr II restriction site and T2SBagro_rev harboring an Age I restriction site and ligating the double digested PCR fragment into the Age I/ Avr II digested vector pAGRO322 to yield the final plasmid named pAGRO:T2SB. The plasmid pAGRO:T2SB was propagated in *E. coli* DH5α for further modifications and transformed in *Agrobacterium tumefaciens* GV3101 pMP90 for infiltration of plants.

3.19 INFECTION OF PLANTS WITH THE RECOMBINANT *TOBACCO MOSAIC VIRUS*

3.19.1 *IN VITRO* RNA SYNTHESIS AND INOCULATION WITH PT2SB(:SPXX) DERIVED IN VITRO RNA

To inoculate plants with infectious transcripts of the TMV cDNA clone, either wildtyp or recombinant carrying fused peptide sequences, *in vitro* RNA synthesis was performed using the RiboMAX Large Scale RNA Production Systems – SP6 and T7 (Promega, Mannheim, Germany). Prior to *in vitro* transcription approx. 20 µg pT2SB(:SPxx) were linearized by Bse YI digestion at 37 °C oN. After digestion the DNA was purified by two P:C:I extraction steps, followed by two C:I extraction steps, precipitated with 2.5 volumes of 100% (v/v) ethanol and finally washed twice with 70% (v/v) ethanol. The air-dryed linearized DNA was resuspended in DEPC-ddH_2O and quantified and analysed for purity using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). On average, 70% of the starting DNA could be recovered with a high purity suitable for *in vitro* transcription.
A 100 µl transcription reaction was set up as follows:

5x SP6 transcription buffer	20.0 µl
25 mM (each) ATP, UTP, CTP	20.0 µl
40 mM m^7G Cap analog	2.5 µl
linearized template DNA	12.0 µg

SP6 Enzym Mix	20.0 µl
nuclease free ddH_2O	ad 100.0 µl

After 5 min of incubation at 37 °C in an incubator 1 µl of 100 mM GTP was added. After additional 30 min of incubation 4 µl of 100 mM GTP was added to yield a final concentration of 5 mM GTP in the reaction and the transcription continued for further 2.5 h at 37 °C. Following *in vitro* transcription the DNA template was removed as recommended by the manufacturer and the result of the reaction analyzed on a formaldehyde containing agarose gel with wildtyp TMV RNA as size reference. The synthesized RNA was diluted with RNA infection buffer and used for infection of plants. Two lower leaves of six to seven weeks old tobacco plants were dusted with silicon carbide as abbrasive and infected by gently rubbing the RNA solution onto the surface (rub inoculation). Afterwards, leaves were washed with ddH_2O and plants continued to grow till either necrotic lesions appeared on resistant cultivars or systemic leaves of susceptible hosts showed typical symptoms of TMV infection.

3.19.2 INOCULATION BY AGROINFECTION WITH PAGRO:T2SB(-SPXX)

For infection of plants with the recombinant TMV cDNA clone using the agroinfection vector pAGRO:T2SB(-SPxx) some well grown colonies of *Agrobacteria* carrying the corresponding plasmid were transferred into 30 ml liquid LB medium supplemented with the appropriate antibiotics and grown oN. The next day, bacteria were pelleted at 3,000 g for 20 min at 4 °C, once washed in infiltration buffer and the suspension adjusted to an OD_{600nm} of 0.3 with the same buffer. Two to three leaves of four to six weeks old plants were infiltrated from the abaxial side with the bacterial suspension using a syringae without a needle. To facilitate the infiltration process the leaves were cut slightly with a scalpel before infiltration. Afterwards, leaves were washed with ddH_2O and plants continued to grow till systemic leaves of susceptible hosts showed typical symptoms of TMV infection.

3.20 EXTRACTION OF VIRAL X-BODIES FROM PLANT MATERIAL

Purification of the viral coat protein carrying the fused peptide sequences was carried out according to Parish and Zaitlin (1966) with some modifications. The described method is used to purify insoluble viral coat protein or so called X-bodies. Standard methods for TMV purification could not be used because the additional peptide sequence converts the coat protein into an insoluble state. About four weeks after inoculation of plants with the recombinant virus severe symptoms of infection were visible. All upper parts of the plants were harvested, frozen in liquid nitrogen and stored at –80 °C till further processing. Frozen material was first homogenized in liquid nitrogen using a pestle and mortar with little acid-washed sand to aid homogenization and grinding was continued after addition of 3 volumes of 100 mM NH_4HCO_3, 5% Triton X-100, 1% ß-ME till the tissue was well macerated. The homogenate was filtered through two layers of Miracloth into 40 ml Beckman centrifuge tubes and insoluble components were pelleted at 23,000 g (14,000 rpm in a Beckman JA-20 rotor) for 30 min at 4 °C. The dark-green supernatent was

discarded, the pellet resuspended in 1.5 volumes of the Triton X-100 containing buffer and again centrifuged. The supernatent was again discarded and the pellet was washed twice in 1 volume of 100 mM NH_4HCO_3, 1% ß-ME to remove remaining Triton X-100 with subsequent centrifugation as mentioned above. The remaining pellet was again resuspended in a little amount of 100 mM NH_4HCO_3, 1% ß-ME and dried in a lyophilizer to drive off buffer components that might interfere with following purification steps. To break viral x-bodies and to split virus-like particles dried insoluble material was resuspended in 1 volume of 70% (v/v) glacial acetic acid and stirred for 1 h at RT on a magnetic stirrer. Recombinant TMV coat protein carrying the fused peptide was now found dissolved in the acetic acid solution and an additional centrifugation step at 23,000 g for 30 min at 12 °C removed all remaining insoluble cell material. An aliquot was taken for analysis of extraction and determination of protein content before the supernatent was completely dried in a lyophilizer.

3.21 REDUCTION OF OXIDIZED METHIONINE RESIDUES

To enhance subsequent chemical cleavage reaction by CNBr to release the fused antimicrobial peptide from the recombinant TMV CP an reduction step was performed to convert possibly oxidized methionine residues into the reduced form. The lyophilized acetic acid extract was resuspended in 1 volume (based on the starting material) of 200 mM NH_4HCO_3, 5% ß-ME, blanket with argon and incubated oN at RT with inverting. The reduced protein extract was divided into 1 ml fractions in 2 ml reaction tubes and dried in a speedvac evaporator with heating to ensure complete removal of the bicarbonate that may form nonvolatile salts during the cleavage reaction with formic acid.

3.22 CHEMICAL CLEAVAGE OF TMV COAT PROTEIN FUSIONS WITH CNBR

The dried 1 ml fractions of the reduction step were dissolved in 800 µl 88% (v/v) formic acid which yields a clear, slightly yellow solution. 200 µl of 5 M CNBr in acetonitrile were added and the reaction was mixed by vortexing. The formic acid concentration in the reaction mixture was finally 70% (v/v) and CNBr was present at circa 100-fold molar excess over methionyl residues. The solution was overlaid with argon, wrapped in aluminium foil and incubated in the dark for 24 - 48 h at RT. When proteolysis was finished an aliquot was taken to analyze cleavage efficiency and samples were completely lyophilized in a speedvac evaporator, resuspended in 500 µl ddH_2O and again dried. Samples were stored at -20 °C till chromatographic separation of digested protein mixture.

3.23 PROTEIN CHROMATOGRAPHY

Chromatographic separation of proteins was performed on an ÄKTA Explorer 10 liquid chromatography system (GE Healthcare, Freiburg, Germany) equipped with a Frac-901 fraction collector and operated by the UNICORN control software version 5.11. All prepacked columns were purchased from GE Healthcare (Freiburg, Germany) and

equilibration, cleaning and maintenance procedures were carried out according to the manufacturer's instructions. Chemicals and organic solvents used for buffer preparation were of HPLC grade, filter sterilized (if possible) and degassed prior use. Only ddH_2O was used for liquid chromatography. Maintenance and cleaning procedures of the ÄKTA Explorer 10 were performed at regular intervals as indicated in the ÄKTA Explorer system manual.

3.23.1 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Lyophilized CNBr digested protein samples were resuspended in 250 µl SEC buffer (5% acetonitrile, 150 mM NaCl, 0.01 M HCl) and clarified by centrifugation at 10,000 g for 10 min at RT. The clear supernatent was manually injected in a 250 µl sample loop and separated according to the size in SEC buffer on a *Superdex Peptide 10/300 GL* column (GE Healthcare, Freiburg, Germany) with following method variables:

Operation temperature	RT
Flow rate	1.2 ml/min
Monitoring wavelength	215 nm, 280 nm
Empty loop	250 µl
Length of elution	1.5 CV

Fractions of 1 ml were collected and analyzed on Tricine-SDS gels for existence of protein bands in the expected size range. Runs with plant protein extracts were compared to blank runs respectively runs with synthetic peptides using the UNICORN 5.11 evaluation tool. Appropriate fractions were frozen in liquid nitrogen and stored at -80 °C till further processing.

3.23.2 REVERSED PHASE CHROMATOGRAPHY (RPC)

Selected SEC fractions were desalted and further separated on a 1 ml self-packed RPC column. *LiChrosorb RP-8* (Merck, Darmstadt, Germany), a C8-substituted silica based matrix with an average particle size of 10 µm, was chosen as separation medium to keep hydrophobic interactions of the desired peptides with the matrix low. SEC fractions were manually injected in a 1 ml sample loop and separated in RPC buffer (5% acetonitrile, 0.01 M HCl – 80% acetonitrile, 0.01 M HCl) using gradient elution with following method variables:

Operation temperature	RT
Flow rate	1.0 ml/min
Monitoring wavelength	215 nm, 280 nm
Empty loop	8 ml
Wash out unbound	4 CV
Length of gradient	20 CV

Fractions of 1 ml were collected and analyzed by MALDI-TOF-TOF mass spectrometry and on Tricine-SDS gels for existence of protein bands in the expected size range and runs with plant protein extracts were compared to blank runs respectively runs with synthetic

peptides using the UNICORN 5.11 evaluation tool. Correct fractions were completely dried down in a vacuum evaporator and stored at -20 °C till use.

3.24 MALDI-TOF-TOF MASS SPECTROMETRY

Mass spectrometric analysis were conducted at the 'Core facility proteomics' of the Helmholtz Center Munich. Purified recombinant peptides were analyzed on a MALDI time-of-flight tandem mass spectrometer (ABI 4700 Proteomics Analyzer, Applied Biosystems) to confirm sequence accuracy and to identify possible plant specific modifications. Peptides were spotted on matrix-assisted laser desorption ionization (MALDI) targets and diluted fourfold in 2.5 mg/ml of a-cyano-4-hydroxycinnamic acid (CCA), 70% acetonitrile and 0.1% TFA, by a Probot liquid handling system (Dionex). Data were analyzed using the GPS Explorer software (Applied Biosystems).

3.25 NMR SPECTROSCOPY

NMR spectroscopic analysis were conducted at the 'Insitute of structural biology' of the Helmholtz Center Munich. NMR measurements were carried out at 298 K on a Bruker Avance III 750-MHz spectrometer equipped with a TXI probe head, a 600-MHz spectrometer equipped with a TCI cryo-probe head or on an Avance 900 instrument equipped with a TXI cryoprobe head. Spectra were processed with NMRPipe and analyzed with Sparky. For measurements 0.5 mg synthetic peptide and tobacco purified peptide, respectively, was desalted by dialysis and measurements were performed in phosphate buffer.

3.26 ANTIBACTERIAL ACTIVITY ASSAY

The minimal inhibitory concentration leading to > 90% inhibition of bacterial growth (MIC^{90}) of synthetic and purified recombinant antimicrobial peptides was determined with a modified version of the microbroth dilution assay of the Clinical and Laboratory Standards Institute (CLSI/NCCLS). *In vitro* inhibition assays were performed in sterile flat-bottom 96-well plates (Greiner bio-one, Frickenhausen). Dilutions of peptides were prepared in peptide dilution buffer or ddH_2O and 10 µl of each concentration was loaded per well. Bacterial colonies from fresh agar plate cultures were transferred into LB medium, grown to an OD_{600nm} of 0.08 – 0.1 and diluted 1:100 into LB medium. 90 µl of this suspension containing about 10^5 CFU/ml of bacteria were added to each well resulting in final peptide concentrations of 0, 0.1, 0.25, 0.5, 1, 2, 2.5, 5, 10, and 20 µg/ml. After incubation at 28 °C oN on a rotary shaker (300 rpm) growth inhibition of the bacteria was analyzed by measuring the OD_{550nm} with an Tecan GENios microplate reader (Tecan, Crailsheim, Germany) after 5 min of intense orbital shaking to ensure evenly distribution in the well. For each peptide concentration two identical inhibition assays were performed with at least two independent replicates.

4. RESULTS

4.1 PRODUCTION OF ANTIMICROBIAL PEPTIDES IN STABLE TRANSFORMED PLANTS

To evaluate whether stable expression of genes encoding for the designed peptides is most suitable for *in planta* peptide production *Nicotiana tabacum* cv. Xanthi NN were transformed using the leaf disc method (Firoozabady and Kuehnle, 1995). Next to a kanamycin resistance gene for selection of positive transformants the transformation construct contained solely the peptide sequence without any fusion partner or signal peptide under the control of the CaMV 35S promoter combined with an Ω-leader sequence for strong expression and the corresponding CaMV 35S terminator. Numerous transgenic plants with normal phenotype were achieved and successful transformation was verified by PCR and Southern blotting (***Appendix, Figures 35-37***). As transformed plants showed normal development toxicity of the antimicrobial peptides to plant cells was not detected. Although expression of the peptide genes could be demonstrated on RNA level by qRT-PCR, no peptides were detected neither by Western blotting analysis using peptide-specific antibodies nor by MALDI-TOF-TOF analysis of total protein extracts (***Appendix, Figure 38***). Since peptide production could not be demonstrated a virus-based expression system was established.

4.2 IN PLANTA PEPTIDE PRODUCTION USING THE TRANSIENT EXPRESSION SYSTEM MAGNICON®

A transient expression system based on a deconstructed tobamovirus with highest expression levels was supplied by Icon Genetics (Marillonnet et al., 2004). This system was used for initial expression studies to analyze in principle the ability of tobacco to synthesize the designed antimicrobial peptides. Experiments with GFP as GOI (gene of interest) revealed that *Nicotiana benthamiana* displays high expression levels at the ideal OD_{600nm} of 0.001-0.01 of the infiltration suspension. In contrast, all tested *Nicotiana tabacum* cultivars showed low transgene expression, strong transprotein degradation two weeks after infiltration and required high amounts of bacteria in the infiltration solution (OD_{600nm} 0.3-0.8). At those bacterial densities the *Nicotiana tabacum* cultivars displayed also strong defense reactions against the *Agrobacteria*, manifested by necrotic lesions in the infiltrated areas. First trials in different *Nicotiana* species with untagged and His_6-tagged peptides targeted to the cytosol failed and no peptide production could be detected by immunoblotting analysis using antibodies raised against the His_6-tag. To overcome possible problems and restrictions that might arise as consequence of the short size of the peptides, e.g. problems during RNA-processing, translation or cytosolic peptide accumulation, fusion to GFP and GST was aspired. Fusion to an other protein should also decrease possible toxic effects of the peptides. To facilitate the potential purification process a His_6-tag was introduced between GFP and the sequence coding for the peptide (SP1). After vacuum infiltration of whole plants with a solution of *Agrobacteria* (OD_{600nm} of 0.001) carrying the expression constructs, strong GFP

fluorescence was visible under UV illumination (***Figure 8 A***). Nickel affinity chromatography, enabled by the internal His_6-tag, was used to enrich the fusion protein. The fraction containing the GFP-SP1 construct was easily detected under UV illumination (***Figure 8 B***). Attempts to produce SP13 fused to GST in *Nicotiana benthamiana* failed. Nonetheless, although the expression level and accumulation of GFP with fused peptide was really high, several points have to be considered using the magnICON® system.

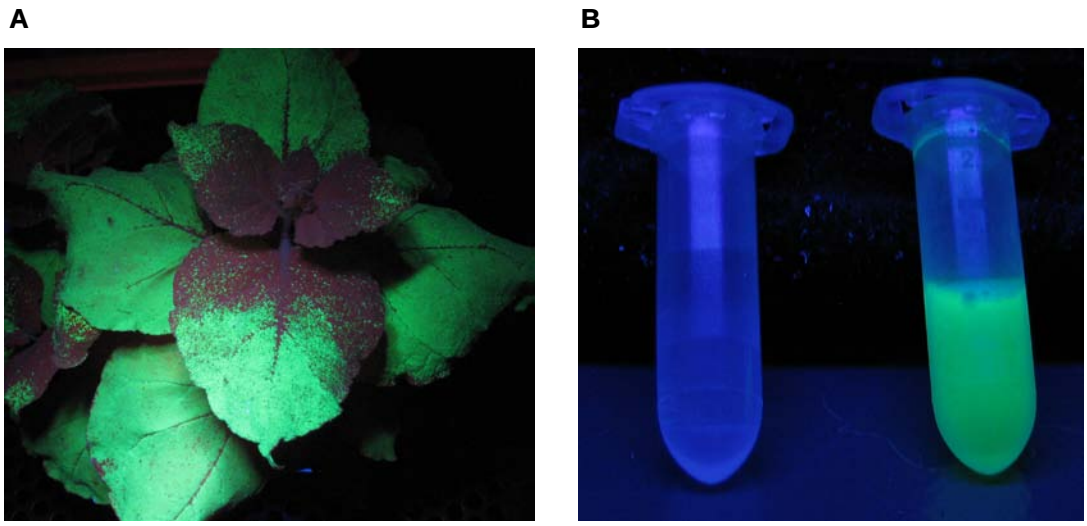

Figure 8 *Nicotiana benthamiana accumulating the peptide SP1 fused to GFP after transfection with the magnICON® expression system. (A) Plants were vacuum infiltrated with a mixture of three Agrobacteria cultures carrying the different expression constructs, whereas each Agrobacteria suspension had an OD600nm of 0.01. Pictures were taken 6 dpi under UV illumination. (B) Partial purification of GFP-His-SP1 on Ni Sepharose. Clarified plant extracts were loaded on a 1 ml HisTrap HP column (GE Healthcare) with a flow rate of 1 ml/min and eluted by gradually increasing the imidazole concentration.*

First, as the expression construct moves not systemic, infiltration of whole plants is necessary to achieve transgene expression in as many parts of the plants as possible. This is a quite time consuming and cumbersome procedure requiring an special apparatus for vacuum infiltration. Another crucial point is the fate of *Agrobacteria* in the plants. Even if this is not the natural habitat for a soil bacterium, they can at least survive for weeks in the infiltrated regions. Consequently, amounts of bacteria are present in the starting material for protein isolation and therewith abolish one advantage of plant based protein production, namely the elimination of bacteria derived compounds in the protein preparation. Hence, the idea was strengthened to generate an own expression system based on TMV that is able to move systemic through the plants thereby producing the peptides in all infected areas.

4.3 ISOLATION OF WILDTYP TMV AND GENOMIC TMV RNA EXTRACTION

Purification of *Tobacco mosaic virus* from infected leaves of *Nicotiana tabacum* cv. Xanthi nn according to Chapman (1998) could be performed without any difficulties and yielded high amounts of TMV virions. The purity was sufficient for the subsequent RNA extraction step, as no contaminating plant proteins were detected by SDS-PAGE showing the 17 kDa TMV coat protein (***Figure 9 A***).

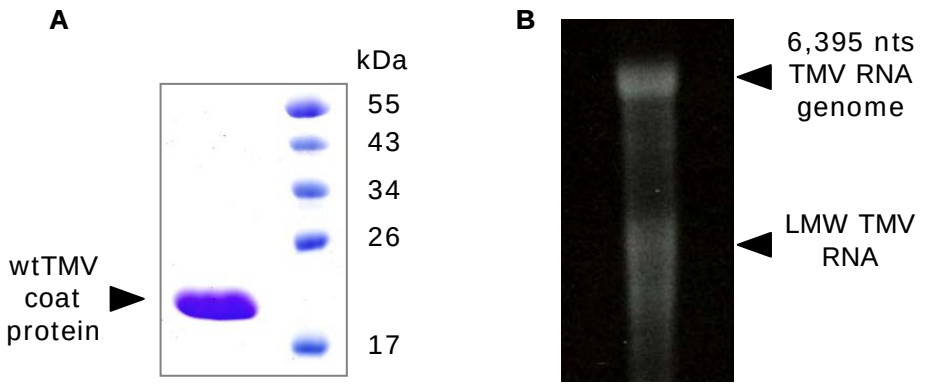

Figure 9 *Analysis of TMV purification. (A) Virus particle were isolated from infected leaf tissue and separated by SDS-PAGE. Relative molecular marker standards are shown on the right. kDA, kilodalton. (B) Denaturating formaldehyde gel electrophoresis of the TMV RNA genome. nts, nucleotides; LMW, low molecular weight.*

To extract viral RNA an aliquot of the virion preparation was adjusted to a concentration of 10 mg/ml and the RNA extraction was performed as described in Chapman (1998). Spectrophotometric analysis of the RNA preparation revealed a high purity, displayed by the 260/230 ratio of 2.3 and the 260/280 ratio of 2.0 and RNA integrity was confirmed by analysis on denaturating formaldehyde gels. The dominant high molecular mass band represents the 6,395 nts (nucleotides) TMV genome whereas the smear in the low molecular mass range is a result of coextracted subgenomic RNAs rather than degradation products (***Figure 9 B***).

4.4 cDNA CLONING OF TMV

To generate the full length cDNA clone of TMV the purified RNA genome was used as template. After Reverse Transcriptase mediated first strand synthesis the second strand was amplified in a standard PCR reaction using a proof-reading polymerase and after separation on a 0.8% agarose gel a 6.4 kb band was detected (***Figure 10***). After purification of the PCR product an A-tailing step was performed to enable the following TA-cloning into the TOPO vector.

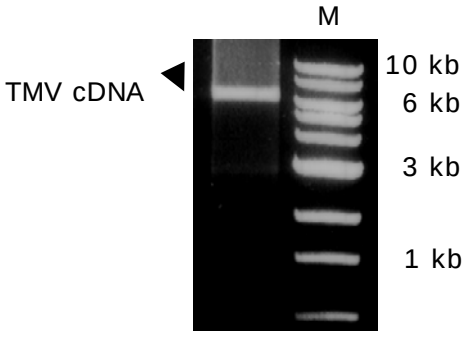

Figure 10 *cDNA cloning of TMV genome. Reaction of the second strand synthesis was analyzed on a 0.8% agarose gel. TMV, Tobacco mosaic virus; M, molecular weight marker; kb, kilobase.*

4.5 CLONING OF THE TMV cDNA INTO pCR-XL-TOPO

The amplified and A-tailed TMV construct was ligated directly into a pCR-XL-TOPO vector (***Figure 11***) which was transformed into *E. coli* DH5α.

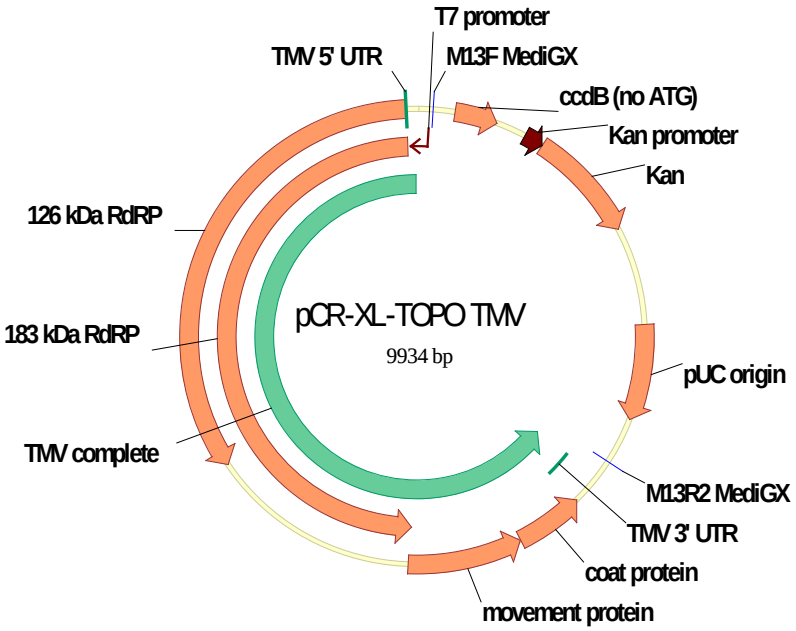

Figure 11 *pCR-XL-TOPO:TMV. UTR, untranslated region; RdRP, RNA dependent RNA polymerase; Kan, kanamycin resistance gene; M13F_MediGX and M13R2_MediGX indicate hybridization sites for standard M13 sequencing oligonucleotides.*

Interestingly, nearly all of the colonies detected after incubation over night (oN) under selective conditions showed a wrong fragmentation pattern after restriction digest (***Figure 12***).

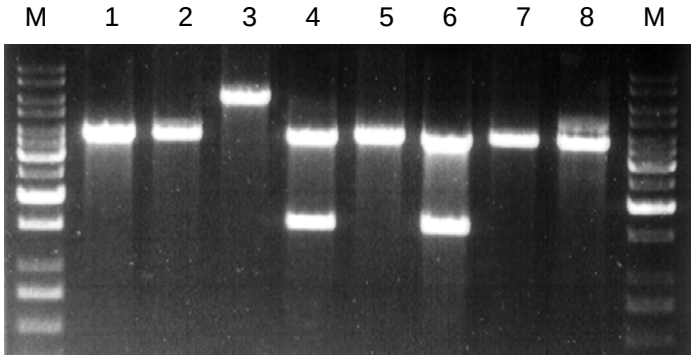

Figure 12 *Pvu I restriction digest of putative pCR-XL-TOPO:TMV (lanes 1-8). Fragments of 5268 bp and 4666 bp for direct orientation or 8363 bp and 1517 bp for complementary orientation were expected, but all clones showed a wrong fragmentation pattern.*

Partial sequencing of the putative TMV cDNA clones with oligonucleotides hybridizing to the viral UTRs revealed that all plasmids contained viral sequences. But mostly the 5'-UTR or 3'-UTR or long internal stretches of the viral genome were lost during cloning, probably as a result of the instability of RNA virus derived sequences in *E. coli* (Boyer and Haenni, 1994). Also the low amount of plasmid DNA found in transformed *E. coli* cells and their restricted and slow growth are indications for the toxicity of the TMV cDNA. Nonetheless, one clone could be detected carrying a complete TMV sequence, but surprisingly, on DNA/RNA level it showed highest sequence similarity to Holmes' masked strain of *Tobacco mosaic virus*, Genbank AF273221.1 (Holt et al., 1990) instead to TMV variant 1, GenBank V01408.1 (Goelet et al., 1982). Most of the 55 detected base changes relative to the common TMV strain variant 1 remained silent (***Appendix, Figure***

39). Only 6 mutations resulted in amino acid changes whereas 3 were located in the RdRP, 2 in the MP and 1 in the CP (***Table 3***).

Table 3 *Amino acid changes of identified TMV clone in comparison to TMV variant 1 GenBank V01408 (Goelet et al., 1982) and Holmes' masked strain (Holt et al., 1990). Corresponding to TMV variant 1, six aa changes were detected – three located in the RdRP, two in the MP and one aa change in the CP. RdRP, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein.*

	Position of aa mutation and ORF beloging						
	186 RdRP	187 RdRP	326 RdRP	361 RdRP	368 RdRP	417 RdRP	588 RdRP
TMV clone (this work)	C	C	S	S	K	A	P
Holt et. al (1990)	E	E	G	T	E	V	A
Goelet et al. (1982)	M	R	S	S	K	A	P
	602 RdRP	669 RdRP	748 RdRP	129 MP	143 MP	228 MP	139 CP
TMV clone (this work)	E	R	A	T	V	S	F
Holt et. al (1990)	K	R	T	S	V	S	S
Goelet et al. (1982)	E	K	A	T	I	N	S

Additionally, although the TA-cloning procedure allows insertion of the TMV cDNA in both orientations into the empty vector plasmid, all clones, independent if mutated or not, showed the insert on the complementary strand.

4.6 GENERATION OF THE TMV RNA EXPRESSION VECTOR PT2SB

To enable the functional *in vitro* transcription of the cloned TMV genome additional vector modifications were necessary. Upstream the 5'-UTR a truncated promoter for the bacteriophage SP6 RNA polymerase was introduced. In contrast to the T3 or T7 promoters the SP6 promoter was best suited for this purpose as the last 3 nucleotides can be neglected and the first incorporated nucleotides nearly match the original TMV sequence (***Table 4***). To avoid additional nucleotides at the 3'-UTR the non-TMV-cutting restriction site Bse YI was introduced downstream the TMV clone.

Table 4 *Introduction of bacteriophage SP6 promoter to enable exact transcription of in vitro RNA wtih pT2SB as template.*

	Sequence 5'-3'	First nucleotides
SP6 promoter	ATTTAGGTGACACTATA **GAA**GNG	GAA GNG
SP6 promoter trunc.	ATTTAGGTGACACTATA **GAA**	GAA
TMV 5'-UTR		GTA TTTTT

After plasmid linearization with the accordant enzyme only one extra nucleotide remains attached to the 3'-end and serves as template during *in vitro* RNA synthesis. The plasmid carrying the functional TMV clone under the control of above mentioned elements was generated by PCR from pCR-XL-TOPO:TMV. The resulting plasmid was named pT2SB, because it carries the **T**MV cDNA and was the **2**nd clone under the control of the **S**P6 promoter and the **B**se YI restriction site that showed the right fragmentation pattern after restriction digest (***Figure 13 and 14***).

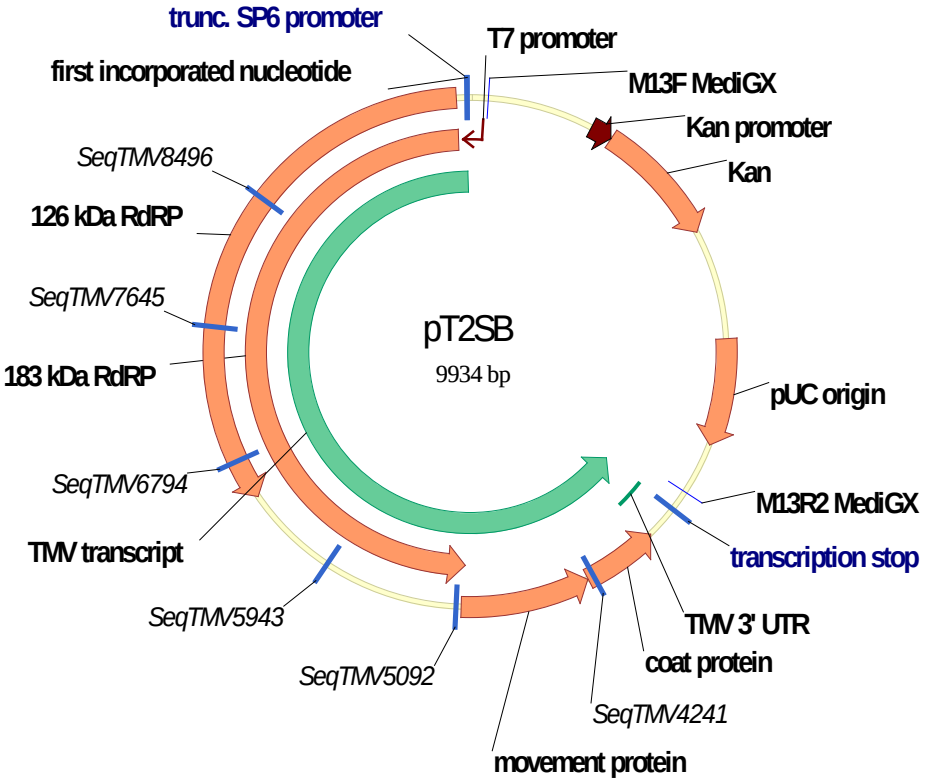

Figure 13 *TMV RNA expression vector pT2SB. UTR, untranslated region; RdRP, RNA dependent RNA polymerase; Kan, kanamycin resistance gene. M13F_MediGX and M13R2_MediGX indicate hybridization sites for standard M13 sequencing oligonucleotides and hybridization sites for TMV-specific sequencing oligonucleotides are indicated in italics. Locations of regulatory sequences are indicated in blue.*

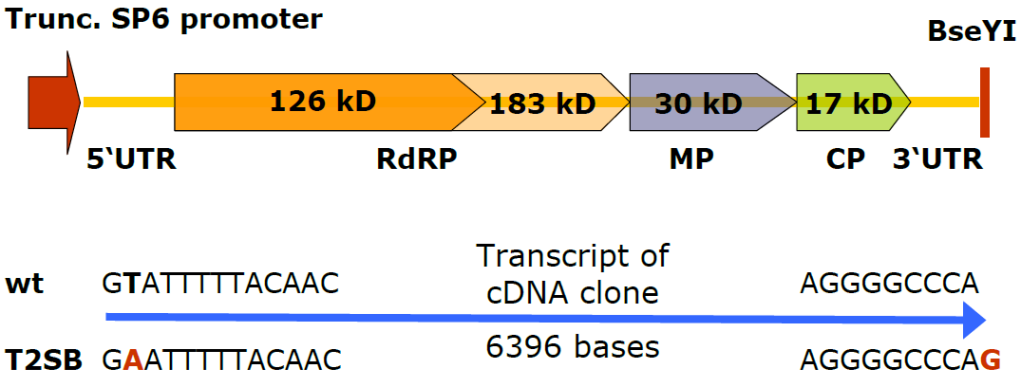

Figure 14 *Functional TMV cDNA clone flanked by the regulatory elements SP6 promoter and Bse YI restriction site. 5'UTR, 5'-untranslated region; 3'UTR 3'-untranslated region; RdRP, RNA dependent RNA polymerase; MP, movement protein; CP, coat protein.*

4.7 INFECTIVITY ANALYSIS OF PT2SB ON TOBACCO

To analyze the proper functioning of pT2SB derived RNA, infection studies were performed with *Nicotiana tabacum* cv. Xanthi NN as local lesion host and *Nicotiana tabacum* cv. Xanthi nn as systemic host. Purified RNA from the *in vitro* transcription reaction or purified wildtyp TMV were used to infect leaves by rub inoculation. Two to three days after infection, the resistant plants showed the typical HR as well known reaction after TMV infection (***Figure 15 A***). The susceptible plants react by developing the typical mosaic symptoms about two weeks after infection (***Figure 15 B***).

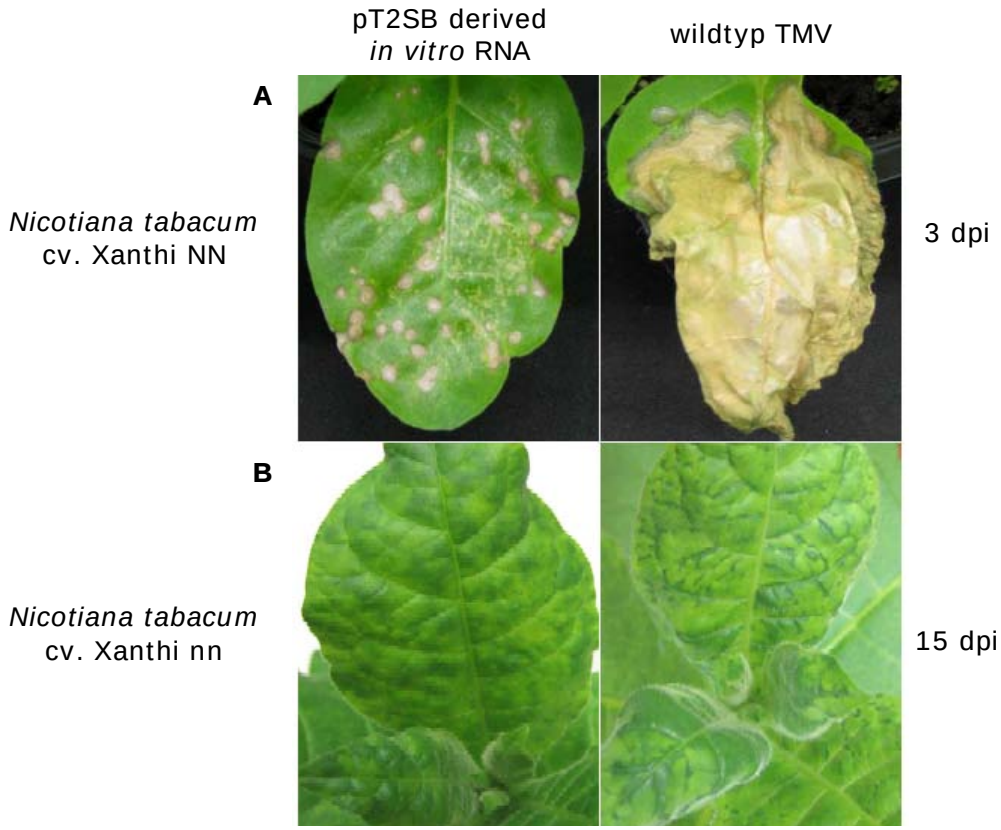

Figure 15 *Infectivity analysis of pT2SB derived RNA on Nicotiana tabacum cvs. Xanthi NN (A) and Xanthi nn (B). Leaves of healthy tobacco plants were dusted with carborundum and rub inoculated with either purified RNA of wtTMV or RNA generated during in vitro transcription with pT2SB as template. After infection, plants are grown under standard conditions. dpi, days post infection.*

A sligth delay in mosaic formation was observed on plants infected with the recombinant viral RNA. This might be due to the lower inoculum concentration compared to the wildtyp virus, probably as consequence of inaccurate synthesized *in vitro* RNA or RNA degradation during infection.

Although the mosaic symptoms induced by the pT2SB derived RNA differ from the symptoms induced by wildtyp TMV, both viruses move systemically and accumulate to similar levels *in planta*. Wildtyp TMV and T2SB accumulation in infected leaves was detected by SDS-PAGE 7 dpi (***Figure 16, lanes 3 and 4***) and 15 dpi the virus was present in systemic leaves (***Figure 16, lanes 8 and 9***). Calculated on the integrated density of the TMV CP band the amount of isolated CP was in all cases 20-23% of total extracted proteins. Therefore, the basis for the use of pT2SB as expression system is given.

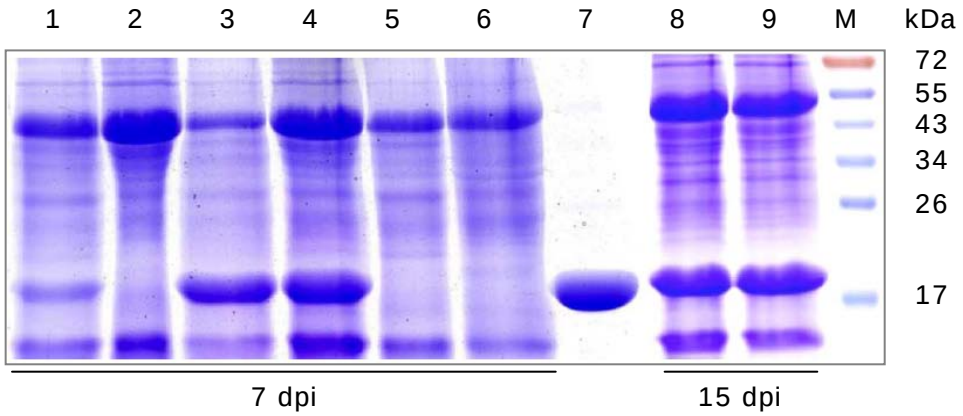

Figure 16 *Expression analysis of pT2SB derived in vitro RNA. Crude protein extracts from infected Nicotiana tabacum cv. Xanthi plants or purified TMV were separated on 15% SDS-PAGE followed by Coomassie staining. Lane 1, pT2SB local leaf; lane 2, wtTMV local leaf; lane 3, pT2SB systemic leaf; lane 4, wtTMV systemic leaf; lane 5, mock local leaf; lane 6, mock systemic leaf; lane 7, purified wtTMV; lane 8, wtTMV systemic leaf; lane 9, pT2SB systemic leaf. Relative molecular marker standards are shown on the right. M, molecular weight marker; dpi, days post infection.*

4.8 PCR-BASED INTRODUCTION OF PEPTIDE SEQUENCES INTO THE TMV GENOME

To avoid inefficient ligation reactions of the short sequences encoding for the antimicrobial peptides and the large pT2SB expression vector, a PCR based approach was used to introduce the sequences for peptide SP1-1, SP6, SP10-2 and SP13 into the recombinant TMV genome (***Appendix, Table 11***). Therefore, long oligonucleotides with overhanging peptide sequences were used to perform a PCR reaction with pT2SB as the template. In this way it was not necessary to further modify the TMV genome by the insertion of an additional cloning site and also the digestion of vector and peptide encoding insert could be avoided. After Dpn I digestion of the PCR reaction the mixture could be used directly for transformation of *E. coli*. The selected peptides were C-terminal fused to the coat protein as described in ***Table 5***. In addition, for SP1-1 a construct was generated carrying the peptide sequence in a loop region of the coat protein between Pro^{63} and Asp^{66} with the intermediate amino acids eliminated. This region is known to tolerate additional amino acids introduced (Turpen et al., 1995).

4.9 INFECTIVITY ANALYSIS OF PT2SB:SP1-1, PT2SB:SP10-2 AND PT2SB:SP13 ON TOBACCO

After insertion of the peptide sequences into plasmid pT2SB, expression analysis were performed as described in chapter 4.7 for the wildtyp TMV clone. In addition, several constructs were also analyzed on *Nicotiana benthamiana*, the most often used *Nicotiana* species for transient expression approaches. *Nicotiana sylvestris* served as additional control. It was expected that resistant cultivars develop lesions similar to the reaction upon wildtyp TMV infection and that susceptible host plants show systemic infection with the recombinant TMV clone carrying the fused peptide sequences. The resistant *Nicotiana tabacum* cultivar showed the designated symptoms two to three days after infection with *in vitro* RNA derived from pT2SB:SP1-1, pT2SB:SP10-2 and pT2SB:SP13 but,

surprisingly, also the susceptible Xanthi nn plants, that should serve as expression platform, showed HR-like lesions (***Figure 17 A and B***). Since also leaves of *Nicotiana sylvestris* reacted with necrotic lesions upon infection with pT2SB:SP1-1, it was supposed that the coat protein-peptide fusion protein itself elicited this HR-like response.

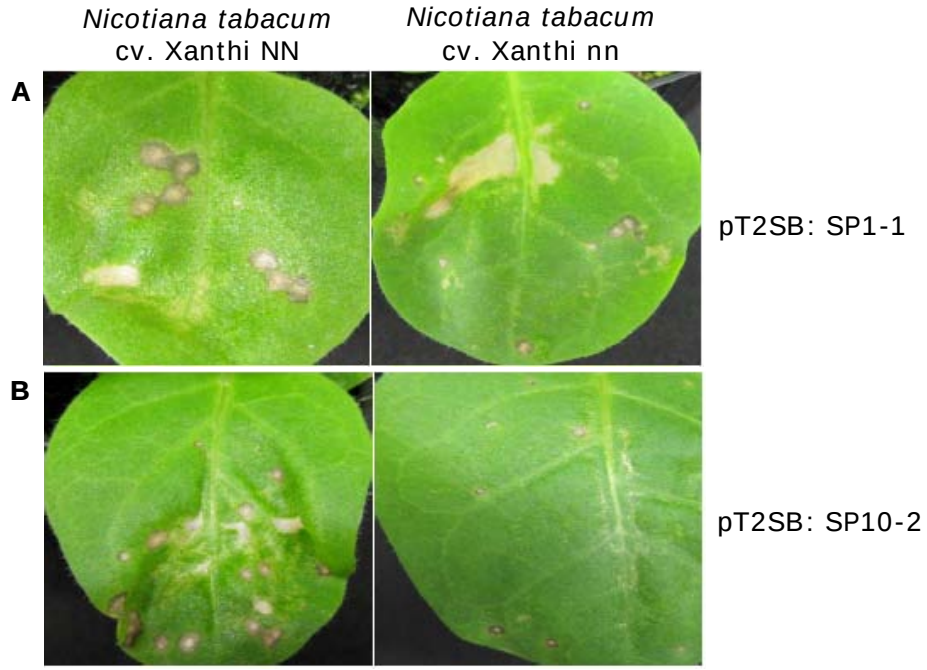

Figure 17 *Nicotiana tabacum cvs. Xanthi NN and Xanthi nn infected with pT2SB:SP1-1 (A) and pT2SB:SP10-2 (B) derived in vitro RNA. Both the resistant and the susceptible cultivar show distinct HR-lesions 6 dpi that restricted the recombinant TMV from local cell-to-cell movement in the leaf and inhibited formation of infection. dpi, days post infection.*

4.10 INTRODUCTION OF CHARGE COMPENSATING AMINO ACIDS TO STABILIZE TMV COAT PROTEIN-PEPTIDE FUSION PROTEINS

The introduction of the hydrophobic and positively charged peptide sequences into the TMV coat protein completely alters its biochemical properties, which might explain the loss of infectivity and the elicitation of a HR-like reaction on the primarily susceptible host. To restore the original coat protein properties additional amino acids were introduced that should compensate at least the charge differences (***Table 5***).

Table 5 *Overview of TMV coat protein-peptide fusions. Numbers above sequences designate location of introduced peptide sequences (red) and charge compensating amino acids (green). pI, isoelectric point; SP, synthetic peptide; cp coat protei; cc, charge compensation.*

Name	amino acid sequence - location of SP in TMVcp	charge at pH 7	pI
	coat protein		
pT2SB	**1 3 158** *SYS . . VWTSGPAT* *	- 2.23	5.09
pT2SB: SP1-1	**1 3 158 171** *SYS . . VWTSGPAT* **M RKKRLKLLKRLL** *	4.77	9.66

pT2SB: SP1-1cc	1 3 158 178 *SYS . . VWTSGPAT* DDEDDEE M RKKRLKLLKRLL *	- 2.22	5.27
pT2SB: ccSP1-1	1 3 4 10 151 158 178 *SYS . .* EDDEDEE *. . VWTSGPAT* M RKKRLKLLKRLL *	- 2.22	5.29
pT2SB: SP1-1 loop	1 3 63 66 170 *SYS . . RFP* M RKKRLKLLKRLL M *DFK . . TSGPAT* *	5.77	9.83
pT2SB: SP10-2	1 3 158 171 *SYS . . VWTSGPAT* M LRFLKKALKKLF *	2.77	9.22
pT2SB: SP10-2cc	1 3 158 176 *SYS . . VWTSGPAT* DDEDD M LRFLKKALKKLF *	- 2.22	5.22
pT2SB: SP13	1 3 152 173 *SYS . . VW* M KRRLIARILRLAARALVKKR * *TSGPAT* *	6.77	10.07
pT2SB: SP6cc	1 3 152 170 *SYS . . VW* E DDDDK ALAHFLKKAIKK * *TSGPAT* *	- 2.14	5.44

The “charge compensating motif” was composed of a varying number of the acidic amino acids aspartic acid and glutamic acid, randomly arranged and introduced preceding the methionine residue between coat protein and peptide sequence. For SP1-1 one construct was generated, harboring the charge compensation near the coat protein N-terminus to separate these additional foreign amino acid residues from the peptide. Infectivity analysis were performed as described in chaper 4.9. All of the recombinant TMV clones induced the HR in *Nicotiana tabacum* cv. Xanthi NN, which demonstrates that the vector in principle works and that at least the RNA-dependent RNA polymerase is proper synthesized *in planta* since its helicase domain elicits the HR in resistant plants.

Figure 18 A showes pT2SB:SP1-1cc infected leaves of both, the resistant and susceptible cultivar. The necrotic lesions induced by the construct are clearly visible. *Nicotiana sylvestris*, a host that reacts only to infections of some TMV strains in a HR-like manner also restricts spreading of recombinant TMV with N-terminal charge compensation (***Figure 18 B***). In addition, the charge compensation was not capable of overcoming the induction of the HR-like response of the susceptible plants upon infection with pT2SB:SP10-2cc (***Figure 18 C***).

The most used tobacco species for transient expression approaches based on viral vectors is *Nicotiana benthamiana* (Goodin et al., 2008). These plants are restricted in their response to plant viruses and therefore designated as hypersusceptible. Probably, the use of this host plant might allow the successful expression of the recombinant TMV clone carrying the fused peptide sequences. *Nicotiana benthamiana* plants were infected in the same way as *Nicotiana tabacum* plants and cultivated under the same conditions. In contrast to the *Nicotiana tabacum* plants, no HR was detected on *Nicotiana benthamiana* and four to five weeks after inoculation typical symptoms of a TMV infection occurred.

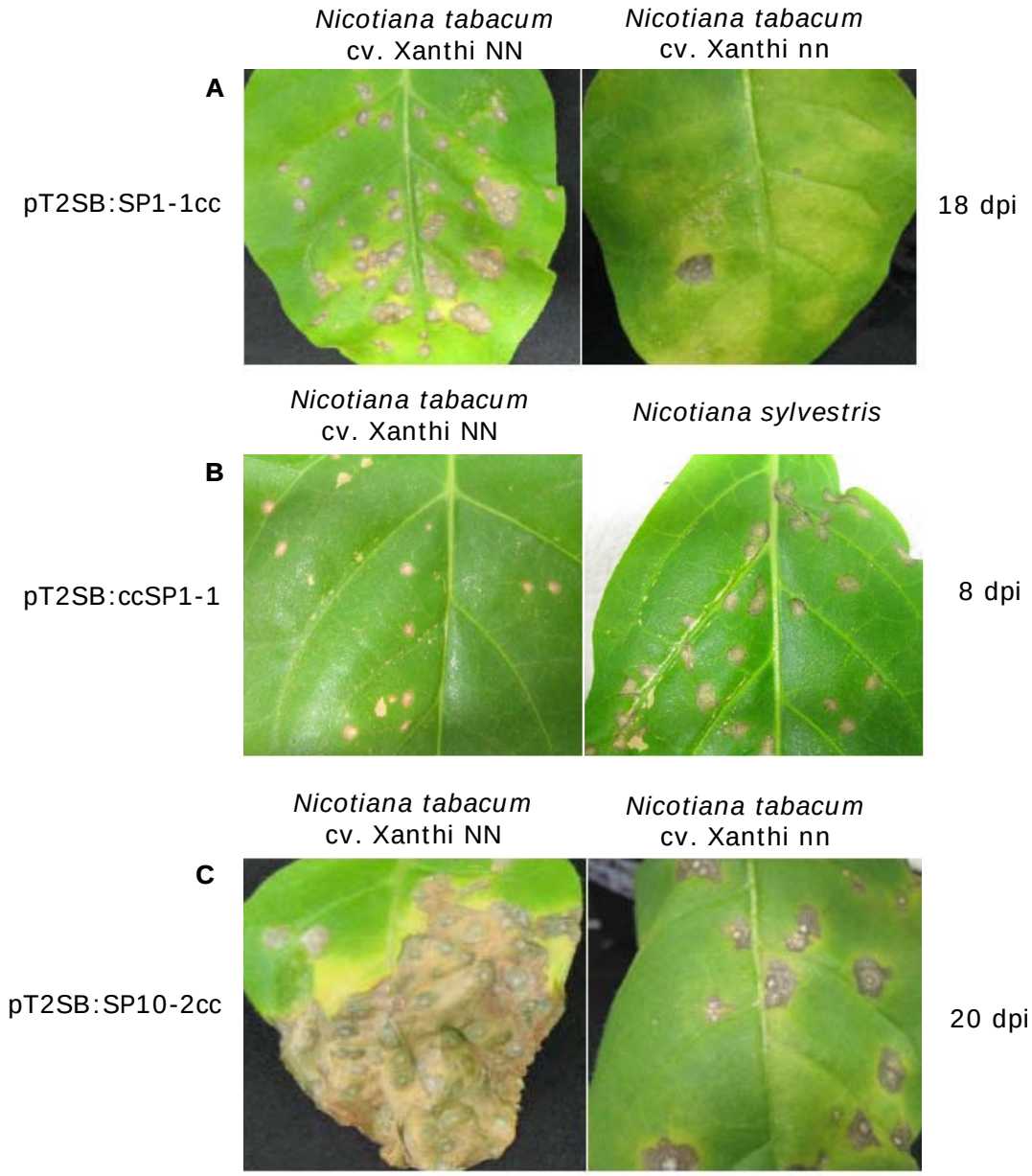

Figure 18 *Effect of charge compensating amino acids on induction of necrotic lesions. Nicotiana tabacum cvs. Xanthi NN and Xanthi nn and Nicotiana sylvestris were infected with pT2SB:SP1-1cc (A) and pT2SB:ccSP1-1 (B) and pT2SB:SP10-2cc (C) derived in vitro RNA. All plants showed HR-like lesions on infected leaves and local movement of recombinant TMV was not detectable even 20 dpi. dpi, days post infection.*

The main disadvantage of *Nicotiana benthamiana* as host for TMV-based full virus expression systems is the high susceptibility leading often to the death of the host plant. Furthermore, upon infection with the recombinant TMV clone carrying the fused peptide stem necrosis could be detected as indication for multiplication and spread of the virus (***Figure 19 A***). Interestingly, when the charge compensating amino acids were introduced directly upstream of SP1-1 the symptoms were more severe and reflected more a wildtyp TMV infection compared to the charge compensation located at the N-terminus of the fusion protein. The latter construct did not induce stem necrosis but resulted solely in strong yellowing and reduced and abnormal growth of the leaves whereas the plants survived for weeks (***Figure 19 B***).

Figure 19 *Nicotiana benthamiana infected with pT2SB:SP1-1cc (A) and pT2SB:ccSP1-1 (B) derived in vitro RNA. 28 dpi the recombinant TMV carrying the charge compensating amino acids and attached SP1-1 at the C-terminus of its coat protein lead to stem necrosis followed by wilting of upper plant parts (A, left) and death of infected leaves (A, right) as indicated by red arrows. In contrast, by arranging the charge compensation at the N-terminus and keeping SP1-1 at the C-terminus of the TMV coat protein symptoms were weakened to a strong yellowing of leaves (red arrow) and infected plants survived even 72 dpi (B). dpi, days post infection.*

Nicotiana benthamiana seems to work well as expression host when SP1-1 is combined with charge compensating amino acids. Therefore, these plants were used for further work and to analyze the production of the designed peptide SP1-1 *in planta*.

4.11 AGROINFILTRATION VECTOR PAGRO:T2SB AS ECONOMIC INFECTION METHOD

The *in vitro* RNA synthesis of the recombinant TMV genome carrying the additional peptide sequences is quite expensive and therefore not useful for large scale infection of plants. In addition, the RNA is instable and has to be handled carefully and yield is not only depending on reaction conditions but also on DNA template quality. Therefore another infection vector was created that relies on *Agrobacteria*'s capability of transferring DNA into plant cells, namely pAGRO:T2SB (***Figure 20***). The *in vitro* RNA synthesis cassette of pT2SB and peptide containing related vectors were transferred into a binary vector plasmid, derived from pBIN19. After exchange of the origin of replication from pUC Ori to pBR322 Ori the stability of pAGRO:T2SB and derivatives in *E. coli* could be increased and enabled easy handling of the agroinfiltration vector. Interestingly, although the regulatory sequences for *in vitro* RNA synthesis were not removed and also transferred into the plants the vector performed well and infection started in the

infiltrated leaves. As for the pT2SB-peptide constructs only *Nicotiana benthamiana* produced recombinant TMV clones. All other tested cultivars and tobacco species failed, although agroinfiltration with the vector carrying the original wtTMV clone lead to successful TMV infection in all susceptible cultivars.

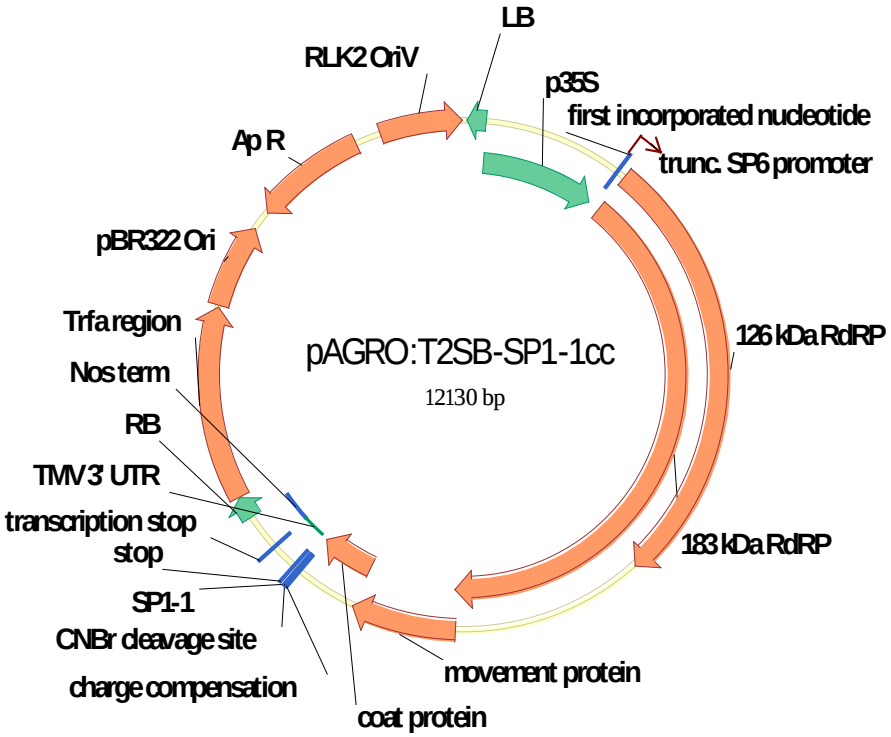

Figure 20 *pAGRO:T2SB-SP1-1cc as example for an agroinfiltration vector. RdRP, RNA-dependent RNA polymerase; p35S, CaMV 35S promoter; Ap R, ampicillin resistance gene; UTR, untranslated region; CNBr, cyanogen bromide; RB, right border; LB, left border; Nos term, nopaline synthase terminator.*

4.12 VERIFYING FUSION PROTEIN EXPRESSION

To ensure that the additional sequence was not lost by e.g. recombination during virus multiplication the expression of the TMV coat protein gene with attached peptide SP1-1 sequence was first confirmed on RNA level. Four weeks after infection of plants with pAGRO:T2SB-SP1-1cc or pAGRO:T2SB-ccSP1-1 symptoms as described in chapter 4.10 were visible and systemic leaves were harvested.

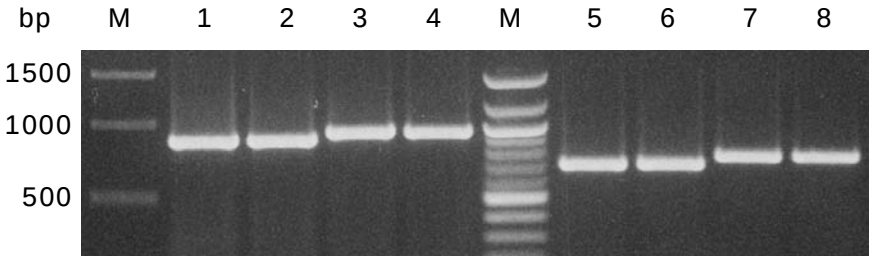

Figure 21 *PCR analysis of fusion protein production. Lanes 1-4, 883 bp and 943 bp PCR product with oligonucleotides TMVmp_for/TMV_rev. Lanes 5-8, 699 bp and 759 bp PCR product with oligonucleotides TMVcp_for/TMV_rev. Lanes 1+5, T2SB; lanes 2+6, wtTMV; lanes 3+7, T2SB:SP1-1cc; lanes 4+8, T2SB:ccSP1-1. M, marker; bp, basepairs.*

Total RNA was extracted and cDNA synthesis was performed with TMV 3'-UTR specific oligonucleotides followed by second strand synthesis with TMV coat protein/movement protein specific oligonucleotides. The product of the PCR reaction was analyzed by DNA gel electrophoresis for correct size (***Figure 21***). The electrophoretic separation of TMV and recombinant TMV derived cDNA clearly shows a difference in size as a result of the fused peptide sequences. To further verify the correctness of the expression construct the PCR product was sequenced and the received sequence was aligned with the predicted expression vector. No difference to the predicted sequence was detected (***Appendix, Figure 40***). Hence, these results reflect that the viral RNA of the recombinant virus is stable *in planta* for weeks and that the sequence encoding for the accordant peptide is not lost during systemic movement through the plant. Hence, one important parameter of a transient expression system is fulfilled.

4.13 ANALYZING FUSION PROTEIN PRODUCTION

The rate of fusion protein production was determined by SDS-PAGE of crude protein extracts of infected leaf material from *Nicotiana benthamiana*. Unfortunately, the recombinant TMV coat protein peptide fusion protein showed the tendency to form largely unsoluble aggregates *in planta* which complicates subsequent purification procedure.

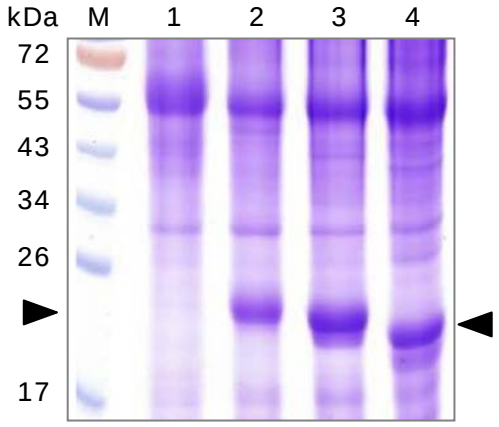

Figure 22 *Analysis of production of recombinant coat protein peptide fusion protein. Proteins were isolated from Nicotiana benthamiana leaves using 8 M urea containing extraction buffer and separated on a 12% polyacrylamide gel. 30 µg protein were loaded per lane. Lane 1, uninfected control; lane 2, T2SB:ccSP1-1 (15%); lane 3, T2SB:SP1-1cc (19%); lane 4, T2SB:SP6cc (12%). Data in brackets indicate amount of isolated recombinant CP [%] of total extracted proteins, calculated on the integrated density of the CP band. Relative molecular marker standards are shown on the left. M, marker; kDa, kilodalton.*

Standard TMV purification protocols are mainly based on extraction using aqueous buffers which could not be applied in this case. ***Figure 22*** shows solubilization of aggregated viral coat protein using a 8 M urea containing extraction buffer. Interestingly, although the constructs T2SB:ccSP1-1 and T2SB:SP1-1cc differ only slightly in charge, a clear difference in the electrophoretic mobility can be detected on reducing SDS-PAGE. Calculated on the integrated density of the recombinant TMV CP bands the amount of isolated T2SB:ccSP1-1 CP was 15% (***Figure 22, lane 2***), of T2SB:SP1-1cc CP 19%

(***Figure 22, lane 3***) and of T2SB:SP6cc CP 12% (***Figure 22, lane 4***) of extracted proteins. Several attempts were conducted to find out the most suitable extraction method and extraction buffer to get the viral "x-bodies" solubilized. Neither high detergent concentrations nor high salt concentrations were capable of dissolving the coat protein aggregates even though the wildtyp virus was solubilized under all tested buffer conditions (***Figure 23, left***). Only under denaturing conditions using at least 4 M urea the x-bodies could be dissolved. In addition, about one third of the coat protein, either wildtyp or recombinant carrying the attached peptide, was not solubilized in the first extraction step and remained in the pellet (***Figure 23, right***). A crucial point with respect to preparative protein extraction.

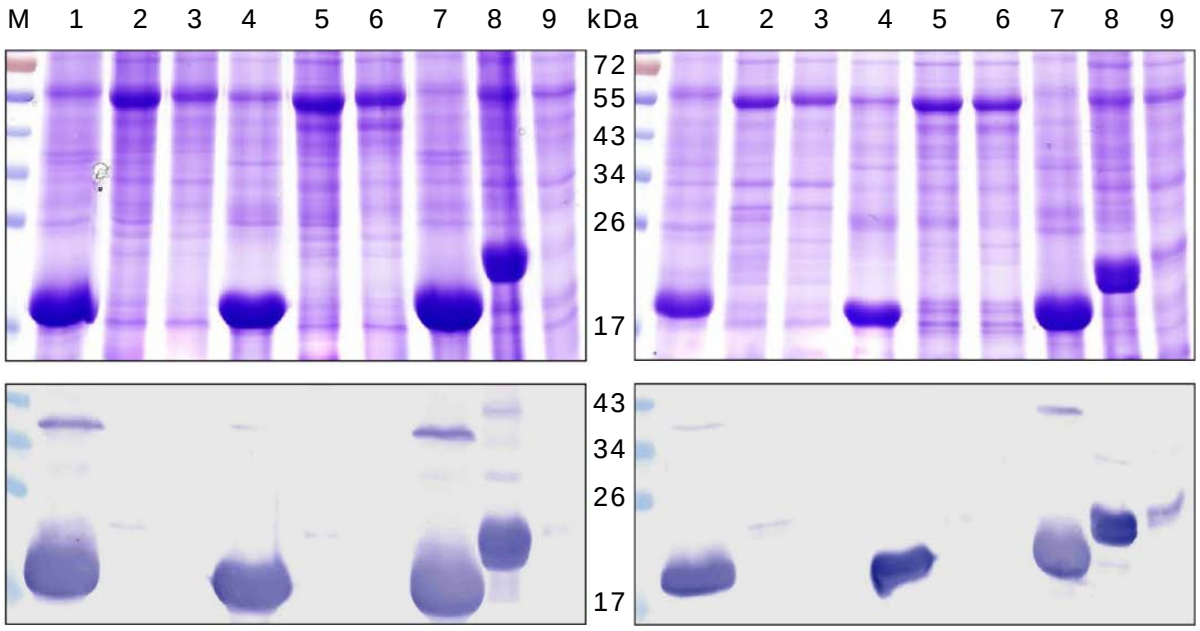

Figure 23 *Solvent screen for recombinant TMV extraction. Upper row, left part showing 12%-SDS-PAGE of proteins extracted with different buffers from Nicotiana benthamiana infected with T2SB (lanes 1 (40/31%), 4 (39/31%), 7 (40/35%)), T2SB:SP1-1cc (lanes 2, 5, 8 (25/25%)), T2SB:ccSP1-1 (lanes 3, 6, 9). Extracts in lanes 1-3 with buffer containing 2% (w/v) Triton X-100, extracts in lanes 4-6 with buffer containing 1.5 M NaCl, extracts in lanes 7-9 with buffer containing 4 M Urea. Upper row, right part showing second extraction, numbering same as left side. Data in brackets indicate amount of isolated CP [%] of total extracted proteins, calculated on the integrated density of the CP band; first value left side, second value right side. Lower row, Western Blots displaying above pictured protein gels, detection with polyclonal anti-TMV-IgG. Relative molecular marker standards are shown on the left, in each case. M, marker; kDa, kilodalton.*

4.14 ATTEMPTS TO ENHANCE TRANSIENT EXPRESSION VECTOR PERFORMANCE

One consequence of the coat protein-attached peptide is the constriction of the recombinant virus to move systemically in the plant, reflected by a very slow infection manifestation. By DNA shuffling, Toth et al. (2002) could show that single point mutations in the gene of the movement protein ORF of TMV can improve the infection rate of handicapped viruses dramatically. To take advantage of this possibility, thymidine at position 5116 was mutated to guanine, resulting in an amino acid change from leucine to valine at position 72 of the movement protein. Both, the wtTMV clone as well as the virus carrying SP1-1 were analyzed for their properties to infect *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Xanthi nn systemically. As described in chapter 4.5, the

isolated TMV clone showed highest similarity to Holmes' masked strain with three amino acid changes located in the RdRP. One position critical for symptom attenuation is A^{2074} (Holt et al., 1990). Because T2SB carries a guanine at this position mutagenesis was performed to restore the wildtyp conditions. Both modifications had no impact on infectivity of wtTMV but, surprisingly, abolished completely the capability of T2SB:SP1-1cc to replicate in plants and to initiate infection (***Figure 24***).

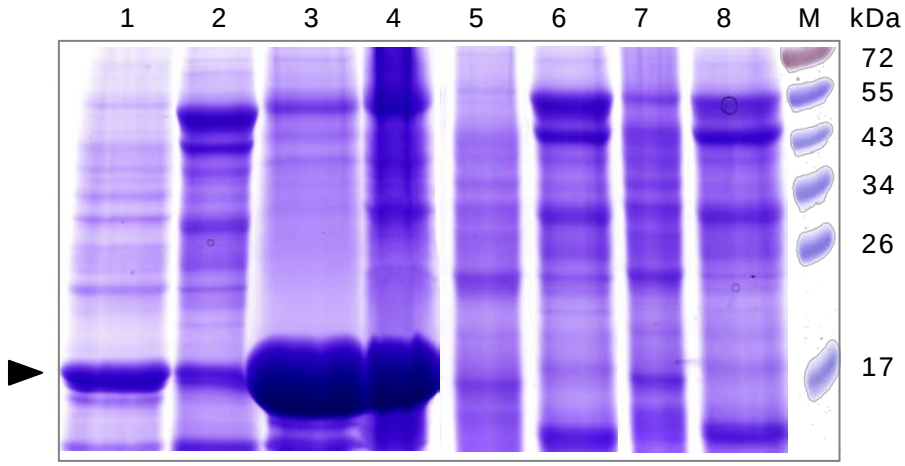

Figure 24 *Effect of single point mutations on expression vector performance. 12%-SDS-PAGE showing total protein extracts of local and systemic leaf of N. benthamiana (1, 3) and N. tabacum cv. Xanthi nn (2, 4) infected with pT2SBΔT5116G. Local leaf of N. benthamiana (5) and N. tabacum cv. Xanthi nn (6) infected with pT2SB:SP1-1ccΔT5116G. Local leaf of N. benthamiana (7) and N. tabacum cv. Xanthi nn (8) infected with pT2SB:SP1-1ccΔG2074A. Relative molecular marker standards are shown on the right. M, marker; kDa, kilodalton.*

Based on these findings and the difficulties during cloning and subcloning steps with the viral sequence further attempts to modify the expression vector were rejected and the initially obtained TMV clone was used for further experiments.

4.15 DEVELOPMENT OF THE PURIFICATION PROTOCOL FOR T2SB:CCSP1-1 AND T2SB:SP1-1CC

Following verification of existence of the TMV coat protein with fused peptide SP1-1 several attempts were conducted to purify the recombinant virus or viral inclusion bodies, respectively. A couple of standard methods for TMV purification were tried but none of them could be applied to isolate the hard to dissolve viral aggregates. ***Figure 25*** shows TMV purification strategies according to Asselin & Zaitlin (1978) and Bendahmane et al. (1999) using ultracentrifugation and selective precipitation with PEG 6000 for T2SB:SP1-1cc and T2SB:ccSP1-1. No viral pellet was detected after ultracentrifugation in 20% sucrose gradient of aqueous protein extracts of *Nicotiana benthamiana* infected with pT2SB:SP1-1cc (***Figure 25, lane 1***) and pT2SB:ccSP1-1 (***Figure 25, lane 2***). Also, attempts to enrich recombinant TMV in total protein extracts by removal of possible aggregation partners by celite failed for pT2SB:SP1-1cc (***Figure 25, lane 3***) and pT2SB:ccSP1-1 (***Figure 25, lane 4***). The standard procedure to precipitate TMV from solution by PEG 6000 failed for pT2SB:SP1-1cc (***Figure 25, lane 5***) and pT2SB:ccSP1-1 (***Figure 25, lane 6***) as the recombinant virus T2SB:SP1.1cc remained in the insoluble

fraction even after low speed centrifugation at 3,000 g (***Figure 25, lane 7***). The latter fraction was composed of about 37% of recombinant CP of total proteins, calculated on the integrated density of the CP band. Apparently, it failed to get the modified virus into solution during the first extraction step and all of it remained in the pellet after low speed centrifugation.

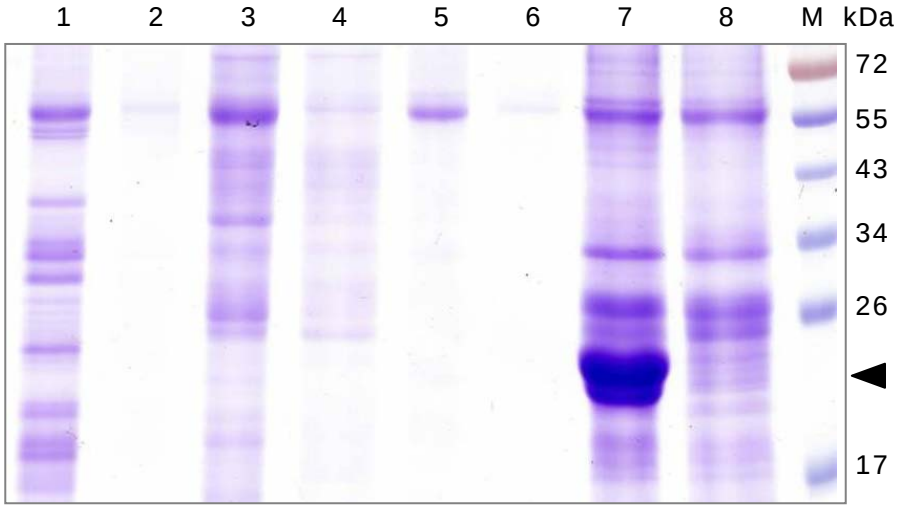

Figure 25 *TMV extraction attempts according to Asselin & Zaitlin (1978) and Bendahmane et al. (1999). Fractions after ultracentrifugation in 20% sucrose gradient of aequous protein extracts of Nicotiana benthamiana infected with pT2SB:SP1-1cc (lane 1) and pT2SB:ccSP1-1 (lane 2). Removal of possible aggregation partners by celite failed for pT2SB:SP1-1cc (lane 3) and pT2SB:ccSP1-1 (lane 4). PEG 6000 precipitation of TMV from solution failed for pT2SB:SP1-1cc (lane 5) and pT2SB:ccSP1-1 (lane 6). The recombinant virus T2SB:SP1.1cc remained in the insoluble fraction even after low speed centrifugation at 3,000 g (lane 7). Lane 8, protein extract of uninfected control plant. Relative molecular marker standards are shown on the right. M, marker; kDa, kilodalton.*

Different buffer compositions and organic solvents were analyzed for their ability to solubilize the viral aggregates and to separate the coat protein from the chlorophyll containing solution to allow liquid chromatography. Furthermore, differing trials to selectively precipitate contaminants or the protein of interest or to separate solubilized compounds were not successful. During removal of chlorophyll by phosphoric acid/sulfuric acid treatment the recombinant coat protein with fused SP1-1 precipitated with contaminants and no protein remained in the supernatent (***Figure 26, lane 1***). Also extraction with methanol failed to get the coat protein-peptide fusion in solution (***Figure 26, lane 2***). After phase separation with butanol (Asurmendi et al., 2007) the recombinant coat protein aggregated with chlorophyll in the interphase (***Figure 26, lane 4***) and was not detectable in the aequous phase (***Figure 26, lane 3***). T2SB:SP1-1cc was completely soluble in DMSO (***Figure, lane 6***) but after dilution with water and centrifugation to remove chlorophyll no recombinant coat protein remained in the supernatent (***Figure 26, lane 5***). Calculated on the integrated density of the CP bands on the protein gel, the interphase of the butanol extraction step and the DMSO solubilized extract were composed to 36% and 39%, respectively, of recombinant CP. As none of the above described extraction attempts was compatible with subsequent purification steps a different purification strategy, modified according to Parish and Zaitlin (1966), was chosen making use of the insolubility of the viral aggregates.

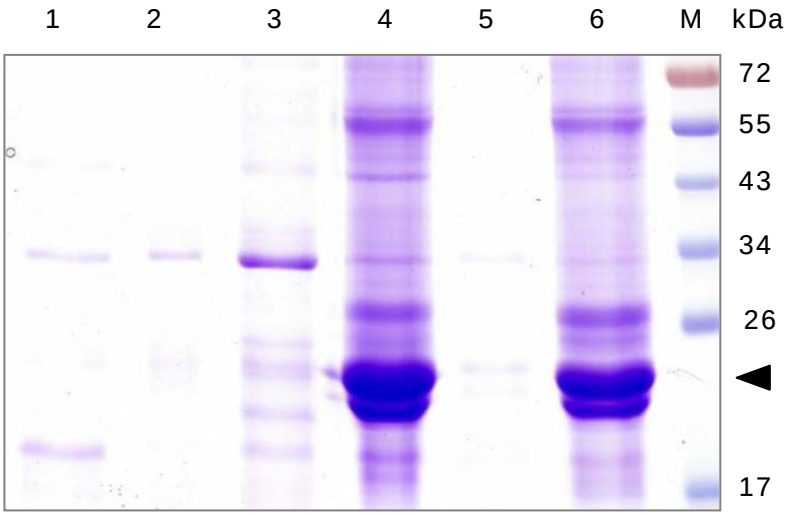

Figure 26 *Solvent screen for selective solubilization of recombinant TMV coat protein carrying peptide SP1-1 from low speed centrifugation pellet (see Figure 25, lane 7). During phosphoric acid/sulfuric acid treatment recombinant coat protein with fused SP1-1 precipitated and no protein remained in the analyzed supernatent (lane 1). Methanol extraction failed to solubilize the coat protein-peptide fusion (lane 2). After phase separation with butanol the recombinant coat protein aggregated with chlorophyll in the interphase (lane 4) and was not detectable in the aequous phase (lane 3). T2SB:SP1-1cc solubilization by DMSO (lane 6) and supernatent after dilution with water and centrifugation to remove chlorophyll no recombinant coat protein (lane 5). Relative molecular marker standards are shown on the right. M, marker; kDa, kilodalton.*

Hence, the homogenized plant material was first washed twice in buffer containing high detergent concentration. This step enabled removal of most of the chlorophyll and further soluble compounds that could interfere with the extraction process. The remaining insoluble fraction containing the protein of interest was washed twice in buffer lacking detergent and finally the viral x-bodies were solubilized and splitted with 70% (v/v) acetic acid (***Figure 27, lane 1***).

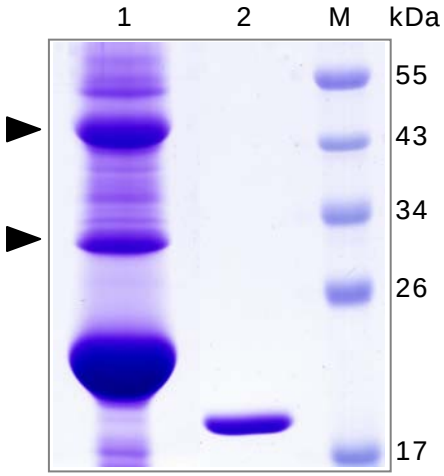

Figure 27 *Acetic acid extraction of T2SB:SP1-1cc. The acid extract was dialysed against water oN and proteins pI precipitated by adjusting the pH to 5.5 with NaOH. Clearly visible precipitate was harvested by centrifugation, resuspended in 1/5 volume of buffer corresponding to the volume of the acetic acid extract and separated on 15%-SDS-PAGE. 1, T2SB:SP1-1cc; 2, wtTMV CP as size control. Relative molecular marker standards are shown on the right. M, marker; kDa, kilodalton.*

Latter procedure not only dissolved the viral coat protein but served, in addition, as virus elimination step, as the TMV RNA precipitated and was simply removed with remaining cell fragments by centrifugation. Bands at ~30 and ~45 kDa (indicated by arrows)

probably represent poly-ubiquitinated coat proteins (Dunigan et al., 1988; Hamacher et al., 2003). Calculated on the integrated density of the CP bands, the acetic acid extract was composed to 55% of recombinant CP, including the ubiquitinated CP forms. Since salt formation during pH adjustment for isoelectric point precipitation would inhibit following cleaving reaction, proteins were recovered by complete lyophilization of the acetic acid extract. Dried proteins were dissolved in reduction buffer to reduce potential oxidized methionine residues that would not be available for CNBr cleavage and incubated oN.

4.16 RELEASE OF FUSED SP1-1 FROM COAT PROTEIN BY CNBR CLEAVAGE

After complete removal of reduction buffer from the protein extracts by speedvac drying SP1-1 was cleaved from its fusion partner by cyanogen bromide in 70% formic acid. The high molar excess of CNBr over methionine residues ensured maximum cleavage even though some of the fusion protein remained uncut. Extension of reaction duration from 24 h to 48 h did not affect cleavage efficiency (***Figure 28, lanes 3 and 4***). The clearly visible mass shift after CNBr digestion indicates the release of SP1-1 from its fusion partner. Calculated on the integrated density of the CP band, about 70% of T2SB:SP1-1cc CP were efficiently cleaved as indicated by arrows (***Figure 28***) and only 30% were not affected during the cleavage reaction also after prolonged incubation. To remove formic acid and CNBr the sample was completely lyophilized, washed by dissolving in ddH_2O and again dried down.

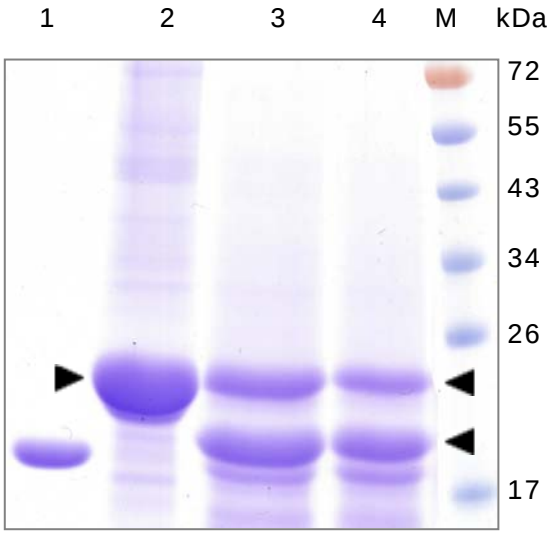

Figure 28 *Determination of CNBr cleavage efficiency depending on reaction duration. 1, wildtyp TMV as size marker; 2, T2SB:SP1-1cc extract after reduction; 3, 24 h CNBr digestion of T2SB:SP1-1cc; 4, 48 h CNBr digestion of T2SB:SP1-1cc. Relative molecular marker standards are shown on the right. M, marker; kDa, kilo dalton.*

4.17 LIQUID CHROMATOGRAPHIC PURIFICATION OF PEPTIDE SP1-1

To isolate the released peptide from the remaining proteins and compounds in the cleaving set-up a two-step chromatographic approach was established, beginning with size exclusion chromatography (SEC). For this purpose the lyophilized mixture was

dissolved in SEC buffer and first separated according to the molecule size. To define the retention time of SP1-1 under given conditions a chromatographic run with a synthetic version of the peptide was performed before purification of the recombinant peptide.

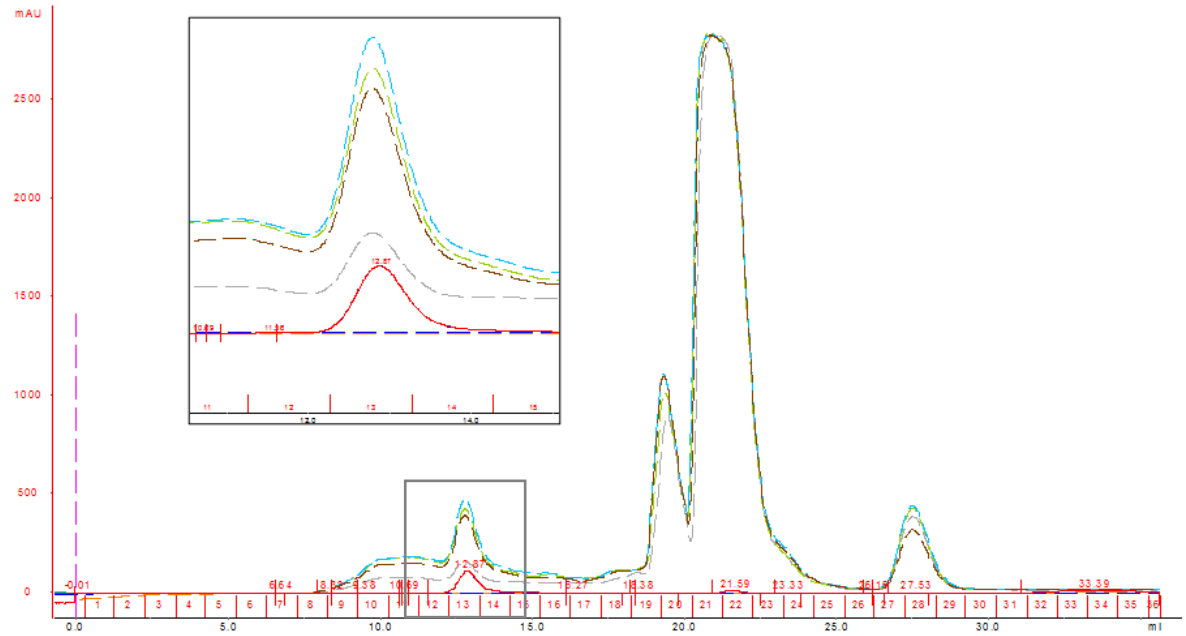

Figure 29 *Typical chromatogram of the SEC. Synthetic peptide SP1-1 or dried CNBr cleaving mixture was dissolved in 250 µl of 5% ACN, 150 mM NaCl in 0.01 M HCl and separated on a Superdex Peptide 10/300 GL column (GE Healthcare, Munich) by applying a flow rate of 1.2 ml/min. Red curve, 50 µg of synthetic SP1-1 as standard; all other curves, repeated runs of tobacco extracted and CNBr digested proteins. Elution was monitored at 215 nm and fractions of defined volumes were collected.*

Figure 29 shows an overlay of different chromatograms and it is obvious that a compound existent in the CNBr digested tobacco proteins displays exactly the same chromatographic behaviour as synthetic SP1-1. Fractions covering this peak were collected and used for further purification via reversed phase chromatography (RPC). Pooled fractions of the SEC expected to contain the peptide of interest were subjected to reversed phase chromatography as polishing step. Again, a run with synthetic SP1-1 as standard was performed to find out the retention time and the concentration of organic solvent necessary to elute SP1-1 from the C8-substituted RP-column. Also ***Figure 30*** shows an overlay of different chromatograms including SP1-1 as standard. The predicted peptide from the SEC fractions elutes slightly earlier from the RP-column in contrast to the standard peptide, probably due to the missing C-terminal amidation that is present in the synthetic peptide. Because the salt was removed during RPC and remaining buffer components are residue-free volatile the selected RPC fractions were completely speedvac dried to yield pure peptide that could be redissolved in buffer of choice. Based on SP1-1 peak area after RPC-baseline correction the estimated purity was above 90% and hence even higher as for the synthetic version of SP1-1. Calculated on the starting plant material the complete purification process offered the isolation of highly pure peptide SP1-1 in the range of 0.2 to 0.5 mg per 20 g of fresh leaf biomass. Depending on the infection status of the plants the final peptide yield differed slightly, but the

purification process was absolutely reproducible as the chromatogram overlays document (***Figure 29 and 30***).

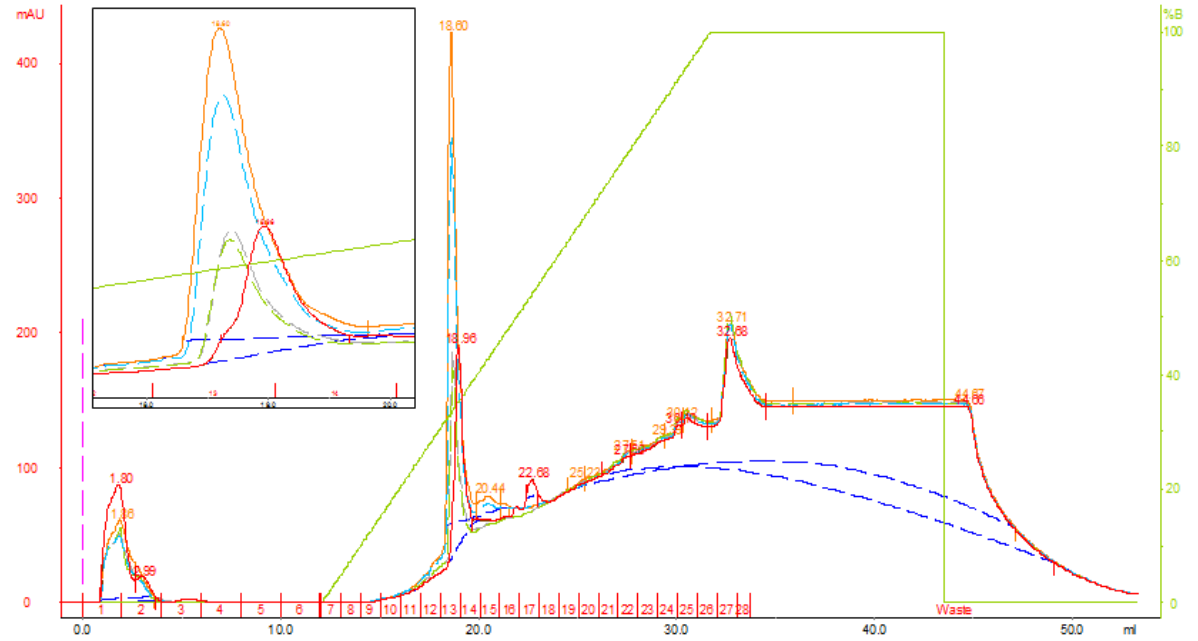

Figure 30 *Typical result of the RPC. Pooled SEC fractions containing either synthetic SP1-1 or predicted recombinant peptide were loaded directly on a self-packed 1 ml Lichrosorb RP-8 column (Merck) and separated in a 5% - 80% ACN gradient in 0.01 M HCl by applying a flow rate of 1 ml/min. Red curve, 50 µg of synthetic SP1-1 after SEC as standard; all other curves, repeated runs of SEC fractions from tobacco extracts. Elution was monitored at 215 nm and fractions of defined volumes were collected.*

The established purification protocol for in tobacco produced SP1-1 is summerized in the following flowchart (***Figure 31***).

Starting material: 20 g fresh leaf biomass of *Nicotiana benthamiana* were homogenized in liquid nitrogen using a pestle and mortar

⇩

2 washing steps with 5% (w/v) Triton X-100 containing buffer
→ *CP-SP1-1 fusion protein remains in the insoluble fraction*

⇩

2 washing steps with detergent-free buffer
→ *CP-SP1-1 fusion protein remains in the insoluble fraction*

⇩

Acetic acid extraction of insoluble fraction to split viral x-bodies and to solubilize CP-SP1-1 fusion protein
→ *7.6 mg ^{B} total protein content in the supernatant, 55% ^{ID} CP-SP1-1 (4.2 mg)*

⇩

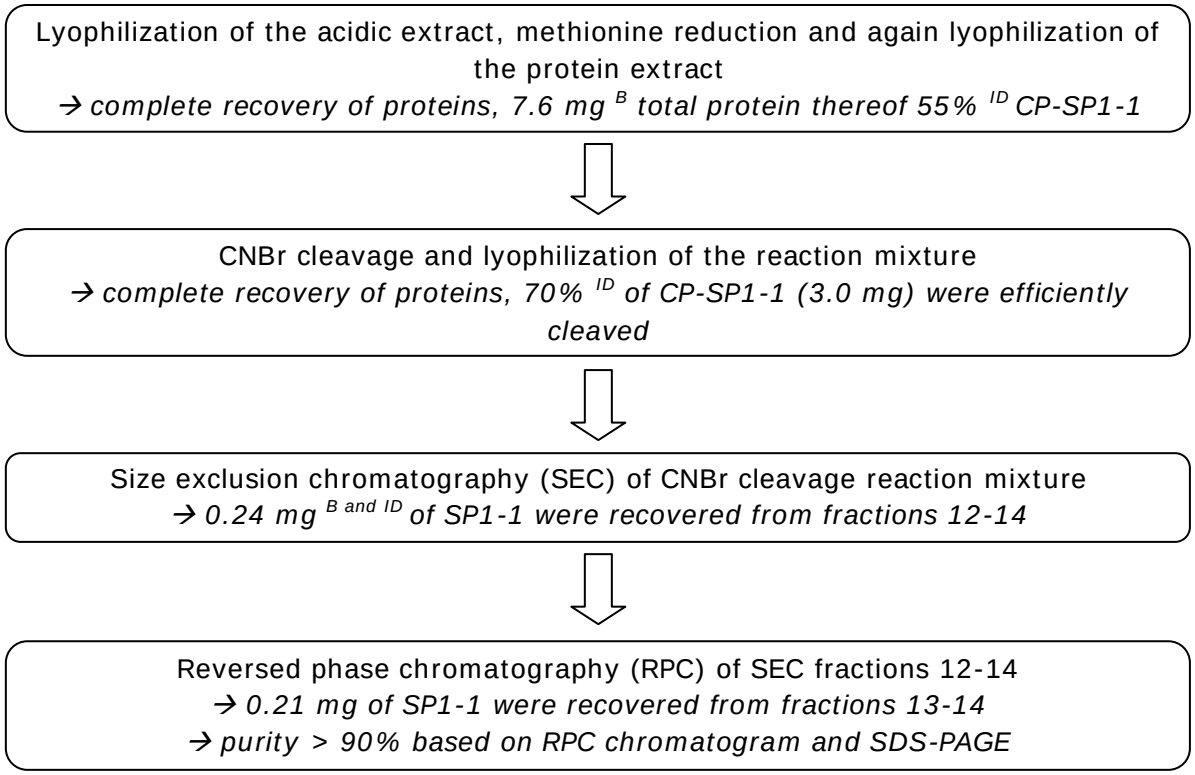

Figure 31 *Flowchart presenting the elaborated purification process for in planta produced SP1-1.* [B] *Protein content was determined using the Bradford protein assay (Bradford, 1976);* [ID] *Proportion of the protein of interest was calculated on the integrated density of protein bands on SDS-polyacrylamide gels.*

4.18 PURITY ANALYSIS AND SEQUENCE CONFIRMATION OF IN PLANTA PRODUCED SP1-1

The dried RPC fractions were dissolved in ddH_2O and first analyzed on Tricine-SDS-PAGE to detect protein impurities in the collected fractions. Even when high amounts of the peptide preparation were loaded onto the gel no contaminating bands could by detected after Coomassie G-250 staining.

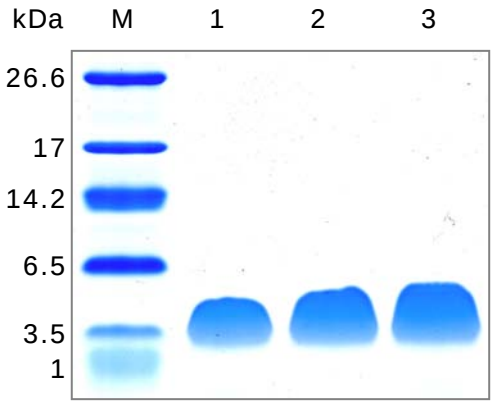

Figure 32 *15%-Tricine-SDS-PAGE to analyze purity of FPLC purified SP1-1 from tobacco. Corresponding RPC fraction was dissolved in* ddH_2O *and protein content determined by the Bradford assay (Bradford, 1976). Lane 1, 1 µg SP1-1; lane 2, 2.5 µg; lane 3, 5 µg; Relative molecular marker standards are shown on the right. M, ultra low molecular weight marker; kDa, kilodalton.*

Surprisingly, the 1.56 kDa peptide runs slightly higher in a range of about 3 kDa, probably in consequence of the high hydrophobicity and the high amount of positively charged amino acids (***Figure 32***). To further ensure that right fractions of the chromatographic separation were collected, Western Blot analysis were performed with RPC eluates and SP1-1 specific mABs (***Appendix, Figure 41***). In order to verify the proper amino acid sequence of the purified peptide and to identify potential plant-mediated posttranslational peptide modifications mass spectrometric analysis using MALDI-TOF-TOF were performed (***Figure 33***). The synthetic version of SP1-1 served as standard. To ensure that harsh conditions during purification do not affect peptide properties, the purification procedure was also performed with the synthetic peptide.

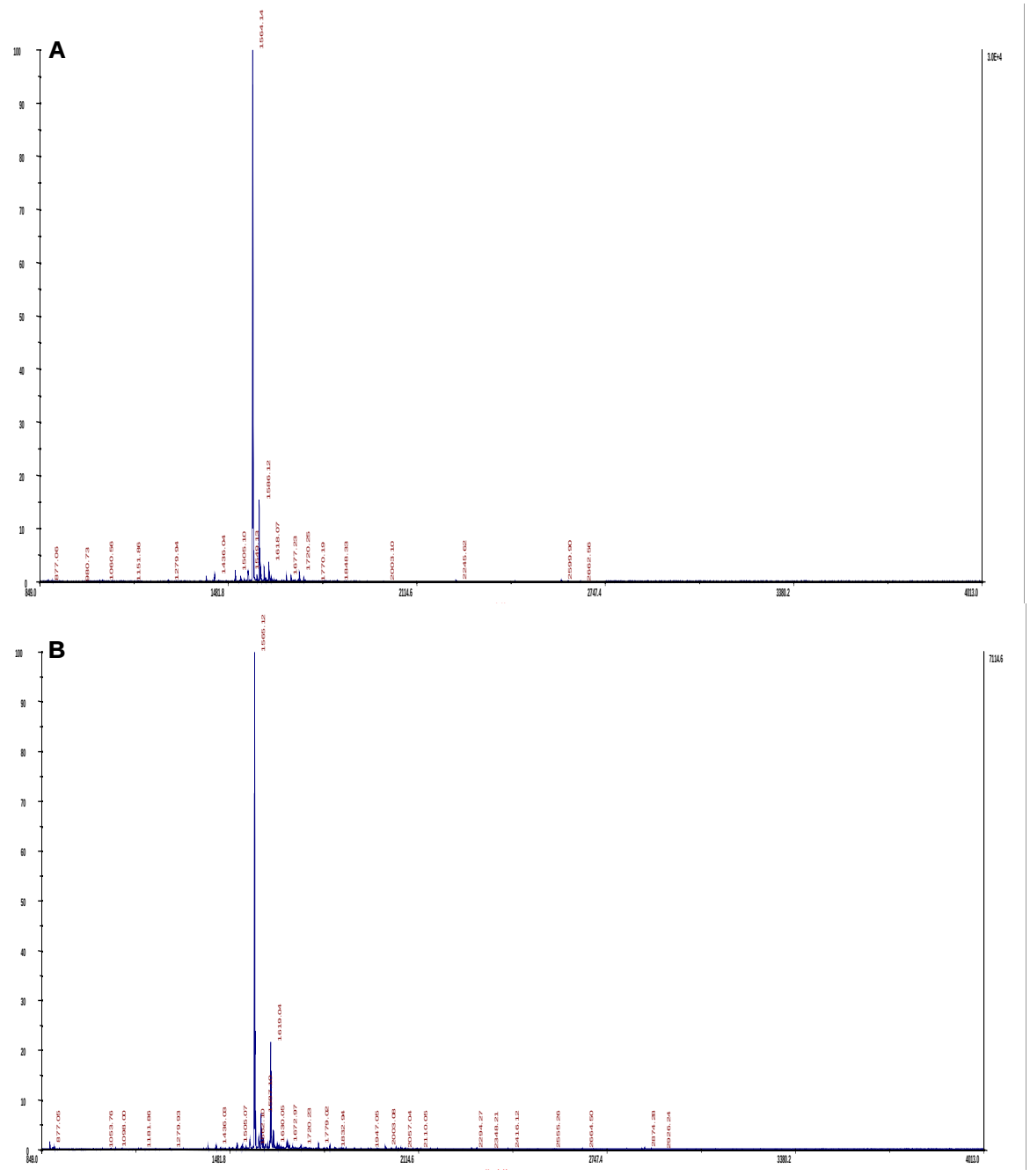

Figure 33 *Maldi-TOF-TOF analysis of (A) synthetic SP1-1 and (B) tobacco purified SP1-1 after chromatographic procedure as described in chapter 4.17. Y-axis showing the signal intensity [%], x-axis the mass [m/z].*

Figure 33 clearly indicates highest purity of tobacco SP1-1 similar to the synthetic version. In addition, MS-analysis confirmed correctness of the amino acid sequence and showed the absence of modifications. The measured m/z value for the synthetic SP1-1 was 1,564 Da and, taking into consideration the C-terminal amidation, is in accordance with the calculated m/z value (***Figure 33 A***). In contrast, the tobacco purified peptide lacks the C-terminal amidation. Hence, the calculated m/z value is 1,565 Da, which is confirmed by the MS-analysis (***Figure 33 B***).

4.19 NMR STRUCTURAL ANALYSIS OF TOBACCO SP1-1

The three-dimensional structure of the synthetic peptide and *in planta* produced SP1-1 was analysed by NMR spectroscopy (***Figure 34 and Appendix, Figure 42***). Unfortunately, beside a varying degree of purity further conclusions are difficult to draw from these measurements. However, it can be concluded that in aqueous solutions lacking micelles or lipid mimicking compounds these peptides are randomly coiled. More detailed structural analysis would be only possible with isotope-labeled peptides. But in consequence of repeating motifs present in the peptides, also labeled peptides will generate a lot of overlap in carbon and proton.

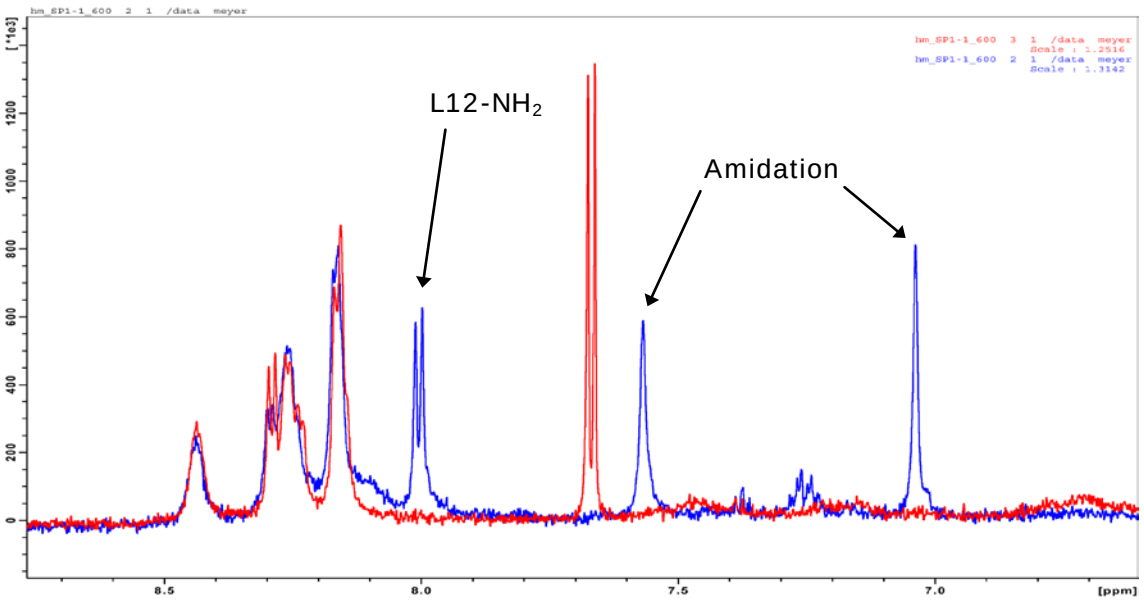

Figure 34 *NMR structural analysis of tobacco SP1-1 and synthetic SP1-1. Red curves represent tobacco purified SP1-1, blue curves the synthetic SP1-1. One-dimensional NMR spectroscopy, sector 6.5 to 9 from total spectrum of 1H overlay of both peptides. Measurements were carried out with 0.5 mg peptide dissolved in phosphate buffer on an Avance 900 instrument equipped with a TXI cryoprobehead.*

4.20 ANTIBACTERIAL ACTIVITY OF TOBACCO PURIFIED SP1-1

To analyze antibacterial activity of the purified peptide, microdilution assays with plant pathogenic bacteria were performed. The synthetic version of SP1-1 served as control.

An initial activity test against *Pseudomonas syringae pv. syringae* (gram-) and *Xanthomonas vesicatoria* (gram-) shows a similar activity range of synthetic SP1-1 and tobacco purified SP1-1. Both peptides inhibit the growth of *P.s. pv. syringae* to more than 90% (MIC_{90}) at 5 µg/ml. In contrast, slight differences in the toxicity against *X. vesicatoria* were observed. At a concentration of 5 µg/ml the tobacco SP1-1 inhibited bacterial growth only by 46% whereas in the assay with synthetic SP1-1 only 10% remaining bacterial growth was detected. At 10 µg/ml also tobacco SP1-1 reached the MIC_{90} for *Xanthomonas vesicatoria* (***Table 6***).

Table 6 *Antimicrobial activity of purified tobacco SP1-1. Shown is the bacterial growth [%] relative to the buffer treated control at indicated peptide concentrations. Peptides were dissolved in sterile ddH2O and activity was determined by measuring the OD550nm after 16 h of incubation at 28 °C with shaking. Assay was performed in 96-well plates with 3 replicates per peptide concentration and standard LB medium was used for growth of bacteria and assay performance.*

Organism	Peptide source	µg/ml			
		1	5	10	20
		bacterial growth [% of buffer control]			
Pseudomonas syringae pv. syringae	synthetic	81	< 10	- - -	- - -
	tobacco	88	< 10	- - -	- - -
Xanthomonas vesicatoria	synthetic	100	10	< 10	- - -
	tobacco	100	54	< 10	- - -

To support above shown results the antimicrobial activity was monitored on further pathogens. Because the measured MIC_{90} was far above previous test results the purified peptides were dissolved in a buffer composed of 0.01% HAc, 0.2% BSA that was used in previous studies to ensure equal assay conditions. The data presented in ***Table 7*** confirm first results for *P.s. pv. syringae* and *X. vesicatoria*. Growth of both bacteria was inhibited by both peptides to more than 90% at 5 and 10 µg/ml, respectively. Also for *Pseudomonas syringae pv. tomato* a distinct growth inhibitory effect was detected with the tobacco purified peptide and the synthetic version. *P. s. pv. tomato* treated with 1-5 µg/ml of synthetic SP1-1 showed a constantly reduced growth to only ~ 30% referred to the mock control. At 10 µg/ml a certain threshold was passed and more than 90% growth inhibition was detected. A similar behaviour was monitored with tobacco SP1-1 where growth was constantly inhibited to more than 50%, but 10 µg/ml were not sufficient to exceed the threshold concentration. To reduce growth of *Pectobacterium carotovorum ssp. carotovorum* to more than 80% 10 µg/ml synthetic SP1-1 were necessary and at 5 µg/ml still 77% growth was measured. The tobacco SP1-1 also reduced the growth by 20% at 5 µg/ml but an increase to 10 µg/ml failed to further reduce bacterial growth. The MIC_{90} of synthetic SP1-1 for *Pseudomonas corrugata* was measured at 5 µg/ml whereas the MIC_{90} of tobacco SP1-1 was at 10 µg/ml. Probably, the photometric determination of peptide concentration was not as accurate as expected leading to slightly different amounts of peptide in the assay and hence to different activity ranges.

Table 7 *Antimicrobial activity of purified tobacco SP1-1 depending on peptide solvent. Shown is the bacterial growth [%] relative to the buffer treated control at indicated peptide concentrations. Peptides were dissolved in sterile 0.01% HAc, 0.2% BSA and activity was determined by measuring the OD550nm after 16 h of incubation at 28 °C with shaking. Assay was performed in 96-well plates with 3 replicates per peptide concentration and standard LB medium was used for growth of bacteria and assay performance.*

Organism	Peptide source	µg/ml			
		1	2.5	5	10
		bacterial growth [% of buffer control]			
Pseudomonas syringae pv. syringae	synthetic	97	48	< 10	- - -
	tobacco	86	66	< 10	- - -
Xanthomonas vesicatoria	synthetic	100	20	< 10	< 10
	tobacco	100	50	20	< 10
Pseudomonas syringae pv. tomato	synthetic	36	33	30	< 10
	tobacco	51	47	45	42
Pectobacterium carotovorum ssp. carotovorum	synthetic	100	85	77	17
	tobacco	100	100	80	80
Pseudomonas corrugata	synthetic	100	75	< 10	< 10
	tobacco	100	100	65	< 10

Beside the high hydrophobicity one important design feature of the created peptides is the strong positive charge. Since the charge is directly connected to the pH, activity of the peptides was measured between differing pH values by buffering the medium used for cultivation of bacteria and the assay procedure. *P.s. pv. syringae* and *P.c. ssp. carotovorum* were selected, having a very sensitive and more resistant bacteria against SP1-1, respectively. However, *P.s. pv. syringae* prefers neutral pH for optimal growth. Within acidic conditions restricted growth of only 2% of the initial proliferation was detected, measured as cell density, and also in basic medium the cell number was below 20% when compared to growth at neutral pH. Based on this circumstances the acidic assay was excluded from the analysis whereas the alkaline one was kept, although the bacteria were inhibited in growth. As expected, buffering the medium to neutral pH had no effect on peptide activity and both peptides inhibited the growth of *P.s. pv. syringae* to more than 90% at 5 µg/ml. However, under basic conditions higher activity was detected with both peptides, but probably just reflecting the already restricted growth of the bacteria. In contrast to neutral conditions, synthetic SP1-1 inhibited bacterial growth to more than 90% already at 2.5 µg/ml and the tobacco SP1-1 inhibited growth to 75% and not only to 50% as in the neutral assay (***Table 8***). Interestingly, different results are obtained with *P.c. ssp. carotovorum*. In contrast to *P.s. pv. syringae* these bacteria show higher tolerance during pH shift and grow even slightly better under acidic conditions (***Table 9***). Latter point is likely the reason for the low antibacterial activity of the peptides in the low pH assay. At 10 µg/ml still 60% bacterial growth was measured when synthetic SP1-1 was added and still 85% when tobacco SP1-1 was used in the assay.

Compared to previous experiments with *P.s. pv. syringae*, buffering the medium to neutral pH increased the activity of the peptides – at least for the synthetic version that had the MIC_{90} at 5 µg/ml. At this concentration still 93% growth was found when the tobacco SP1-1 was used.

Table 8 *Antimicrobial activity of purified tobacco SP1-1 against Pseudomonas syringae pv. syringae depending on assay pH. Shown is the bacterial growth [%] relative to the buffer treated control at indicated peptide concentrations. Peptides were dissolved in sterile ddH2O and activity was determined by measuring the OD550nm after 16 h of incubation at 28 °C with shaking. Assay was performed in 96-well plates with 3 replicates per peptide concentration and buffered ½ LB medium with differing pH was used for growth of bacteria and assay performance.*

Medium composition	Bacterial growth [%] pH 7.2	Peptide source	µg/ml 1	2.5	5	10
			bacterial growth [% of buffer control]			
½ LB 25 mM MES pH 5.7	2.1	synthetic	n.d.	n.d.	n.d.	n.d.
		tobacco	n.d.	n.d.	n.d.	n.d.
½ LB 25 mM MOPS pH 7.2	100	synthetic	85	37	< 10	- - -
		tobacco	83	50	< 10	- - -
½ LB 25 mM Tricine pH 8.6	18.9	synthetic	27	< 10	- - -	- - -
		tobacco	51	25	< 10	- - -

Table 9 *Antimicrobial activity of purified tobacco SP1-1 against Pectobacterium carotovorum ssp. carotovorum depending on assay pH. Conditions as described above.*

Medium composition	Bacterial growth [%] pH 7.2	Peptide source	µg/ml 1	2.5	5	10
			bacterial growth [% of buffer control]			
½ LB 25 mM MES pH 5.7	110	synthetic	93	89	75	60
		tobacco	100	100	93	85
½ LB 25 mM MOPS pH 7.2	100	synthetic	82	67	< 10	- - -
		tobacco	100	100	93	80
½ LB 25 mM Tricine pH 8.6	19.6	synthetic	61	24	< 10	- - -
		tobacco	100	90	50	10

At high pH the cell proliferation was markedly reduced whereas a general growth inhibition at this pH has to be kept in mind. For the tobacco peptide the MIC_{90} was detected at 10 µg/ml and already 5 µg/ml were sufficient to halve bacterial growth rate. Better performance was measured with synthetic SP1-1 that already inhibited proliferation by nearly 40% at 1 µg/ml and inhibiting activity increased to more than 75% and more than 90% at 2.5 and 5 µg/ml, respectively.

Table 10 *Antimicrobial activity of purified tobacco SP1-1 against Pseudomonas syringae pv. syringae depending on the availability of mono- and divalent cations. Shown is the bacterial growth [%] relative to the buffer treated control at indicated peptide concentrations. Peptides were dissolved in sterile ddH2O and activity was determined by measuring the OD550nm after 16 h of incubation at 28 °C with shaking. Assay was performed in 96-well plates with 3 replicates per peptide concentration and MOPS-buffered ½ LB medium supplemented with 1.5 mM MgCl2 and 1.5 mM CaCl2 and differing NaCl concentrations was used for growth of bacteria and assay performance.*

Medium composition	Bacterial growth [%] NaCl 80 mM	Peptide source	µg/ml			
			1	2.5	5	10
			bacterial growth [% of buffer control]			
½ LB 25 mM MOPS pH 7.2 40 mM NaCl	110	synthetic	100	100	94	67
		tobacco	100	100	100	88
½ LB 25 mM MOPS pH 7.2 80 mM NaCl	100	synthetic	100	100	76	68
		tobacco	100	100	100	90
½ LB 25 mM MOPS pH 7.2 160 mM NaCl	95	synthetic	100	100	72	57
		tobacco	100	100	100	100
½ LB 25 mM MOPS pH 7.2 240 mM NaCl	70	synthetic	100	100	100	78
		tobacco	80	80	80	80

One important parameter for microdilution assay-based activity tests is the strong influence of mono- and divalent cations on peptide performance (Hancock and Sahl, 2006). To accommodate this, another assay was performed on *Pseudomonas syringae pv. syringae* with fixed concentrations of $MgCl_2$ and $CaCl_2$ and varying amounts of NaCl included in the buffer that all are antagonizers to the cationic peptide to diverse extents. The concentration of Mg^{2+} and Ca^{2+} in total was 3 mM and thereby near physiological conditions and Na^{+} ranged from 40 mM to 240 mM. The maximum growth inhibition by synthetic SP1-1 was 43% at 10 µg/ml when 160 mM NaCl were present in the assay. For the tobacco SP1-1 growth inhibition in all assays was only between 10% and 20% at 10 µg/ml (***Table 10***).

5. DISCUSSION

For the application in plant protection and human medicine novel peptides were developed at the Institute of Biochemical Plant Pathology that are much more active against plant and human pathogens than natural occurring antimicrobial peptides. But the synthetic production of these peptides is very expensive. Hence, the economic production in different *Nicotiana* species serving as inexpensive green factories was evaluated during this study. A *Tobacco mosaic virus* (TMV) derived expression system following the 'full virus vector strategy' with the viral coat protein as fusion partner was the method of choice for *in planta* production of the short peptides. After successful purification, the *in vitro* antimicrobial activity was proved under varying assay conditions and emerged to be similar to the activity of the synthetic analogue.

5.1 EVALUATION OF THE MOST SUITABLE EXPRESSION SYSTEM FOR *IN PLANTA* PEPTIDE PRODUCTION

5.1.1 TRANSGENIC *NICOTIANA TABACUM* EXPRESSING PEPTIDE GENES

The generation of transgenic plants expressing foreign genes of interest is the standard method for recombinant protein production in plants and meanwhile well established for manufacturing of biopharmaceuticals (Kusnadi et al., 1997; Giddings et al., 2000; Ma et al., 2003; Fischer et al., 2004; Goldstein and Thomas, 2004; Horn et al., 2004). To analyze, if this method can be used for the production of the *de novo* designed peptides, plants of *Nicotiana tabacum* cv. Xanthi NN were stable transformed to express the genes for peptides SP1-1, SP10-2 and SP13 (***Appendix, Table 11***). For plant codon usage optimized sequences of the 12 or 20 amino acid long peptides were under the control of a duplicated CaMV 35S promoter and translation should be enhanced by including the TMV Ω -sequence upstream the peptide genes. Many transformants could be easily generated and the existence of the particular transgene was proofed by PCR and Southern blot analysis. By qRT-PCR expression on RNA level was demonstrated (***Appendix, Figures 35-37***). Transgenic plants showed normal growth and development *extra vitrum* and no toxicity of putative transpeptides to plant cells was detected. However, it failed to show any of the three peptides on protein level in transgenic plants, neither by immunodetection with specific antibodies against the peptides nor by MALDI-TOF analysis (***Appendix, Figure 38***). There are already numerous studies reporting the stable expression of antimicrobial peptide genes in different plant species. These approaches were mainly aimed to enhance the resistance of plants against certain pathogens and to lesser extent for production purposes or exogenous use after peptide purification. For instance, Bhargava and colleagues (Bhargava et al., 2007) produced transgenic *Nicotiana tabacum* cv. Xanthi nn expressing indolicidin variant genes and could thereby enhance resistance against several pathogens including *Erwinia amylovora* and TMV. The expression vectors used were similar to the vectors used in this work. Namely, the genes encoding for the 13 amino acid peptides were set under the control of a duplicated CaMV 35S promoter followed by an *Alfalfa mosaic virus* derived translational enhancer and termination of transcription was mediated by the Nos terminator. Genomic

integration of indolicidin variant genes and their expression was proofed on DNA and RNA level, respectively, but detection of the produced peptide failed. They speculate that peptide amount in the plant cell is too low and that immunodetection is hindered by the small size and high positive charge of the peptides that make it difficult to raise efficient antibodies. Comparable findings were obtained when *MSI-99*, a synthetic substitution analogue of magainin, was expressed in transgenic tobacco and banana (Chakrabarti et al., 2003). *MSI-99* expression was driven by an *Arabidopsis thaliana* ubiquitin promoter and the peptide was directed to the extracellular space by connection to a secretory signal peptide. Although antimicrobial activity was measured in detached leaf assays and in assays with plant extracts and the transgene expression was veryfied by RT-PCR the produced peptide could not be shown. There are more reports on plants expressing genes for cationic antimicrobial peptides either with or without signal peptides where expression can be clearly detected on RNA level. However, although transgenic plants are more resistant against a whole set of different pathogens the detection of the produced peptide was not possible. Probably, low peptide accumulation levels in the cell or aggregation due to their amphipathic character or low attraction for antibodies used during immunodetection complicate peptide detection (Cary et al., 2000; Li et al., 2001; Rajasekaran et al., 2005; Yevtushenko et al., 2005; Xing et al., 2006). Anyway, there are some reports where the transpeptide in stable transgenic plants could be detected. LJAMP1, a heat stable 7.8 kDa peptide first identified in seeds of motherwort, was found in transgenic tobacco by comparing the HPLC chromatograms of plant extracts with chromatograms of a synthetic peptide (Yang et al., 2007). Also Ponti et al. (2003) were able to detect a modified version of esculentin-1 in transgenic plants by immunoblotting with esculentin-specific antibodies. When the cecropin-melittin chimera *MsrA1* was transformed in potato an estimated peptide concentration of 3 µg/g of raw tissue tuber was sufficient to protect them from bacterial attack (Osusky et al., 2004). This peptide concentration might be high enough to increase endogenous resistance of transgenic plants against selected pathogens but for production purposes and subsequent activity studies, this is far below the economic range. It has to be kept in mind that during purification steps a certain amount of peptide will be lost and that a lot of material has to be processed if the peptide accumulation in plants is too low.

5.1.2 MAGNIFECTION – A PROMISING TRANSFECTION METHOD FOR TRANSIENT PROTEIN EXPRESSION IN PLANTS

In 2004 the group of Yuri Gleba presented an efficient transient expression system for recombinant protein production in plants that follows the deconstructed virus vector strategy and enables highest yields of produced proteins (Gleba et al., 2004; Marillonnet et al., 2004). This system is based on the crucifer-infecting TMV and turnip vein-clearing virus (TVCV), both belonging to the well studied tobamoviruses. The viral genomes were separated on two different binary vector plasmids and optimized by introducing numerous plant introns, single point mutations and removal of cryptic splicing sites (Gleba et al., 2005; Marillonnet et al., 2005). When these vectors were introduced into plants by agroinfiltration together with an additional vector carrying a bacteriophage

integrase, a functional viral amplicon was formed by recombination in the plant cell. They estimated the obtained protein yield in the case of nontoxic proteins in a range of 0.5-1 g/kg of fresh weight biomass and even toxic proteins should be produced in amounts that allow application in research assays (Marillonnet et al., 2004). This modular system was used for the *in planta* production of the *de novo* designed peptides SP1, SP13 and SP15. First experiments were designed to produce the peptides either untagged to evaluate, if this system can be used in general for production purposes or as His_6-tag fusion peptide to allow easier purification. An expression vector for cytosolic peptide accumulation was chosen because an additional secretory signal peptide preceding the desired antimicrobial peptides should be avoided. Such a signal peptide would have altered the physico-chemical characteristics of the short peptide sequences and might reduce their antimicrobial activity. Anyway, both attempts failed and no peptide was detected in transfected plant leaves neither with specific antibodies against the peptides nor with antibodies against the His_6-tag. There might be several reasons why this expression approaches were not succcessful. The expression vectors are based on RNA viruses and replicate in the cytosol. But during the infection process they have to undergo an nuclear stage as they are transferred into the plant cells via *Agrobacteria* and the sequences are exposed to the nuclear pre-mRNA processing machinery (Marillonnet et al., 2005). It just can be speculated that the unnatural sequences encoding the designed peptides harbor cryptic splicing sites that are not proper processed during RNA maturation and hence leading to incorrect transcripts or that unfavorable formation of secondary pre-mRNA structure perturbs RNA processing (Simpson and Filipowicz, 1996). Also premature cleavage of the primary transcripts by the ribozyme before transport from the nucleus or inefficient cleavage by the ribozyme in the cytosol might account for the low infectivity of viral constructs and the failure to produce the peptides (Fujiki et al., 2008). A well known defense mechanism against viral infections which is conserved among plants, animals, fungi and insects is RNA silencing (Waterhouse et al., 2001; Soosaar et al., 2005). The trigger for RNA silencing of viruses is double stranded RNA (dsRNA) and sequences homologous to the dsRNA are effectively degraded. Like most plant viruses (Nienhaus, 1985) TMV is a RNA virus and in the early infection stage many long dsRNA species are generated during genome replication by the RNA-dependent RNA polymerase. But also during the ongoing infection process when late genes are transcribed from subgenomic promoters parts of the viral RNA are double stranded and hence might induce the silencing of the peptide encoding mRNA before accumulation of the peptides can be detected (Buck, 1999; Shaw, 1999). Transgene silencing is often observed in stable transgenic plants as well as during transient expression upon infection with viral vectors (Voinnet et al., 2003). In principle, the level of transgene expression peaks 60-72 hpi and declines rapidly afterwards with the onset of post-transcriptional gene silencing. To prolong transgene expression and to enhance infectivity of viral expression vectors and consequently to increase yield of recombinant proteins, viral silencing suppressors are often co-infiltrated into plant leaves. Common suppressor proteins are the HCPro suppressor from potyviruses, the p21 suppressor from beet western yellow virus or the p19 suppressor from tomato bushy stunt virus (Baulcombe, 2004). When the *p19* suppressor under the control of CaMV 35S promoter was co-infiltrated with a *GFP*

encoding binary vector plasmid into leaves of *Nicotiana benthamiana* the expression of several proteins was 50-fold or even more enhanced (Voinnet et al., 2003). In addition, Bayne et al. (2005) could show that silencing suppressor p25 from *Potato virus X* was even necessary to enable viral cell-to-cell movement. Also the TMV 126 kDa protein is expected to act besides viral RNA replication as suppressor of RNA silencing (Ding et al., 2004). The effect of silencing suppressors on magnICON® mediated production of the designed peptides was not analyzed, as on the one hand such suppressors were not available at the institute and on the other hand the developer of magnICON® reported no positive influence of p19 or HCPro on the performance of their viral constructs (Marillonnet et al., 2004). Thus, it remains unknown why this approached failed to produce the peptides. In sum, although viral expression systems are powerful tools to produce recombinant proteins in plants, this system has restrictions and does not work for every protein (Wagner et al., 2004).

Of course, next to possible reasons on transcript level also degradation on protein level may account for the difficulties during peptide production. All peptides that were tried to produce *in planta* carry the amino acids arginine, lysine, leucine or histidine, in the case of the His_6-tagged peptides, at their N-terminus. According to the N-end rule of regulated proteolysis, these residues are classified as destabilizing residues and may enhance peptide degradation (Varshavsky, 1997; Mogk et al., 2007). Also the lack of disulfide crosslinking within the peptides might increase the attack of plant proteases, that use relatively unspecific cleavage sites and can produce fragments as small as six amino acids (Doran, 2006). Furthermore, the high amount of lysine residues in the peptides makes the peptides prone to degradation via the ubiquitin 26S proteasome pathway, the most elaborate regulatory mechanism in plants (Smalle and Vierstra, 2004). Also the overall properties of the designed peptides, namely the high hydrophobicity and the strong positive charge due to the high arginine and lysine content, reflects signal peptide character (Faye et al., 2005). Probably the peptides are degraded by proteases of the secretory pathway between the endoplasmatic reticulum and the Golgi apparatus or are directed to cellular compartments with high proteolytic activity, like the vacuole or the apoplast (Doran, 2006).

To circumvent all these problems that might arise during transcription or peptide synthesis *in planta* the production as fusion protein to GFP was aspired. The successful expression of GFP in *Nicotiana benthamiana* using magnICON® was already described (Gleba et al., 2005). The peptide SP1 was fused C-terminal to GFP and strong production was monitored between 5 and 14 dpi under UV light and on protein gels (***Chapter 4.2, Figure 8***). But as already found in initial GFP expression studies, *Nicotiana benthamiana* was the only plant species, where recombinant protein yield was satisfactory and where the bacterial density in the infiltration solution could be kept in a range of OD_{600nm} 0.001 to 0.01. In contrast, all tested *Nicotiana tabacum* cultivars and *Nicotiana sylvestris* showed low GFP expression mainly only at the lower side of the leaves and required high amounts of bacteria, OD_{600nm} of 0.1 to 1.0, in the infiltration solution. High concentrations of *Agrobacteria* can induce necrosis, resembling the hypersensitive response as component of the plant defense, in Solanaceous species what is problematic and counterproductive during transient expression assays (Joh et al., 2005; Wroblewski et

al., 2005). Additionally, *Agrobacteria* can survive in the intercellular space for weeks in plants (Firoozabady and Kuehnle, 1995). Hence, contamination with bacterial compounds in the peptide preparation can not be excluded.

As for most viral expression systems, the only acceptable host for magnICON® was *Nicotiana benthamiana* (Wagner et al., 2004; Lico et al., 2008). Unfortunately, *Nicotiana benthamiana* is a poor host plant with a plump growth, constantly flowering and high susceptibility to all kind of pathogens. But especially the hypersusceptibility to viruses, strongly linked to a mutation in the RNA-dependent RNA polymerase gene *NbRdRP1m* (Goodin et al., 2008), and thus connected to a limitated capability for virus induced gene silencing makes *Nicotiana benthamiana* the model organism for viral expression systems. When Sheludko and colleagues (Sheludko et al., 2007) compared several *Nicotiana* species as hosts for the *Agrobacterium*-mediated transient expression system magnICON® they found that next to *Nicoatiana benthamiana* also *Nicotiana excelsior* has good characteristics in regard to biomass yield and recombinant protein accumulation. Several other Australian *Nicotiana* species come not into consideration, as growth or protein production ability was not satisfactory. Large Scale Biology Corporation was for long time the only company, that used a viral expression system on industrial scale for recombinant protein production in plants. Their Geneware® technology (http://www.kbpllc.com/GENEWARE/tabid/86/Default.aspx) is based on a functional TMV carrying an additional ORF where the GOI is introduced. Since *Nicotiana benthamiana* is only suitable for greenhouse production they developed a new plant species by crossing *Nicotiana benthamiana* and *Nicotiana excelsior*. This interspecific hybrid, named *Nicotiana excelsiana*, combined the high protein production capability and susceptibility to viral expression vectors of *Nicotiana benthamiana* and high biomass yield and field cultivation characteristics of *Nicotiana excelsior* and served as new expression host (Smith et al., 2009). Taken together, the narrow expression host range of magnICON®, the unavoidable remaining bacteria in the plant material and the need for a stable fusion partner to produce the *de novo* designed peptides, that directs most of the plant's metabolic energy in fusion partner production strengthened the idea, to create an own viral expression system following the full virus vector strategy.

5.2 THE TMV-BASED FULL VIRUS VECTOR EXPRESSION SYSTEM FOR *IN PLANTA* PEPTIDE PRODUCTION

In 2007 Lindbo described a TMV-based overexpression vector for high-efficiency protein production in plants that makes use of a functional virus (Lindbo, 2007a, b). A TMV cDNA clone was introduced into a binary vector plasmid and set under the control of the CaMV 35S promoter. To produce transproteins, the GOI was either inserted by replacing the TMV coat protein or as additional ORF between the TMV movement protein and the coat protein. After agroinfiltration of *Nicotiana benthamiana* leaves, strong expression was detected in the infiltrated areas for the vector lacking the coat protein and even systemic movement and thus systemic transprotein production was measured for the vector still harboring the TMV coat protein (Lindbo, 2007a). Especially the sustainment of TMV's

ability for systemic movement might be useful during drug production, as systemic tissue was not in contact with *Agrobacteria*, which could lead to contaminations of the final protein preparation. In addition, reduced infection effort is necessary, because the virus spreads systemically in the plant from a few lower infected leaves.

As already mentioned, a fusion partner appears to be essential for recombinant peptide production in plants. When using a functional *Tobacco mosaic virus* as expression vector it suggests itself to directly use a viral protein for this purpose and the TMV coat protein seems to be well suited as fusion partner. It is known that TMV tolerates up to 20 additional amino acids attached to either the N- or C-terminus of its coat protein and also an internal loop region was identified, where additional amino acids can be inserted (Turpen et al., 1995; Pogue et al., 2002; Smith et al., 2009). The adaptability of this approach to display peptides on the surface of TMV was already proofed in numerous reports, mainly with the aim to produce antigens for vaccination. Two T-cell epitopes of 10 and 12 amino acids in length providing protection against tumor challenge in mice were genetically fused to TMV and successful produced in *Nicotiana benthamiana*. When mice were vaccinated with the recombinant TMV they showed improved protection from tumor challenge, thereby confirming that TMV can effectively produce and deliver small peptides on its surface (McCormick et al., 2006). An enkephalin derived pentapeptide (ENK) was introduced directly before the TMV coat protein stop codon. When tobacco protoplast were infected with RNA of the modified virus, the coat protein-ENK fusion protein was the major protein that accumulated in the cell (Takamatsu et al., 1990). Also the presentation of a 17 amino acid L2 capsid epitope of the *Cutaneous papillomavirus* on the surface of TMV was successful, when the peptide encoding sequence was introduced between amino acids 154 and 155 of the 158 amino acids counting coat protein (Palmer et al., 2006). Based on already described reports about successful peptide display on the surface of recombinant TMV combined with the high accumulation of TMV coat protein in tobacco up to 10 mg/g of fresh weight (Culver, 2002) this approach should be optimal for the production of the designed peptides.

5.2.1 GENERATION OF AN INFECTIVE TMV CDNA CLONE

The biggest problem was to isolate an infective cDNA clone of wildtyp *Tobacco mosaic virus*. Although RT-PCR with purified genomic TMV RNA as template provided large amounts of full-length cDNA, the cloning into standard plasmids for DNA propagation in bacteria turned out to be a crucial point (***Chapter 4.5, Figure 11***). However, reported limitations during first and second strand synthesis in the wake of hampered primer annealing or disabled polymerization by strong secondary structures of the viral template RNA were not detected (Boyer and Haenni, 1994). During first cloning attempts, the PCR amplified TMV genome should be cloned into a standard pBluescript (Stratagene) cloning vector and propagated in *E. coli*. The oligonucleotides used in the PCR contained unique restriction sites and after double digestion of PCR product and vector, numerous ligation conditions were tried. All trials failed and after transformation of *E. coli* no correct plasmid was identified. It is known that problems may occur while creating full-length cDNA clones of plant viruses. One reason is the potential toxicity of particular viral

sequences for bacteria, leading to instability of cloned cDNA in *E. coli* and thus to mutations or complete loss of the sequence (Boyer and Haenni, 1994; Nagyova and Subr, 2007). To overcome those problems the cloning strategy was changed and TA-cloning into a different vector, pCR-XL-TOPO (Invitrogen), was applied. This approach was successful and plasmids containing a full-length cDNA clone were identified, even though many plasmids carried just parts of the viral sequence. Partial DNA sequencing revealed that mostly large parts of the RdRP sequence were deleted. Interestingly, although insertion into the plasmid in both directions was possible, only plasmids harboring the cDNA clone in complementary direction were identified. Furthermore, numerous clones were found with several mutations – either single point mutations or deletions of single nucleotides. Not only sequence instability in bacteria may account for this, but also variation and spontaneous mutations in the virus population might be a reason (Boyer and Haenni, 1994; Nagyova and Subr, 2007). It is well known that all RNA viruses have extremely high mutation rates, resulting in the generation of quasispecies mutant swarms even when the population has just arisen from one single clone (Holland and Domingo, 1998). The mutation frequency of TMV movement protein was estimated as 0.02-0.05, which is at a lower range. Anyway, 35% of all sequenced mutants showed two or more mutations. Furthermore, based on the detection of mutations lethal for cell-to-cell movement, a mutation rate per genome of 0.1-0.13 was reported (Malpica et al., 2002; Garcia-Arenal et al., 2003). Taken into account the rapid replication of the TMV RNA genome, probably a must as consequence of the chemical instability of the RNA (Garcia-Arenal et al., 2003), and the lack of proofreading activity of the viral RdRP it is not surprising that the viral RNA in infected plants is highly heterogeneous (Tao and Ye, 2010). This is not cumbersome for TMV itself, but during cloning large amounts of mutated RNA molecules can serve as templates during amplification what may complicate successive subcloning steps. If mutations in the obtained TMV cDNA clone are due to spontaneous viral mutations or bacteria induced mutations during cloning remains speculative, but derived amino acid sequence indicated no misfunction of translated proteins. To allow *in vitro* RNA synthesis on the correct TMV cDNA clone, a truncated bacteriophage SP6 promoter was inserted directly upstream the viral genome. This enabled the production of *in vitro* transcripts without additional nucleotides at the 5'-terminus and only thymidine at position 2 was changed to adenine (***Chapter 4.6, Table 4***). Even short 5'-extensions at the viral genome can dramatically decrease or even abolish infectivity of cDNA derived TMV transcripts (Boyer and Haenni, 1994) whereas slight mutations in the leader sequence are well tolerated (Buck, 1999). To terminate *in vitro* transcription by plasmid linearization the unique restriction site Bse YI was integrated directly downstream the 3'-terminus resulting in only one additional guanidine at the transcripts. Infectivity of viral transcripts appears relatively insensitive to short 3'-extensions of 1 to 7 nucleotides (Boyer and Haenni, 1994; Nagyova and Subr, 2007). To obtain transcripts with optimum infectivity a m^7GpppG cap structure had to be included (Buck, 1999; Okada, 1999). Rub inoculation of *Nicotiana tabacum* cultivars with infectious transcripts from pT2SB resulted eiher in the typical mosaic disease formation on susceptible hosts or HR necrotic lesions on resistant cultivars (Dawson et al., 1986; Meshi et al., 1986; Dawson, 1992; Culver et al., 1994; Dawson, 1999; Erickson et al.,

1999a; Scholthof, 2004). In contrast to infection of plants with purified viral RNA from isolated TMV virions the infectivity was much lower, relating to the number of lesions or a slight delay in mosaic development and varied from experiment to experiment (***Chapter 4.7, Figure 15***). Probably, the heterogenity of transcript population, or the presence of point mutations caused by inaccuracy of template DNA- and RNA-synthesizing enzymes might be the reason for lower infectivity (Hamilton and Baulcombe, 1989; Domingo and Holland, 1992).

To resolve such drawbacks and to avoid the need for expensive material like the m^7GpppG cap for *in vitro* RNA synthesis an agroinfection-compatible vector was created, similar as described in Lindbo (2007a, b). The complete pT2SB *in vitro* transcription cassette including the 5'-extending 17 nucleotides counting SP6 promoter and the Bse YI restriction site as 3'-extension was inserted into a standard binary vector plasmid. Thus, in pAGRO:T2SB the T-DNA region covered following components in this order: duplicated CaMV 35S promoter, TMV Ω-sequence, SP6 promoter, TMV cDNA clone, Bse YI restriction site, Nos terminator (***Chapter 4.11, Figure 20***). When this construct was agroinfiltrated into two lower leaves of *Nicotiana tabacum* cv. Xanthi nn, about 10 dpi mosaic development was observed in young leaves and systemic disease formation took place. Also in the hypersusceptible host *Nicotiana benthamiana* about 14 dpi plants showed severe stem necrosis and wilting and started to die off. These findings are interesting, as the TMV genome is, counting from the CaMV 35S promoter transcription start, elongated at the 5'-terminus by 118 nucleotides due to the additional Ω-sequence and the SP6 promoter region. As the Nos terminator follows directly the 3'-UTR of TMV this sequence is only extended by the few nucleotides of Bse YI. Turpen and colleagues (1993) created a comparable vector, but transfection of tobacco failed when they attached a 80 bp *mas* leader sequence upstream the TMV 5'-UTR. They also found that growth conditions of bacteria drastically effects transfection efficiency and that TMV sequences can reduce virulence of *Agrobacterium*. In this work a different *Agrobacterium tumefaciens* strain was used, although exhibiting the same C58 chromosomal background, containing a different helper plasmid. Maybe this accounts for the higher transfection efficiency of pAGRO:T2SB. The transfection vectors described in Marillonnet et al. (2004; 2005) make use of the *Arabisopsis thaliana Actin 2* promoter instead of a CaMV 35S promoter to drive transcription in the plant nucleus. Also these constructs cause a 5'-extension at the crucifer infecting TMV cDNA clone, but nevertheless high transfection efficiency is reported, as already discussed in chapter 5.1.2. Further interesting findings are reported by Dagless et al. (1997) who directly used a CaMV 35S promoter driven cDNA of TMV for inoculation of plants. When this fragment was manually inoculated on leaves of *Nicotiana benthamiana* leaf distortion and systemic necrosis occured as typical symptoms of TMV infection, but no disease was manifested in several tested *Nicotiana tabacum* cultivars. It is also mentionable, that agroinfiltration of pAGRO:T2SB into leaves of resistant *Nicotiana tabacum* cv. Xanthi NN did not induce HR necrotic lesions in the infiltrated areas. In contrast, in the susceptible cultivar Xanthi nn TMV accumulated to high amounts – clearly reflected by a dominant coat protein signal on protein gels. This phenomenon can be attributed to *Agrobacterium tumefaciens*' capacity to induce host defense reponses in the infiltrated leaf zones. Infiltrated regions display unusually high level of resistance against

TMV infection and formation of local lesions remains absolut absent (Pruss et al., 2008). Taken together, the created transfection vector pAGRO:T2SB enables the successful infection of *Nicotiana tabacum* cv. Xanthi nn and *Nicotiana benthamiana* with a functional TMV cDNA clone. In addition, by replacing the original high copy pUC ORI of the backbone plasmid with the low copy pBR322 ORI the stability in *E. coli* cells was dramatically increased, probably due to reduced sequence toxicity (Boyer and Haenni, 1994; Nagyova and Subr, 2007), and hence large amounts of plasmid DNA could be produced. As also regulating sequences for *in vitro* transcription are still present in the vector, inoculation with infectious transcripts is retained as alternative infection method. After proof of function of pAGRO:T2SB and pT2SB these vectors formed the basis for the development of a production method for the *de novo* designed peptides.

5.2.2 *IN PLANTA* PRODUCTION OF DESIGNED PEPTIDES FUSED TO RECOMBINANT TMV

Several possibilities are described to attach peptide sequences to the TMV CP, mostly resulting in the display of the peptides on the surface of the assembled virions. Direct fusion to the C-terminus, insertion near the N-terminal end or into a loop region between Pro^{63} and Asp^{66} was shown to be tolerated by TMV when peptide length does not exceed 22 amino acids (Turpen et al., 1995; Smolenska et al., 1998; Beachy, 1999). Due to possible alterations of their physico-chemical properties and potential activity loss, no additional amino acids neither at the N-terminus nor at the C-terminus of the designed peptides were tolerated. Therefore, C-terminal fusion to the coat protein with an additional methionine N-terminal of the peptide was aspired. The methionine allows the exact release of the designed peptides by CNBr cleavage (Yuan et al., 1986; Smith, 1994; Kaiser and Metzka, 1999; Crimmins et al., 2005). An additional advantage of CNBr cleavage in this case was the innately nonexistence of further methionine residues in the fusion partner that simplified purification procedures (Glover and Wilson, 1982; Goelet et al., 1982; Culver et al., 1993). In a first trial the 12 amino acids counting peptides SP1-1 and SP10-2 plus the preceding methionine were inserted directly between Thr^{158} and the TMV coat protein stop codon. The 20 amino acids counting peptide SP13 plus the preceding methionine was inserted after Trp^{152} and the recombinant coat protein subunits lacked the terminal peptides Thr^{153}-Thr^{158} due to early translation termination by an inserted TGA stop codon. The last amino acids are not essential for correct CP function and the possibility to shorten the fusion partner should allow the attachment of longer peptides. Using this approach foreign transmembrane domains of up to 25 amino acids were successful produced in the *Nicotiana tabacum* cvs. Xanthi nn and Samsun nn (Li et al., 2006). For production of shorter peptides like ENK or T-cell epitops (already discussed in chapter 5.2) the truncation of the CP was shown to be not essential (Takamatsu et al., 1990; McCormick et al., 2006). When *Nicotiana tabacum* cv. Xanthi NN was treated with these constructs by rub inoculation of *in vitro* transcripts, local lesions were detected not later than 3 dpi equal to wtTMV treated plants. Unfortunately, the susceptible cultivar Xanthi nn, the primary selected expression host, reacted in the same way as the resistant plants and HR-like necrotic lesions were found in the inoculated regions (***Chapter 4.9, Figure 17***). Similar results were obtained when Li and

colleagues fused foreign peptides to the TMV CP (Li et al., 2006). They analyzed for more than 30 peptides of 5 to 25 amino acids in length the applicability of TMV to produce these peptides as C-terminal CP fusions (Wu et al., 2003). Approximately half of their recombinant viruses retained the ability for propagation, systemic movement and disease induction in the *Nicotiana tabacum* cvs. Xanthi nn and Samsun nn, whereas the other half induced local lesions. Various criteria are discussed that might affect viral infectivity or viral particle formation, including amount of inserted amino acids, fusion site or the pI/charge value of foreign peptides. But a prediction from their used peptide sequences and a correlation of definite criteria to the host response was not possible. Certainly, the charge and pI of foreign peptides has a rigorous impact on virus-host interaction during epitope display on the surface of TMV. Bendahmane et al. (1999) fused 14 to 24 amino acids spanning epitopes of the rabies virus glycoprotein and the murine hepatitis virus S-glycoprotein to TMV CP. For the TMV recombinants CP-G5.24 and CP-5B19 they observed by 15 days delayed systemic infection in *Nicotiana tabacum* cv. Xanthi nn and symptoms were more mild compared to wtTMV infection. But the TMV recombinants CP-31D and CP-RB19 either failed to induce any symptoms in Xanthi nn or induced HR-like local necrotic lesions that restricted virus spreading. In contrast to the present work, they inserted the peptide sequences between Ser^{154} and Gly^{155}, but mainly for cloning reasons as they had a TMV clone with unique Spe I and Ppu MI restriction sites at this position (Bendahmane et al., 1997). To exclude position effects they inserted the RB19 epitope in frame after Thr^{158}, the last CP amino acid residue. Also this construct induced local lesions in the susceptible host. Hence, the inserted peptide itself and not its position is responsible for induction of defense reaction. They speculate that especially highly positively charged and hydrophobic peptides may destabilize plant membranes or might anchor the recombinant coat proteins within cell membranes and hence leading to the formation of local lesions (Fitchen et al., 1995). But when they introduced charge compensating amino acids upstream the RB19 and 31D epitopes to adapt charge and pI of the recombinant CP to the values of the wtCP, the resulting TMV recombinants behaved similar to the wildtyp virus. These viruses were capable to propagate and to move systemic in susceptible plants without local lesion induction.

The *de novo* designed peptides possess high hydrophobicity and high positive net charge (Epand and Vogel, 1999; Zasloff, 2002; Zeitler et al., 2010). Consequently, these peptides have enormous influence on CP characteristics referring to pI and charge. The wtCP has its pI at 5.09 and a charge of -2.23 at neutral pH (Nedoluzhko and Douglas, 2001; Asurmendi et al., 2007). But recombinant CP-peptide fusions have a pI around 9.8 and a charge at pH 7 in the range of +4.7 to +6.7 and this might account for the loss of infectivity and induction of HR-like lesions in susceptible host plants (***Chapter 4.10, Table 5***). It is assumed that such alterations affect the stability of tertiary and quaternary CP structures leading to mis-assembled virions and might influence recognition by the host (Ehrenfeld et al., 2008). To reconstitute the original CP properties a charge compensating domain was introduced into the recombinant CP-peptide fusion constructs. The insertion is located preceding the methionine residue for CNBr cleavage, composed of 5 to 7 randomly arranged aspartic and glutamic acid residues (Bendahmane et al., 1999). To investigate position effects, the charge compensation was also inserted

N-terminal between Ser^{3} and Ile^{4} of the SP1-1 harboring construct to separate the charge compensating domain and SP1-1. Additionally, in another construct SP1-1 was inserted into the CP loop region between Pro^{63} and Asp^{66}. To make it short, neither the insertion of the charge compensating domain, independent of position, in the SP1-1, SP6 and SP10-2 carrying TMV recombinants nor the translocation of SP1-1 into the CP loop region restored viral infectivity and all constructs induced local necrotic lesions in the susceptible *Nicotiana tabacum* cv. Xanthi nn (***Chapter 4.10, Figure 18***). It might be that recombinant CP is produced at least in initially infected cells, but instead of assembling with viral RNA to build virions it forms unordered aggregates. Those structures that are also found in transgenic tobacco plants stable expressing the TMV CP confer resistance against TMV infection, named CP-mediated resistance, and may inhibit spread of the recombinant TMV (Bendahmane et al., 1997; Asurmendi et al., 2007). Actually, reasons for infection failure remain speculative, but likely there are still many factors unknown that result in local lesions on susceptible tobacco plants by TMV recombinants (Li et al., 2006).

Against initial intentions, infection studies were conducted with the hypersusceptible *Nicotiana benthamiana* as expression host. That *Nicotiana benthamiana* is well suited for peptide display was already proofed with several epitopes (McCormick et al., 2006) and the overall qualification of *Nicotiana benthamiana* for use with viral expression vectors was already discussed (Turpen, 1999; Gleba et al., 2007; Lindbo, 2007b; Goodin et al., 2008; Lico et al., 2008). All treatments of plants with TMV recombinants lacking the charge compensation either by rub inoculation or by agroinfection remained symptomless. The existence of recombinant viral coat protein was not detectable and also the typical TMV induced systemic necrosis was omitted. But when recombinant TMV carrying both the charge compensation and SP1-1 or SP10-2 or SP6 at the C-terminus was used for infection typical stem necrosis and wilting occurred. Infection spread from inoculated leaves to the closest branch to induce necrosis and finally reached young, systemic leaves to induce distortion and yellowing. About 6 to 7 weeks after infection the plants died. In contrast, plants infected with wt TMV survived maximum 2 weeks. Interestingly, when the charge compensating motif in the SP1-1-CP construct was located at the N-terminus, the symptoms were drastically attenuated. Systemic stem necrosis remained absent and plants showed only severe leaf yellowing and distortion, but survived for more than 10 weeks after infection. For SP1-1 and SP6 the recombinant CP was clearly visible on protein gels, whereas the CP-SP10-2 was only slightly detectable (***Chapter 4.13, Figure 22***). If systemic infection of plants relies really on formation of virions that are able to move remains unclear. Probably, just some infectious RNA is transported in plant tissue from cell-to-cell as *Nicotiana benthamiana* can even serve as host for movement-deficient TMV mutants (Karger et al., 2003; Fujiki et al., 2006; Goodin et al., 2008). However, infectivity of putative recombinant virions was tested on different *Nicotiana* species. Infected leaves were homogenized without addition of buffer and plant sap was incubated 48 h at room temperature. Dilutions thereof were rub inoculated on leaves of *Nicotiana tabacum* cv. Xanthi NN and *Nicotiana sylvestris*. In both cases typical local lesions developed 3 dpi. It can be assumed that uncoated viral RNA was degraded during the incubation step and that amounts of, at

least protected, recombinant viral genomes are present in the cell, although probably assembled in a wrong way. Another explanation would be high titers of undegraded viral RdRP in the plant sap. Since it was shown that a helicase domain elicits the HR in plants harboring the *N* gene (Padgett et al., 1997; Erickson et al., 1999b; Padmanabhan et al., 2005). In *Nicotiana sylvestris* the HR is permitted by the *N'* gene that recognizes domains of the coat protein (Knorr and Dawson, 1988; Culver et al., 1994) and hence numerous undegraded recombinant CP subunits would have been present in the sap, what would at least proof their *in vivo* stability. When the infectious sap was used to inoculate *Nicotiana benthamiana,* disease symptoms developed equal to infection with the vector constructs.
Noteworthy, there are further possibilities to recover viral infectivity of TMV coat protein mutants. The insertion of the leaky stop codon of the RdRP 126 kDa protein (Buck, 1999) between CP and attached peptide might increase the ability to form functional virions that behave similar to wtTMV. As the six nucleotides of the leaky stop allow a read-through in the ratio of 1:20 much more wtCP is synthesized than recombinant CP and the overall properties of the virion are only slightly changed (Okada, 1999; Shaw, 1999). This approach was successfully applied to display the angiotensin-I-converting enzyme inhibitor peptide (ACE1) on TMV and recombinants were capable to infect tobacco and tomato (Hamamoto et al., 1993). Also different kind of epitopes from the influenza virus hemagglutinin and the HIV-1 envelope protein could be presented on the surface of TMV. Recombinant virions moved systemically in *Nicotiana tabacum* cv. Samsun nn and after virus purification the attached peptides were detected in a precipitin reaction with specific antibodies (Sugiyama et al., 1995). When malarial epitopes where inserted into the coat protein, high titers of recombinant TMV were observed in infected tobacco and chimeric particles could be purified in high yield. The recombinant viruses were stable and could be easily stored and hence offer the potential for effective production of subunit vaccines (Turpen et al., 1995). Inserting a leaky stop codon might be indeed useful for epitope display when studies are aimed to use complete recombinant virions and only small amounts of fused peptides are sufficient, e.g. for vaccination. But the read-through sequence drastically decreases peptide production. The present work was aimed to use only the released and purified designed peptides and hence this approach came not into consideration as the recombinant peptide production is strongly reduced.
Contrary to previous findings, Werner et al. (2006) reported the production of a 133 amino acid fragment of protein A on the surface of TMV by the insertion of a 15 amino acid Gly or Ala linker between CP and the fragment of protein A. The helical or flexible linker replaced the need for a leaky stop codon and they estimated to yield 3 g of CP-fusion protein per kg of fresh leaf biomass of *Nicotiana benthamiana* when the fusion protein was attached C-terminal to the CP. This illustrates a really interesting and promising approach and it would be worth to apply this for production of the *de novo* designed peptides. Anyway, if the use of a linker is transferable to produce any peptide sequence on TMV remains unclear and it can not be predicted to what extent peptide sequences influence plant response to infection with TMV recombinants (Ehrenfeld et al., 2008).

5.3 PURIFICATION OF *IN PLANTA* PRODUCED PEPTIDES

As CP with attached SP10-2 was hardly detected in infected plant material, but CP-SP1-1 fusion protein accumulated to high levels the main focus was on development of a purification protocol for SP1-1 (summerized in ***Figure 31***). A simple TMV purification protocol is described by Chapman (1998) and makes use of virion precipitation with PEG from aqueous solution in the presence of high amounts of salt. This method should yield at least 20 mg pure TMV from 20 g fresh leaf material. Although this protocol was successfully used to isolate wtTMV, it failed to get the TMV recombinant into solution. Probably, the CP-SP1-1 fusion construct forms insoluble aggregates and hence this method could not be applied. When Smith et al. (2006) purified their mutated TMV particle with high yield they used a modified procedure according to Gooding and Hebert (1967). They supplemented the aqueous extraction buffer with sodium metabisulfite to maintain reducing conditions and included a pH adjustment step to pH 5.0 and heating in their protocol. When elevated temperatures are applied to acidified plant extracts most membranes aggregate and can be easily removed by centrifugation. The resulting clarified protein extract still contains the recombinant virions and can be subjected to ultrafiltration, column chromatography or further methods to separate the virus (Pogue et al., 2002). Using this method TMV recombinants presenting cutaneous papillomavirus L2 capsid epitopes on their surfaces were successful recovered from infected *Nicotiana benthamiana* (Palmer et al., 2006). In addition, McCormick et al. (2006) applied this protocol to effectively purify TMV-peptide mutants. Furthermore, Bendahmane (1999) created a poorly soluble recombinant TMV, named RB-19. They used low speed centrifugation to remove cell debris from initial plant extract that was afterwards clarified by supplementation with diatomaceous earth and again low speed centrifugation. Ultracentrifugation of the supernatent enabled the pelleting of TMV RB-19 with high purity. However, when this application was applied for TMV SP1-1 purification it resulted in the sedimentation of the recombinant TMV (***Chapter 4.15, Figures 25 and 26***). It is known that TMV coat protein mutants have the tencendy to assemble wrong in infected cells and to form insoluble aggregates. By mutating Thr42Trp Asurmendi and colleagues (2007) created a CP-mutated virus that forms large aggregates, named virus-like particles (VLP). Also in this case, standard purification protocols failed. They separated initial plant extracts by adding a mixture of chloroform and butanol and low speed centrifugation. The TMV mutant accumulated in the aqueous phase and could be precipitated with PEG. But, also this method failed to purify the TMV mutant carrying SP1-1. The formation of insoluble aggregates of TMV CP mutants is not a new finding and was already described years ago. Kassanis and Milne (1971) called amorphous, dense inclusions that they found by light microscopy in epidermal cells of TMV mutant infected plants X-bodies. These results were proofed by electron microscopy that also demonstrated the lack of normal TMV virions (Jockusch, 1966). And even large amounts of wtTMV CP exist in infected leaves as aggregates that are often ubiquitinated (Hamacher et al., 2003). An interesting study was performed with the defective TMV strain PM1 that widely reflects the findings obtained in this work and represents the basis for the developed purification protocol. Parish and Zaitlin (1966) found in the 12,000 g

fraction from leaf homogenates of plants infected with TMV strain PM1 most of the viral coat protein. This fraction is usually discarded during virus purification and extraction of the sediment with either sodium deoxycholate or Triton X-100 did not solubilize the CP. When the initial extraction step was performed with aqueous buffers the CP always remained in the pellet after centrifugation for extract clarification. Differing buffer pH, salt concentrations and homogenization conditions had no effect on solubility. They speculate that mutant CP was not adsorbed to or within cell organelles as detergent treatment in concentrations usually used to solubilize or disrupt chloroplast, nuclei and mitochondria did not affect mutant CP insolubility. These results are in accordance with the observations achieved in this work. However, treatment of the sediment with 67% acetic acid was capable of solubilizing the CP and it remained in the acidic solution even after high speed centrifugation (***Chapter 4.15, Figure 27***). After pH adjustment to pH 7 by addition of NaOH the mutated CP precipitated and could be recovered by centrifugation to yield viral pellet insoluble in water, salt solutions and various buffers. Extraction of TMV with 67% acetic acid is the standard method to split virions and to separate coat protein subunits (Fraenkel-Conrat, 1957; Scheele and Lauffer, 1967). The TMV CP tolerates this harsh treatment and after buffer neutralization the single coat protein subunits are even able to assemble *in vitro* in the presence of TMV RNA to functional virions what was shown in numerous reconstitution studies (Fraenkel-Conrat and Williams, 1955; Durham, 1972). This approach in general harbors another advantage, namely the precipitation of the viral RNA during acetic acid treatment (Fraenkel-Conrat, 1957; Fraenkel-Conrat et al., 1957). As the purifed peptides are intended to be used as antimicrobial agents against plant pathogens a contamination with pathogenic RNA is absolutely undesirable. After TMV extraction with 67% acetic acid for one hour no remaining infectivity of the CP preparation was detected (Fraenkel-Conrat, 1957; Fraenkel-Conrat et al., 1957; Fraenkel-Conrat and Singer, 1958). For purification of the recombinant TMV carrying SP1-1 it was taken advantage of its insolubility. Repeated extraction steps with 5% Triton X-100 containing buffers removed contaminants like chloroplasts, chlorophyll and further compounds and subsequently the detergent was removed by washing with buffers lacking the Triton X-100. In contrast to previous reports (Parish and Zaitlin, 1966; Hariharasubramanian and Zaitlin, 1968), only volatile buffers were used and the pellet was dried under vacuum to drive off remaining buffer after the last washing step. Surprisingly, after extraction with differing buffers, virion splitting by acetic acid and methionine reduction more than 50% of the obtained protein fraction was recombinant CP-SP1-1. After CNBr cleavage about 30% of the CP-SP1-1 fusion protein remained unaffected, even after 48 h of incubation (***Chapter 4.16, Figure 28***). Although this cleavage method is very efficient (Sambrook and Russell, 2001; Crimmins et al., 2005), incomplete cleavage can occur for several reasons. Probably not all methionine residues were reduced during the reduction step and therefore were not available for cleavage (Kaiser and Metzka, 1999) or complexation with LMW compounds occurred already in the plant cell leading to cleavage failure (Jervis and Pierpoint, 1989). Furthermore, the formation of unfavorable protein structures might inhibit the access of CNBr to the cleavage site and thus reduce cleavage efficiency (Fontana and Gross, 1986). The subsequent liquid chromatographic purification of SP1-1

was based on the high resolution separation techniques size exclusion chromatography (SEC) and reversed phase chromatography (RPC) and followed standard procedures (***Chapter 4.17, Figures 29 and 30***). Noteworthy, SEC was chosen as first step because the inclusion of salt during chromatographic separation was mandatory to avoid unspecific matrix interactions as consequence of the high hydrophobicity and charge of peptide SP1-1. Furthermore, it served as additional virus elimination step because the TMV virion as well as the TMV genome are not able to enter the pores of the matrix and would elute in the void volume (Kumpalume and Ghose, 2003). Final RPC served not only as polishing and concentration step but also removed salt from the SEC fractions. As completely volatile buffers were used, the pure peptide SP1-1 could simply be lyophilized for storage to avoid proteolytic degradation. Because of the small size of SP1-1 removal of salts by desalting colums is not possible.

Based on the RPC chromatogram and SDS-PAGE the purity was estimated to be above 90% (***Chapter 4.18, Figure 31***). When hepcidin was recombinant produced in *E. coli* and purified via IMAC and RPC a similar purity in the range of 95% was detected (Zhang et al., 2005). Kim et al. (2006) produced the antimicrobial peptide lactoferricin fused to an anionic peptide in *E. coli.* They also applied CNBr cleavage to release the peptide but used ion exchange chromatography as polishing step during purification. Purity was analyzed on Tricine-PAGE where they could detect a single band in the range of 2 kDa. There are more studies dealing with production and purification of antimicrobial peptides in bacteria, mainly as fusion proteins. The purification is often based on metal-affinity and ion exchange chromatography and final purities in the range of 80 to 95% are reported (Uteng et al., 2002; Chen et al., 2006; Huang et al., 2006; Xu et al., 2006a; Xu et al., 2006b). However, the production of antimicrobial peptides in plants was mainly done in the context of plant resistance and less for further applications after extraction. Donini and colleagues (2005) produced an engineered killer peptide in *Nicotiana benthamiana* using a *Potato virus X* expression system. They used purified recombinant viral particles for use in antibacterial assays and the release of the peptide from the fusion partner and its purification was not done. The larvicide *Aedes* trypsin-modulating oostatic factor was expressed on the surface of TMV in *Nicotiana tabacum* using the leaky stop codon approach. Also in this case, the complete virions were used to analyze larvizidal activity in feeding experiments and the separation of peptide and TMV CP was not necessary for testing the activity (Borovsky et al., 2006).

To confirm the peptide mass of the purified SP1-1 mass spectrometric analysis by MALDI-TOF was performed (***Chapter 4.18, Figure 33***). The detected m/z values of the tobacco SP1-1 as well as of the synthetic version are in accordance with the calculated m/z values. Measured peptide masses show a difference of 1 Da. This is cleary attributed to the C-terminal amidation pattern. To protect the synthetic peptide its C-terminus was amidated and this modification is not performed in plants (Walsh; Walsh and Jefferis, 2006). Hence, the synthetic SP1-1 has the main peak at 1564.1 Da whereas the *in planta* produced peptide has the main compound at 1565.1 Da. This mass shift of 1 Da correlates with the free C-terminus of the tobacco peptide. In addition, for both peptides no impurites were detected in the LMW range, although these results need careful interpretation as the ionization properties of potential contaminations are not known.

The three-dimensional structure of the peptides were analyzed by NMR spectroscopy (***Chapter 4.19, Figure 34***). When measurements were performed in ddH_2O the synthetic peptide as well as the tobacco SP1-1 exists as random coil, although the amino acid sequence implies the adaption of an alpha-helical structure (Epand and Vogel, 1999; Brogden, 2005). Also the antimicrobial peptides MSI-78 and MSI-594 occur unstructured in aqueous solutions but adopt an alpha-helix when measured in the presence of micelles (Bhattacharjya and Ramamoorthy, 2009; Bhunia et al., 2009). It was shown for many amphoteric helical peptides with antimicrobial activity that secondary structure adoption is forced in the presence of lipids or detergent micelles mimicing bacterial membranes (Imamura et al., 2008; Marcos et al., 2008; Haney et al., 2009).

5.4 *IN VITRO* ACTIVITY OF *IN PLANTA* PRODUCED SP1-1

To finally proof the validity of this study *in vitro* inhibitory assays were performed to analyze antibacterial activity of the *in planta* produced peptide SP1-1. The main intention was to compare the antimicrobial activity of synthetic peptide *versus* tobacco purified peptide under different assay conditions. First experiments were conducted with the plant pathogenic bacteria *Pseudomonas syringae pv. syringae* and *Xanthomonas vesicatoria* (Agrios, 2005) and revealed that both peptides inhibit bacterial growth to more than 90% (MIC_{90}) at concentrations of 5 and 10 µg/ml, respectively. The peptides were dissolved just in ddH_2O in this experiment and to evaluate whether peptide solvent has an influence on antibacterial activity an acidified peptide solvent was used for next experiments (***Chapter 4.20, Table 7***). This might affect the conformation adopted by the peptides in solution before interaction with bacterial membranes and thus might affect antibacterial activity. The extent of alpha-helical structure was shown to be positively related to bacterial killing properties against gram-positive and gram-negative bacteria (Johansson et al., 1998; Giuliani et al., 2008). In contrast to the results of the first experiment, a slightly reduced toxicity against *X. vesicatoria* was detected with the tobacco produced SP1-1 compared to the synthetic peptide. Also the growth of *Pseudomonas corrugata* was inhibited to more than 90% by both peptides at 10 µg/ml, but again the synthetic peptide showed slightly higher activity. Probably, this accounts to the missing C-terminal amidation of tobacco SP1-1 which might be more sensitive to proteolytic degradation (Ostrowska and Gacko, 1998). Especially for application as antibiotic therapeutics the susceptibility to proteolysis has to be reduced. Noteworthy, for this reason clinical trials with peptides are mainly focused on topical applications (van 't Hof et al., 2001). Synthetic derivatives in which amino acids are substituted with their D-isomers are often as active as the parent peptides. Since proteolytic enzymes only recognize L-amino acids, the D-derivatives may represent proteolysis-insensitive variants. Hence, Wade et al. (1990) demonstrated that all D-enantiomeric forms of cecropin A, melittin and magainin show the quantitatively equivalent activity. Furthermore, the peptide KLKLLLLLKLK-NH_2 was capable to inhibit the growth of methicillin-resistant *Staphylococcus aureus* for 12 h at 30 µg/ml, but after 24 h the growth was restored nearly 100% (Alvarez-Bravo et al., 1994). Interestingly, the D-

enantiomer was not only more resistant to tryptic digest, but also two-times more active. No significant bacterial growth was detected after 24 h. But sometimes different effects of the L-amino acid composed peptides and their D-amino acid containing counterparts are detected what imparts the possible existence of specific peptide targets (Brogden, 2005). Both peptides failed to reach the MIC_{90} for *Pectobacterium carotovorum ssp. carotovorum* but equal inhibition of growth was detected at 5 µg/ml for the synthetic peptide as well as for tobacco SP1-1. Masen and colleagues (2006) performed an interesting study with the designed histidine rich peptide LAH4. At neutral pH LAH4 derivates do not disrupt membranes and have no bacteriolytic effect on *E. coli* or *Bacillus megaterium*. In contrast, the growth of *Staphylococcus aureus* was inhibited under these conditions. However, at acidic pH the antibiotic activities are drastically enhanced and even membrane destabilizing properties were detected. Furthermore, although ineffective against *Pseudomonas aeruginosa* at neutral pH, at acidic pH the peptides were highly active. To analyze whether the pH of the assay has an effect on peptide activity, the inhibition assay was performed under different pH-conditions (***Chapter 4.20, Tables 8 and 9***). Therefore, the medium for bacterial growth and assay procedure was buffered to pH 5.7, pH 7.2 and pH 8.6. Acidity or alkalinity of the medium can have profound effects on bacterial growth. Particularly, pH extremes can lead to denaturation of surface proteins and enzymes and depolarization of membrane transport for essential ions and nutrients. But these effects are often the consequence of salts in the medium that can precipitate or change to a toxic state (Yaganza et al., 2009). Under the present low-salt conditions a narrow pH shift should only slightly effect bacterial growth. Interestingly, under basic conditions both, the tobacco produced and the synthetic peptide, showed higher activities and already at 1 µg/ml a growth reduction of 73% and 49% was detected for the synthetic SP1-1 and the tobacco peptide, respectively. *P.c. ssp. carotovorum* showed a higher pH range of tolerance and grew normally under acidic conditions. But antibacterial activity of both peptides was quite low and at 10 µg/ml growth reduction of only 40% and 15% was detected for the synthetic SP1-1 and the tobacco peptide, respectively. Surprisingly, buffering the assay to neutral and basic pH dramatically increased antibacterial activity of the peptides. Under both conditions the synthetic SP1-1 reached the MIC_{90} already at 5 µg/ml and the tobacco peptide reduced the growth to 50% at 5 µg/ml and to 90% at 10 µg/ml in the basic assay.

The initial binding of the positively charged peptides to negatively charged membranes of microbes is a simple electrostatic interaction. Hence, this event is very sensitive to the ionic strength of the medium. Most microdilution assays are performed in the presence of low-ionic-strength buffers. But only the use of higher salt concentrations will detect peptides that are relatively insensitive to ionic disturbance (van 't Hof et al., 2001). For example, the activity of histatin 5 against *C. albicans* is nearly abolished in 50 mM phosphate buffer, whereas in 1 mM buffer the LC_{50} is at only 2 µM (Helmerhorst et al., 1997). Hancock and Sahl (2006) assume that virtually every peptide sequence bearing a positive net charge and some hydrophobic residues will have antimicrobial activity if assayed in standard low-ionic-strength buffers or dilute medium. The antagonizing effect of divalent cations like Mg^{2+} or Ca^{2+} and monovalent cations like Na^{+} or K^{+} is often underrated. To take this into account, another assay was performed on *Pseudomonas*

syringae pv. syringae with fixed concentrations of $MgCl_2$ and $CaCl_2$ and varying amounts of NaCl included in the assay buffer. The concentration of Mg^{2+} and Ca^{2+} in total was 3 mM and thereby near physiological conditions and Na^+ ranged from 40 mM to 240 mM. In this experiment the activites of both peptides were dramatically decreased (***Chapter 4.20, Table 10***). This reflects clearly the difficulties that may arise during drug development, since under physiological conditions the antimicrobial activity is dramatically decreased. Anyway, the topical application of AMPs is surely conceivable (Chopra, 1993; Marr et al., 2006). Independent of the NaCl concentration, at 5 µg/ml only the synthetic SP1-1 was able to inhibit 28% of bacterial growth. At 10 µg/ml the maximum growth inhibition was 43% whereas under standard conditions the MIC_{90} of both peptides was found to be at 5 µg/ml. The impact of NaCl on bactericidal activity of recombinant human beta defensin 2 was shown to be also dramatic. At concentration of 150 mM NaCl the peptide was almost inactive and 50 mM NaCl were already sufficient to halve the antibacterial activity against *E. coli* K12D31 (Xu et al., 2006a).

The measured antibacterial activities of the synthetic SP1-1 as well as of tobacco produced SP1-1 are comparable to reported antimicrobial activities of other studies. Powell and colleagues (1995; 2000) also developed antimicrobial peptides for plant protection and presented activities against *Pseudomonas syringae* in the range of 38-42 µg/ml. Some peptides designed to kill *Pseudomonas aeruginosa* strain PAO1 were active in concentrations between 7.8 and 31.3 µg/ml whereas several peptides failed to exhibit antibacterial activity even at concentrations above 500 µg/ml (Jiang et al., 2008b). Also Chen et al. (2005) designed a whole set of antimicrobial peptides and reported MIC_{90} values of 1.6-16 µg/ml against several *E. coli* strains and MIC_{90} values between 5 and 33 µg/ml against different *Pseudomonas aeruginosa* strains. Jiang and colleagues (2008a) showed that several peptides which are active against gram-negative bacteria in a range between 8 and 16 µg/ml are only in higher concentrations of 16-64 µg/ml active against gram-positive bacteria. Numerous short peptides were generated synthetically to inhibit growth of *Streptococcus* mutants. Most of them were active at 8 µg/ml, but other peptides failed to show antibacterial activity up to 125 µg/ml (He et al., 2007). Some of the short cationic peptides presented by Makovitzki et al. (2007) were capable of inhibiting the growth of *Pseudomonas syringae* or *Agrobacterium tumefaciens* in concentrations of 3.1 and 6.2 µg/ml, respectively. The same peptides also inhibited fungal spore germination of *Alternaria alternata* and *Botrytis cinerea*, but concentrations up to 25 µg/ml were necessary.

Hence, the activity range of the *de novo* designed antimicrobial peptide SP1-1 is similar to reported MIC_{90} values of other studies and the detected antimicrobial activity of tobacco produced SP1-1 differs only slightly from activity of the synthetic version. Probably, by rational computational design the salt sensitivity as crucial drawback can be reduced (Juretic et al., 2009). Furthermore, future studies dealing with the design of antimicrobial peptides might take marine peptides as templates that are adapted to salt-rich environments and less sensitive to ionic strength (van 't Hof et al., 2001).

6. CONCLUDING REMARKS

Multiple antibiotic resistant pathogenic bacteria are on the advance and drug resistance presents an ever-increasing global public health threat. Disease agents that were once thought to be controlled by antibiotics are returning in new leagues resistant to these therapies. Although mainly bacteria causing severe problems, it should be mentioned that antimicrobial resistance is also evident in other microorganisms, like parasites, fungi and viruses (Levy and Marshall, 2004; Barrett, 2005). Big pharmaceutical industry dropped off quickly efforts to support drug discovery for competing priorities. Although marketed antibiotics are generally safe, they have weak values as marketable goods because medication durations are short (Vicente et al., 2006). Antimicrobial peptides might fill this gap by providing simple templates for the *de novo* design of antimicrobial therapeutics. When rational designed, their activity spectrum can be extended, the cytotoxicity reduced and the application rate decreased what was also shown in previous studies (Zeitler et al., 2010). In addition, it was proofed that their membrane targeting mode of action delays resistance formation in microorganisms (Perron et al., 2006). In this work it was demonstrated that also the recombinant production of AMPs can be performed with low capital input when plants were used as green factories. The originally plant pathogenic *Tobacco mosaic virus* was engineered to enable foreign gene transfer into plants to permit highest expression levels and to represent a favorable fusion partner. This fits into the meanwhile accepted conclusion that plant viruses are much more than just infectious molecules. Actually, they found their way into several biotechnological applications, including the use as biotemplates in the nanotechnology (Young et al., 2008). If *in planta* peptide production indeed is preferable the chemical peptide synthesis was not apparent from this study as multiple factors have to be considered. Thus, production success is not only dependent on peptide length and sequence but also on its influence on the fusion partner. Furthermore, the final outcome differs between plant species and has to be evaluated for every peptide individually. At least the solid-phase synthesis of short peptides improved strongly during the last years with regard to reaction efficiency and hence reduction of process charges (Guzman et al., 2007; Morrow, 2010). Nevertheless, improving viral vectors and recruiting new viruses for generation of expression systems should provide an efficient platform for recombinant peptide production in plants. Bioinformatic tools might help to optimize peptide sequences and viral fusion partners to enhance viral vector performance and to enlarge the production host species. Removal of plant contaminants by high resolution purification methods, as it was done in this work, enables recovery of pure and biologically acitve AMPs from transfected plant material that can be used in many applications, including human and animal medicine or agriculture.

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8. APPENDIX

8.1 SCREENING OF PUTATIVE TRANSGENIC *NICOTIANA TABACUM* PLANTS

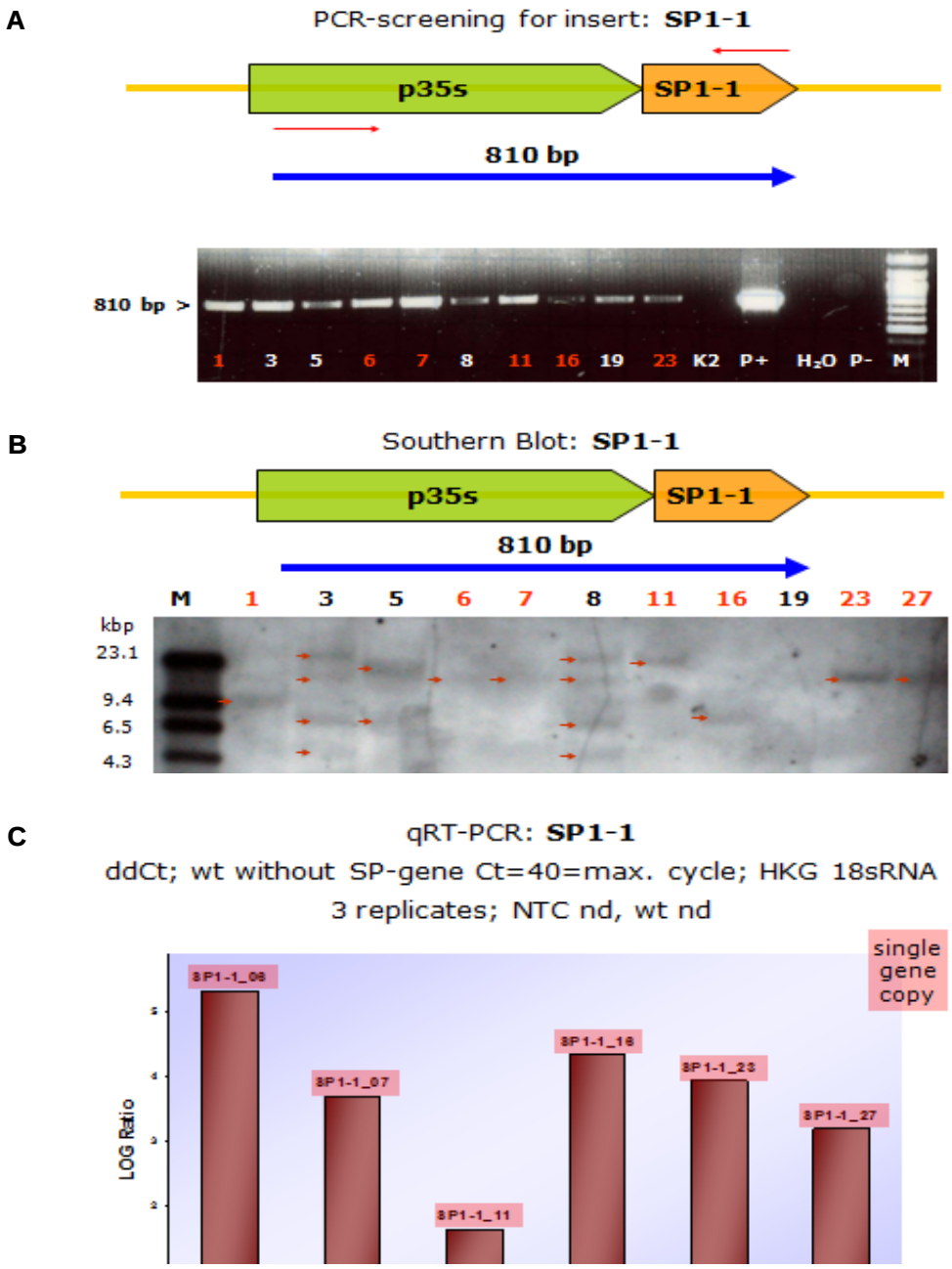


Figure 35 *Screening of transgenic Nicotiana tabacum cv. Xanthi NN for insertion and expression of SP1-1. (A) DNA was extracted from transformed plants and untransformed control plants and PCR analysis were performed with CaMV 35S promoter- and SP1-1-specific oligonucleotides. A single 810 bp band indicates presence of the SP1-1 gene in plants 1, 3, 5, 6, 7, 8, 11, 16, 19, 23. The plasmid used for transformation (P+) served as positive control. No PCR product was generated in the control reaction with DNA from untransformed plants (K2) and in the negative controls with H2O instead of DNA (H2O) and the empty vector used for transformation (P-). Relative molecular marker standards are shown on the right. M, marker. (B) 20 µg DNA of transformed plants were Xba I digested, separated on a 0.8% agarose gel and blotted on a nylon membrane. A 810 bp DIG-labeled probe covering the CaMV 35S promoter and SP1-1 sequence was used in Southern analysis to determine SP1-1 copy numbers in transgenic plants. Only weak signals were generated but in plants 1, 6, 7, 11, 16, 23, 27 only one SP1-1 gene should be present (indicated by red arrows). Relative molecular marker standards are shown on the left. M, marker. (C) Total RNA was extracted from transgenic plants harboring a single gene copy of SP1-1 and its expression was determined by qRT-PCR. The 18S RNA served as control.*

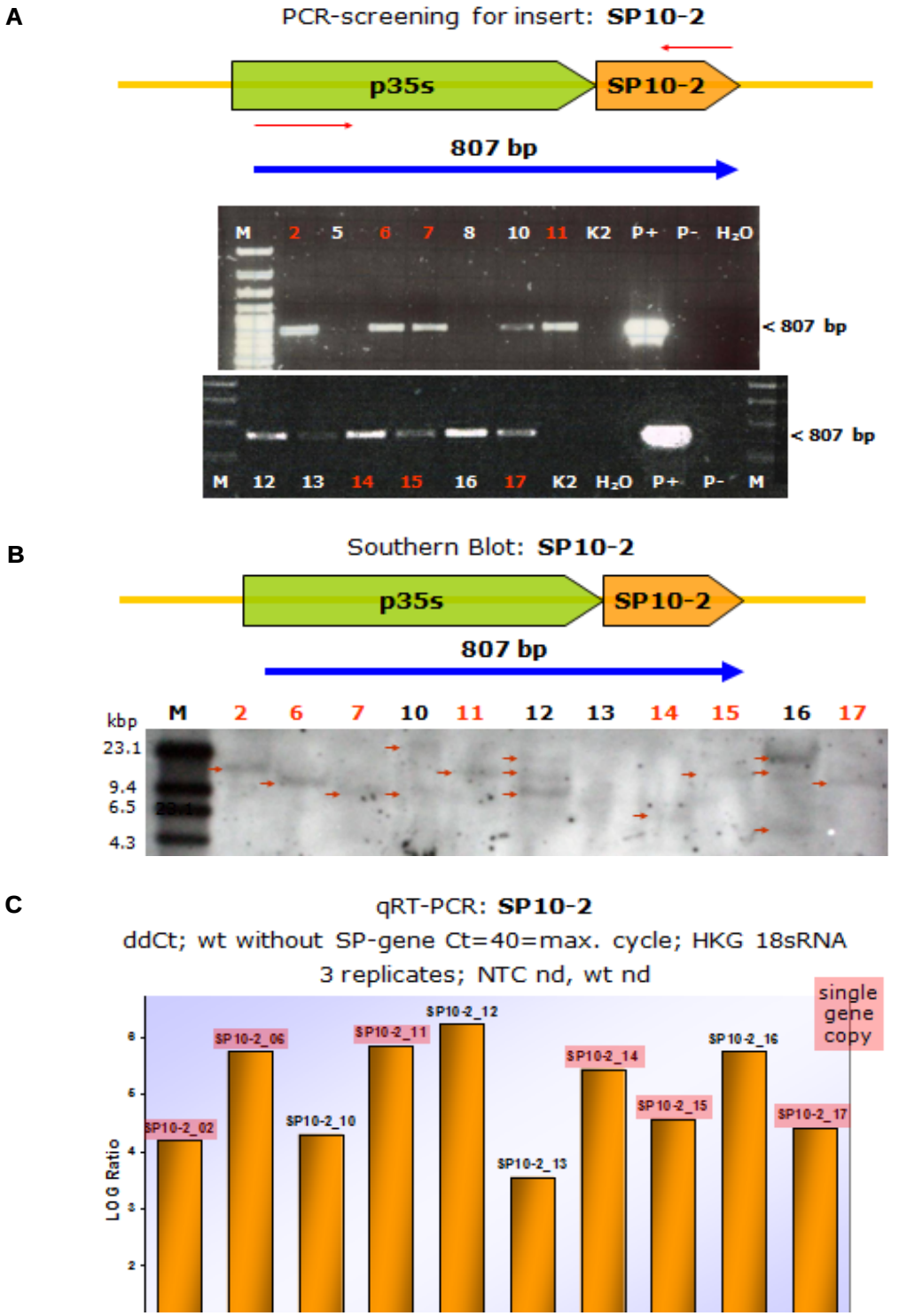


Figure 36 *Screening of transgenic Nicotiana tabacum cv. Xanthi NN for insertion and expression of SP10-2. (A) DNA was extracted from transformed plants and untransformed control plants and PCR analysis were performed with CaMV 35S promoter- and SP10-2-specific oligonucleotides. A single 807 bp band indicates presence of the SP10-2 gene in plants 2, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17. The plasmid used for transformation (P+) served as positive control. No PCR product was generated in the control reaction with DNA from untransformed plants (K2) and in the negative controls with H2O instead of DNA (H2O) and the empty vector used for transformation (P-). Relative molecular marker standards are shown on the left. M, marker. (B) 20 µg DNA of transformed plants were Xba I digested, separated on a 0.8% agarose gel and blotted on a nylon membrane. A 810 bp DIG-labeled probe covering the CaMV 35S promoter and SP10-2 sequence was used in Southern analysis to determine SP10-2 copy numbers in transgenic plants. Only weak signals were generated but in plants 2, 6, 7, 11, 14, 15, 17 only one SP10-2 gene should be present (indicated by red arrows). Relative molecular marker standards are shown on the left. M, marker. (C) Total RNA was extracted from transgenic plants harboring a single gene copy of SP10-2 and its expression was determined by qRT-PCR. The 18S RNA served as control.*

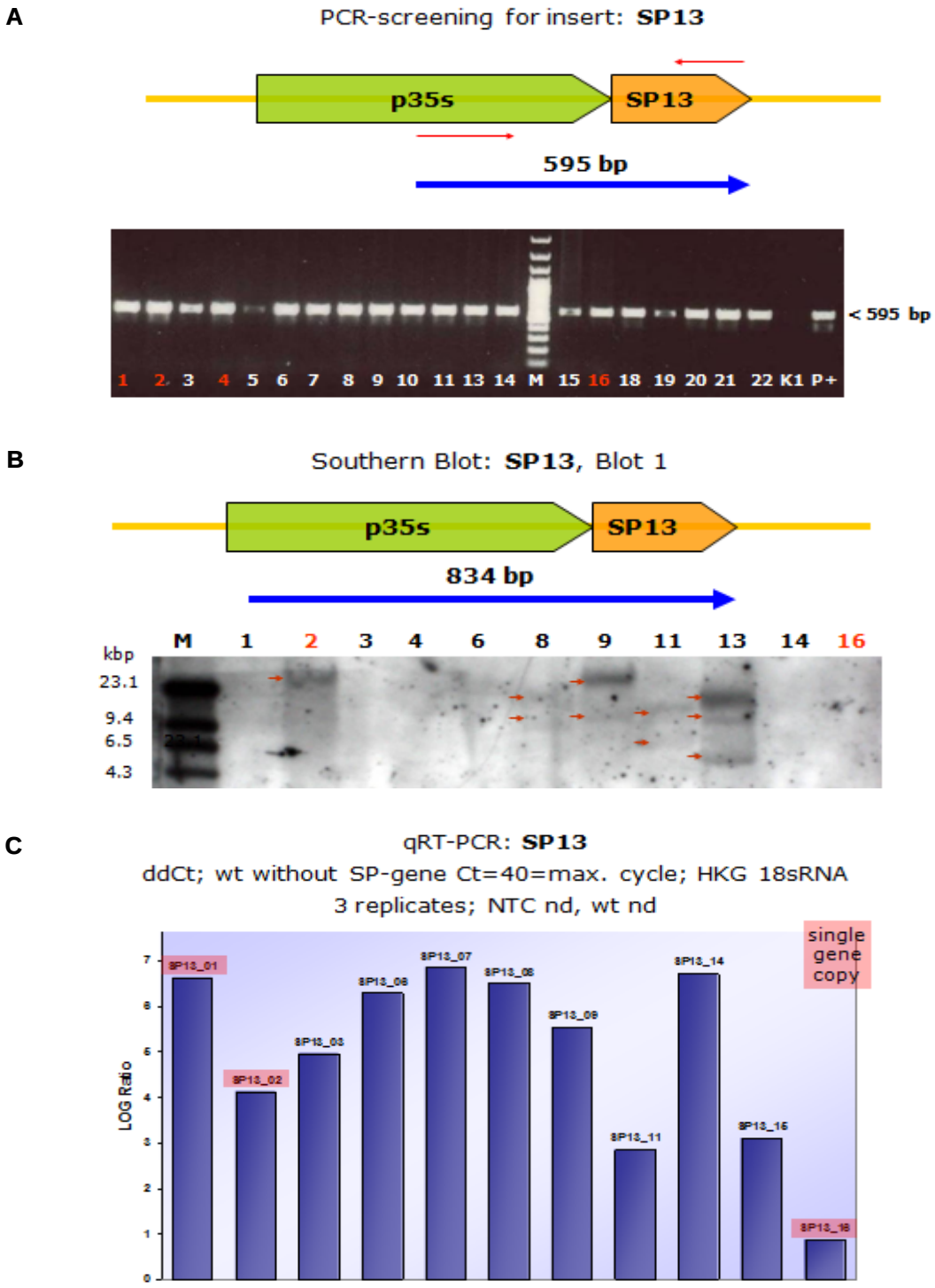


Figure 37 *Screening of transgenic Nicotiana tabacum cv. Xanthi NN for insertion and expression of SP13. (A) DNA was extracted from transformed plants and untransformed control plants and PCR analysis were performed with CaMV 35S promoter- and SP13-specific oligonucleotides. A single 595 bp band indicates presence of the SP13 gene in plants 1-11, 13-16, 18-22. The plasmid used for transformation (P+) served as positive control. No PCR product was generated in the control reaction with DNA from untransformed plants (K1). Relative molecular marker standards are shown on the middle. M, marker. (B) 20 µg DNA of transformed plants were Xba I digested, separated on a 0.8% agarose gel and blotted on a nylon membrane. A 834 bp DIG-labeled probe covering the CaMV 35S promoter and SP13 sequence was used in Southern analysis to determine SP13 copy numbers in transgenic plants. Only weak signals were generated but in plant 2 only one SP13 gene should be present (indicated by red arrows). Relative molecular marker standards are shown on the left. M, marker. (C) Total RNA was extracted from transgenic plants harboring a single gene copy of SP13 as well as multiple SP13-inserts and its expression was determined by qRT-PCR. The 18S RNA served as control.*

8.2 MASS SPECTROMETRIC ANALYSIS OF PROTEIN EXTRACTS OF TRANSGENIC *NICOTIANA TABACUM*

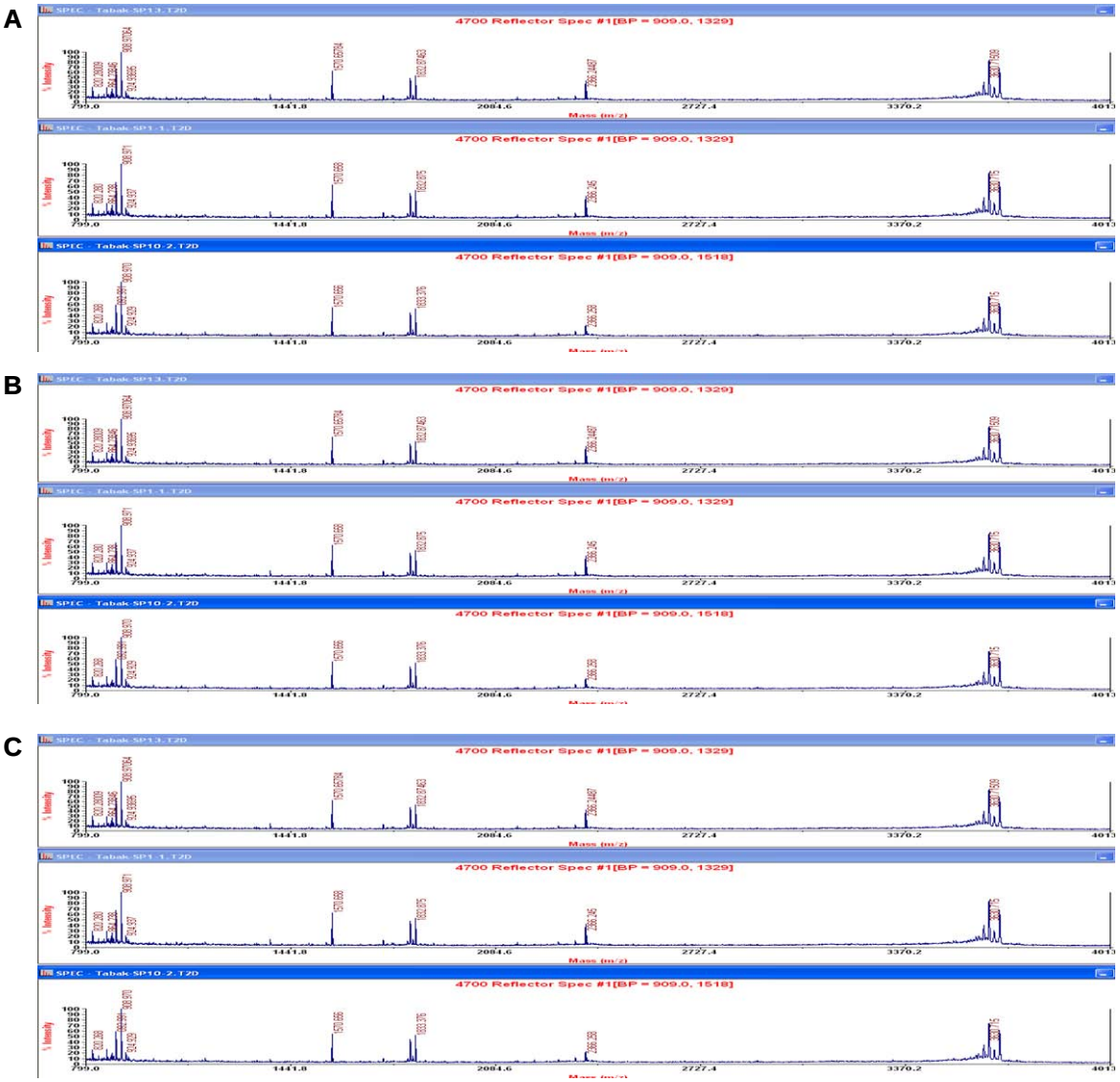
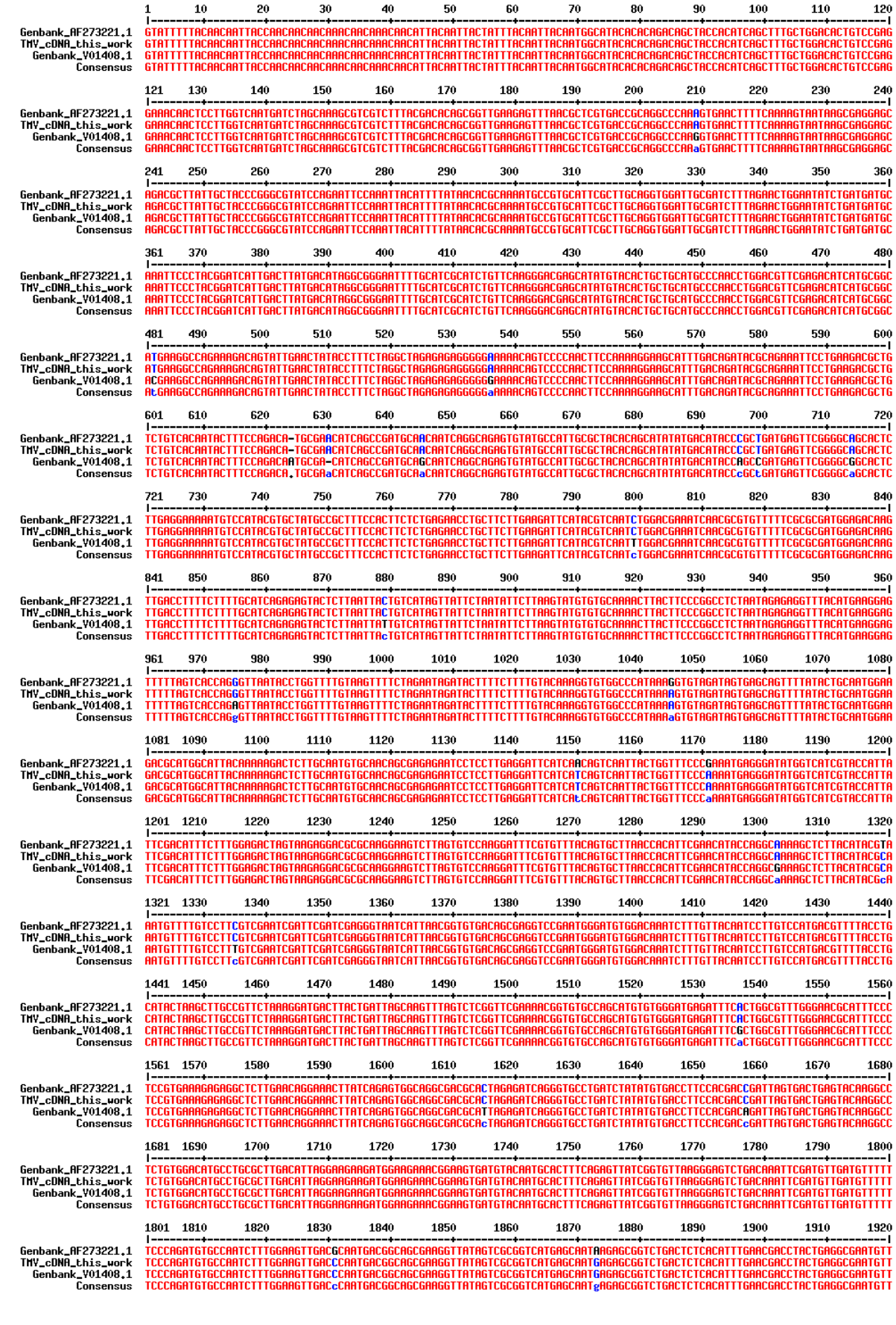
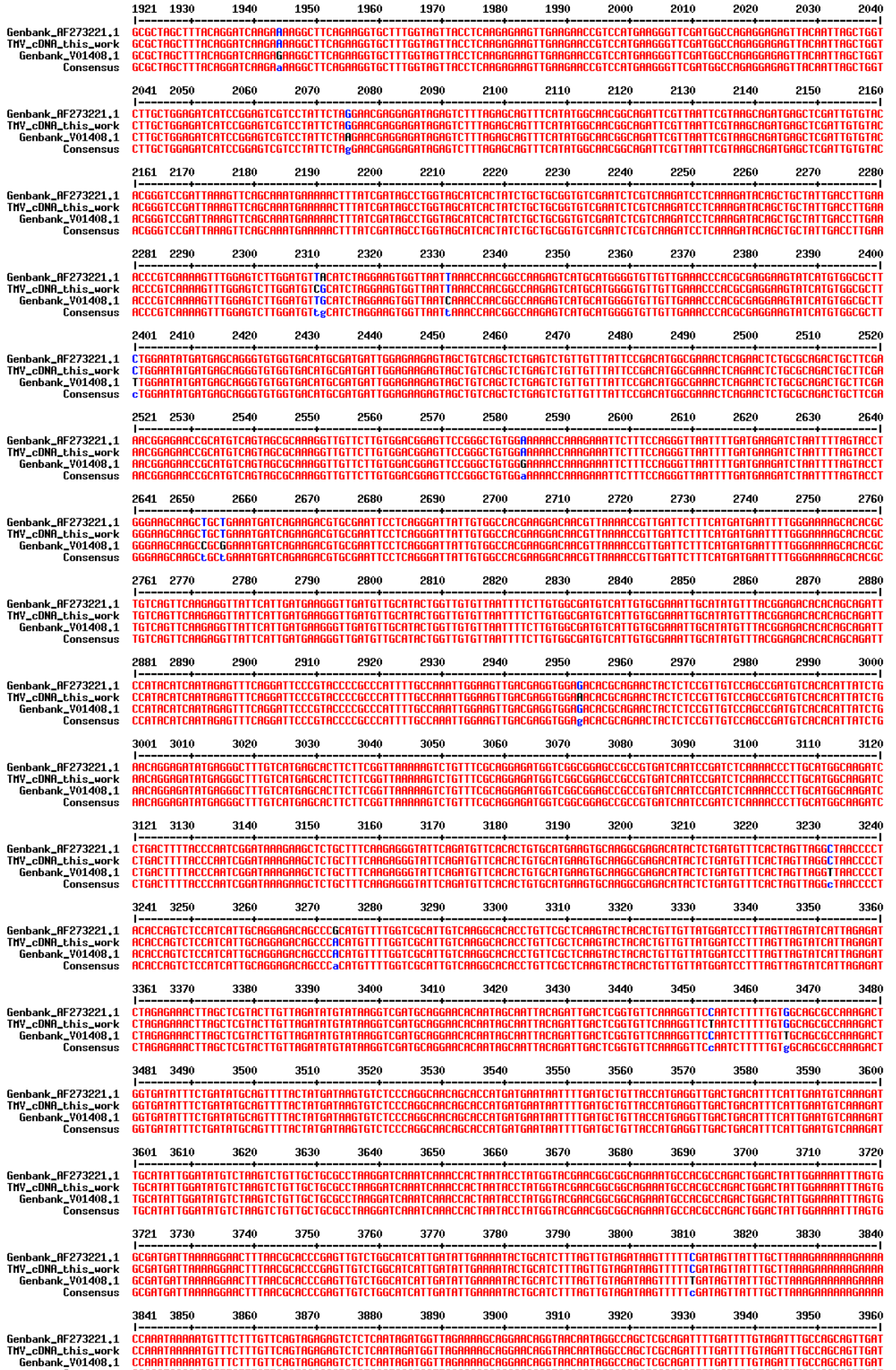


Figure 38 *MALDI-TOF analysis were performed with protein extracts of transgenic Nicotiana tabacum plants to detect peptides SP1-1, SP10-2 and SP13. Proteins were isolated from leaves as described in chapter 3.9. After desalting and buffer exchange to 0.1% TFA with a BioRad Micro Bio-Spin column analysis were conducted as described in chapter 3.21. (A) For SP1-1 plant 6 was chosen for measurements as a single gene copy of SP1-1 and strong expression was detected by Southern and qRT-PCR analysis, respectively (see Figure 35). However, for the calculated m/z value of 1565.1 no signal was detected. (B) For SP10-2 plant 14 was chosen for measurements as a single gene copy of SP10-2 and strong expression was detected by Southern and qRT-PCR analysis, respectively (see Figure 36). However, for the calculated m/z value of 1504.9 no signal was detected. (C) For SP13 plant 1 was chosen for measurements as a single gene copy of SP13 and strong expression was detected by Southern and qRT-PCR analysis, respectively (see Figure 37). However, for the calculated m/z value of 2401.9 no signal was detected. The signal at the m/z value of 1570.6 reflects the internal standard included in the measurements. Y-axis, signal intensity [%]; x-axis, mass [m/z].*

8.3 DNA SEQUENCE ALIGNMENTS

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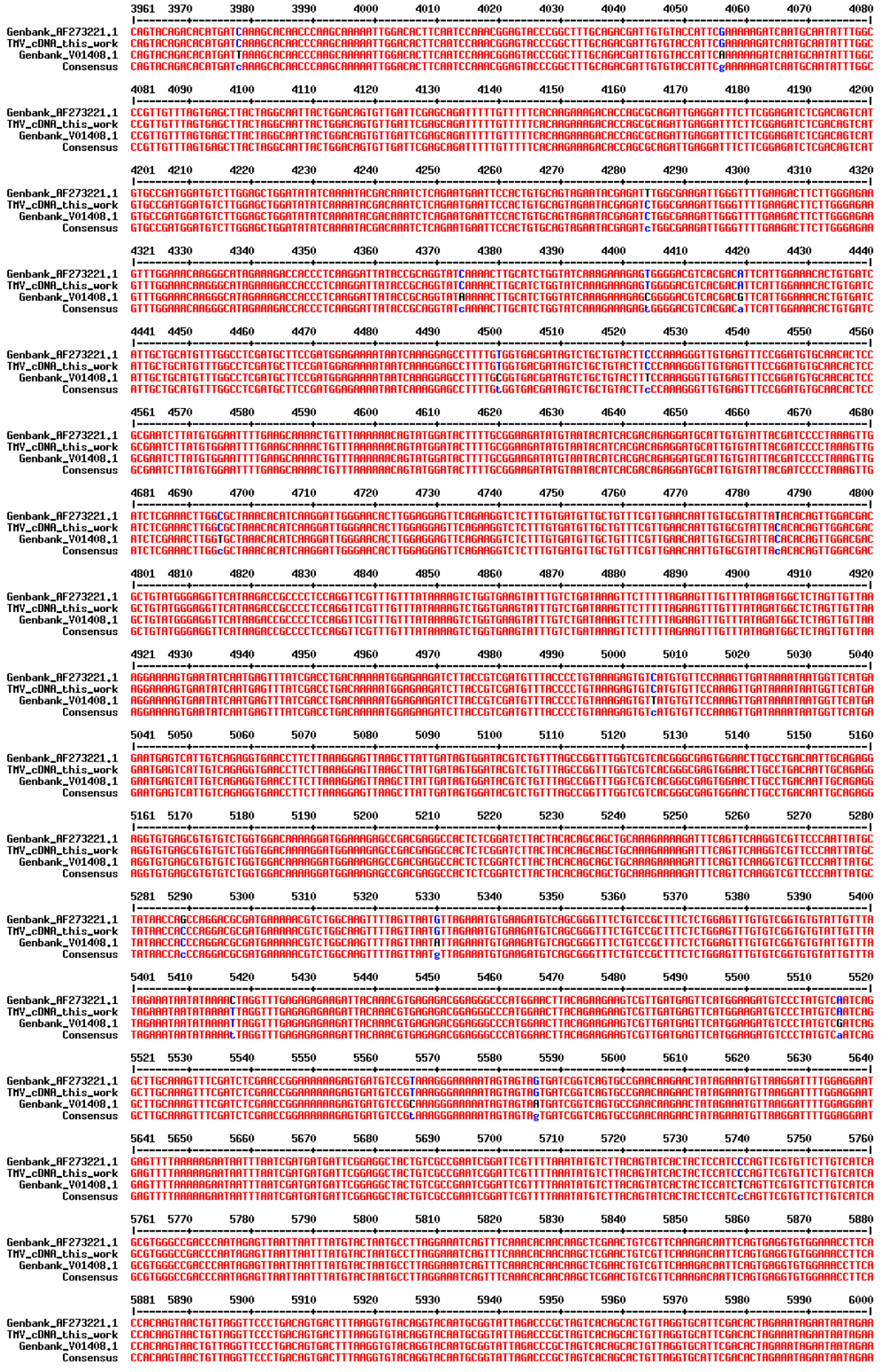
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Genbank_V01408.1 GTGCCGATGGATGTCTTGGAGCTGGATATATCAAAATACGACAAATCTCAGAATGAATTCCACTGTGCAGTAGAATACGAGATCTGGCGAAGATTGGGTTTTGAAGACTTCTTGGGAGAA
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Figure 39 *Alignment of identified TMV cDNA clone (this work) with sequences of Holmes' masked strain of TMV, Genbank AF273221.1 (Holt et al., 1990) and TMV variant 1, Genbank V01408.1 (Goelet et al., 1982). The isolated viral genome shows highest similarity to AF273221.1 and relative to V01408.1 55 base changes were detected. Most of these mutations remained silent, but only 6 lead to amino acid changes (see chapter 4.4, Table 3). Alignment was performed using MultAlin (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988).*

Figure 40 *Alignment of cDNAs derived from recombinant TMV clones carrying C-terminal the peptide SP1-1 and charge compensating amino acids in different positions with the predicted sequences. (A) Charge compensation located at the N-terminus . (B) Charge compensation located at the C-terminus of the TMV coat protein. Alignment was performed using MultAlin (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988).*

8.4 DNA SEQUENCES AND OLIGONUCLEOTIDES

Table 11 *DNA sequences of antimicrobial peptide genes used for stable transformation and transient expression approaches. Start codon marked by underline and amino acid sequences are indicated above DNA sequence. Red, translation start and CNBr cleavage site; green, charge compensating amino acids.*

SP1-1	M R K K R L K L L K R L L * ATG CGC AAA AAA CGC CTG AAA CTG CTG AAA CGC CTG CTG TGA
SP1-1cc	D D E D D E E M R K K R L K L GAT GAT GAA GAT GAT GAA GAA ATG CGC AAA AAA CGC CTG AAA CTG L K R L L * CTG AAA CGC CTG CTG TAA
ccSP1-1	E D D E D E E M R K K R L K L … GAA GAT GAT GAA GAT GAA GAA … ATG CGC AAA AAA CGC CTG AAA CTG L K R L L * CTG AAA CGC CTG CTG TGA
SP10-2	M L R F L K K A L K K L F * ATG CTG CGC TTT CTG AAA AAA GCG CTG AAA AAA CTG TTT TAA
SP10-2cc	D D E D D M L R F L K K A L K K GAT GAT GAA GAT GAT ATG CTG CGC TTT CTG AAA AAA GCG CTG AAA AAA L F * CTG TTT TAA
SP6	M A L A H F L K K A I K K * ATG GCG CTG GCG CAT TTT CTG AAA AAA GCG ATT AAA AAA TGA
SP13	M K R R L I A R I L R L A A R A ATG AAA CGC CGC CTG ATT GCG CGC ATT CTG CGC CTG GCG GCG CGC GCG L V K K R * CTG GTG AAA AAA CGC TGA

Oligonucleotides for qRT-PCR

rtSP1-1_for	5’-GCAGGCTTAATGAGAAAGAAGAGACT-3’
rtSP1-1_rev	5’-TGTACAAGGAAAGCTGGGTATCAA-3’
rtSP10-2_for	5’-GGCGGCCGCACTAGTG-3’
rtSP10-2_rev	5’-GGAAAGCTGGGTATCAGAAAAGC-3’
rtSP13_for	5’-GCAGGCTTAATGAAGAGAAGATTGA-3’
rtSP13_rev	5’-CATCTCTTCTTAACCAAAGCTCTAGCA-3’
rt18s_for2	5’-ATGGTGGTGACGGGTGACG-3’
rt18s_rev2	5’-GCCTGCTGCCTTCCTTGG-3’

8.5 WESTERN BLOT ANALYSIS OF PURIFIED SP1-1

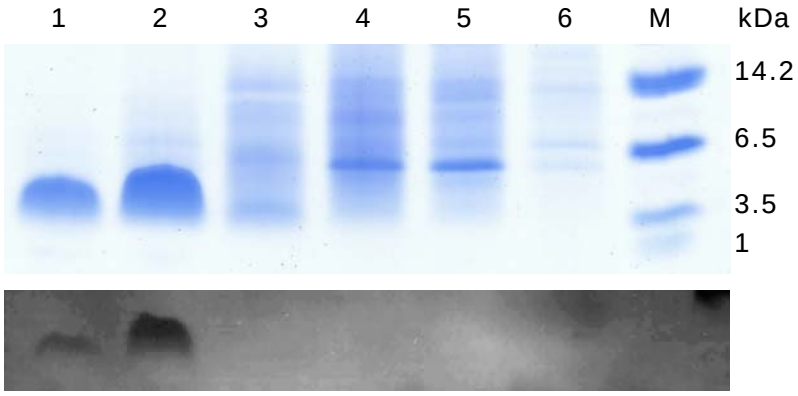

Figure 41 *Western Blot analysis of RPC fractions after NBT/BCIP detection. Lanes 1 + 2, differing amounts of fraction 13 (see Figure 30); lanes 3 – 6, minor peaks eluting at higher organic solvent concentration. Immunodetection was performed with SP1-1-specific monoclonal antibodies. Relative molecular marker standards are shown on the right. M, ultra low molecular weight marker; kDa, kilo dalton.*

8.6 NMR STRUCTURAL ANALYSIS

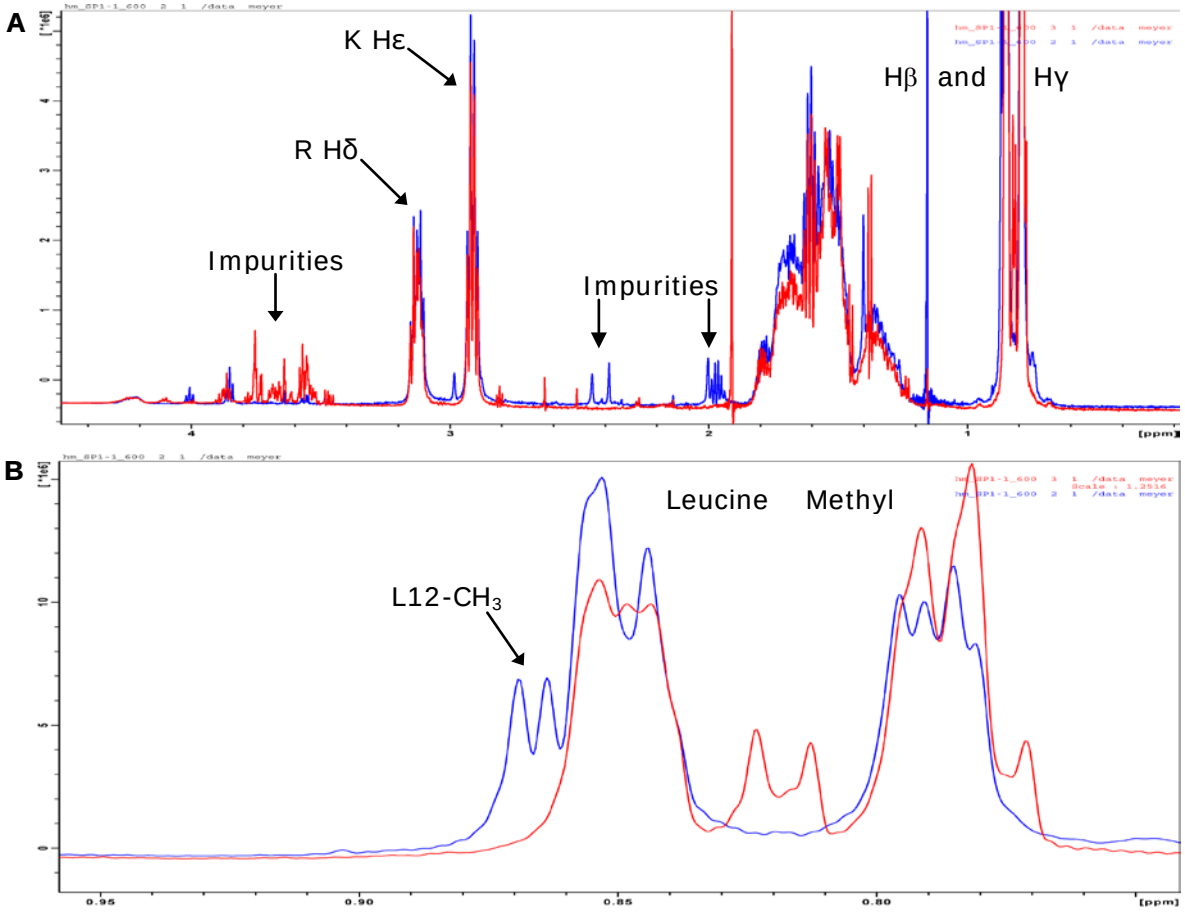

Figure 42 *NMR structural analysis of tobacco SP1-1 and synthetic SP1-1. Red curves represent always tobacco SP1-1, blue curves always synthetic SP1-1. (A) Sector 0.5 to 4.5 from one-dimensional NMR spectroscopy of total spectrum of 1H overlay of both peptides. Impurities appear as strong background peaks. The aliphatic amino acids arginine and lysine appear as Balmer lines Hδ and Hε. Balmer lines Hβ and Hγ are also indicated. (B) Sector 0.75 to 0.95 from one-dimensional NMR spectroscopy of total spectrum of 1H overlay of both peptides indicates the methyl groups of leucine that are present in the peptide. Measurements were carried out with 0.5 mg peptide dissolved in phosphate buffer on an Avance 900 instrument equipped with a TXI cryoprobehead.*

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