A comparative testing of *Cucumber mosaic virus* (CMV)-based constructs to generate virus resistant plants in tobacco species

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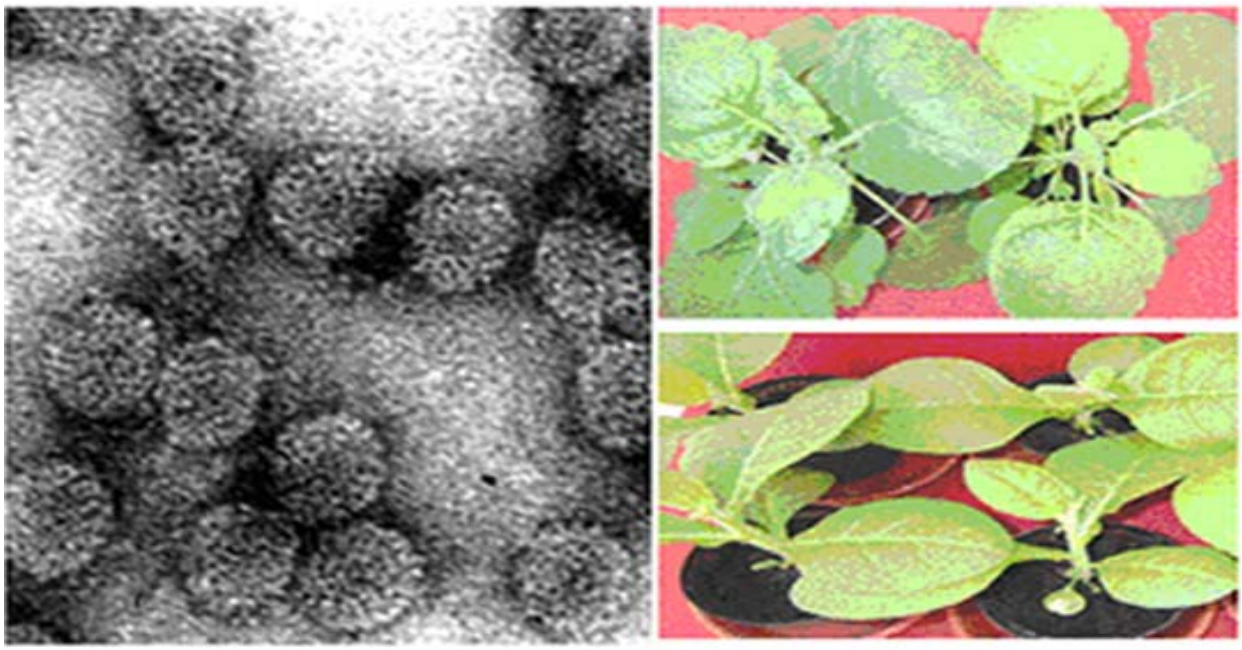

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Hiermit bestätige ich die weitestgehende Korrektheit des Englisch in der mir vorliegenden Arbeit: "A comparative testing of *Cucumber mosaic virus* (CMV) - based constructs to generate virus resistant plants in tobacco species"

Mit freundlichem Gruss und den besten Wünschen an Herrn Tan für seine berufliche Laufbahn.

Sylvia K. Green
Head, virology unit

To my parents Feiyou Tan and Weilian Man

To my wife Fenglian Zhang

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Abbreviations

°C	centigrade
%	percent
χ2	Statistical chi-square test
aa	amino acid
AC4	Suppressor of gene silencing from *African cassava mosaic virus*
AGO1	Argonaute 1 protein
Amp	Ampicillin
AS	Acetosyringone
BAP	6-Benzylaminopurine
2bIR	inverted repeat of 2b gene
bp	base pairs
BYDV	*Barley yellow dwarf virus*
CaCV	*Capsicum chlorosis virus*
CaMV	*Cauliflower mosaic virus*
CCMV	*Cowpea chlorotic mottle virus*
cDNA	complementary DNA
Cefo	Cefotaxime sodium
CGMMV	*Cucumber green mild mottle mosaic virus*
CIAP	Calf intestinal alkaline phosphatase
CMV	*Cucumber mosaic virus*
CP	coat protein
CPIR	inverted repeat of coat protein
CSNV	Chrysanthemum stem necrosis virus
DCL	RNase III-like enzymes (Dicer like)
DI RNA	defective-interfering RNA
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	mixture of the four deoxynucleotide triphosphates
d.p.i	days post inoculation
dsRNA	double-stranded RNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
et al.	et alii
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
Genta	Gentamicin sulphate
GFP	Green fluorescent protein
GM	genetically modified crops
gRNAs	genomic RNAs
GUS	β-Glucuronidase gene
GV3101	Strain of *Agrobacteria tumefaciens*

HCl	Hydrochloride
HC-Pro	Suppressor of gene silening from *Potato virus* Y
hpt	Hygromycin phosphotransferase
HR	hypersensitive response
IPM	integrated pest management
IPTG	Isopropylthio-β-D-galactoside
IR	inverted repeat
Kan	Kanamycin monsulfate
KD	Kilo Dalton
LBA4404	Strain of *Agrobacteria tumefaciens*
LB media	Luria-Bertoni-Medium
MES	morpholinoethansulfonacid
mg	milligram
min	minute
miRNA	micro RNA
ml	milliliter
mM	millimolar
MP	movement protein
mRNA	messenger RNA
MS	Murashige and Skoog media
NAA	1-Naphtalene acetic acid
NBT	Nitroblue tetrazolium chloride
ng	nanogram
NLS	Nucleus local signals
nm	nanometer
N protein	Nucleocapsid protein
nptII	Neomycin Phosphotransferase □
nt	nucleotide
OD	optical density
ORF	open reading frame
p19	suppressor of gene silencing from *Tombusviruses*
p21	suppressor of gene silencing from *Beet yellows virus*
p122 subunit	suppressor of gene silencing from *Tobacco mosaic virus*
P1-Hc-Pro	helper-component-protease from *Potyvirus*
PBS	phosphate-buffered saline buffer
PBS-T	PBS Tween
PCR	polymerase chain reaction
PDR	pathogen-derived resistance
PEG	polyethylene glycol
PLRV	*Potato leaf rolling virus*
PPV	*Plum pox virus*
PSV	*Peanut stunt virus*
PTGS	post-transcriptional gene silencing
PVP	Polyvinylpyrrolidone

RT-PCR	reverse transcriptase and polymerase chain reaction
PVY	*Potato virus* Y
QTL	quantitative trait loci
RdRP	RNA-dependent RNA polymerases
R gene	resistance gene
Rif	Rifampicin
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
rt	room temperature
RT	reverse transcriptase
35S	promoter from *Cauliflower mosaic virus*
2x35S	double 35S promoter from *Cauliflower mosaic virus*
SA	salicylic acid
SatRNA	satellite RNA
SbDV	*Soybean dwarf virus*
SDS	Sodiumdodecylsulfate
sgRNAs	subgenomic RNAs
siRNA	small interfering RNA
Spec	Spectinomycin· 2HCl
ST-LS1 IV2	intron 2 from the ST-LS1 gene of potato
Strep	Streptomycin sulfate
TAV	*Tomato aspermy virus*
T-DNA	transfer DNA
TE	Tris-EDTA
Tetra	Tetracycline hydrochloride
TMV	*Tobacco mosaic virus*
ToMV	*Tomato mosaic virus*
TSWV	*Tomato spotted wilt virus*
TuMV	*Turnip mosaic virus*
TYMV	*Turnip yellow mosaic virus*
TYLCV	*Tomato yellow leaf curl virus*
TRIS	Tris(hydroxymethyl) aminomethane
µ	micro (10^{-6})
U	unit
V	Volume
VIGS	virus induced gene silencing
VSR	viral suppressor of RNA silencing
v/v	volume/volume
WVRDC	World Vegetable Research and Development Center
w/v	weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

1 INTRODUCTION

Cucumber mosaic virus (CMV) is worldwide among the five most important plant viruses, infecting vegetable and ornamental species (Palukaitis *et al*., 1992; Palukaitis and Garcia-Arenal, 2003). The virus has a natural host range exceeding 1000 plant species, which belong to 85 plant families and is transmitted in a non-persistent manner by over 80 aphid species. CMV is also infecting chili or peppers (*Capsicum annuum*), which belongs to the *Solanaceae* family. Chili a good source of many essential nutrients and provide the basis for some medical, pharmacological and food processing applications. More than one billion people consume chili in one or another form on a daily basis. The Chili production has an economically impact in the income in local as well as export markets in Asia and in other parts of the world. Worldwide more as 23.7 million tons of chilies are produced on around 1,650,000 ha (FAOSTAT data, http://faostat.fao.org)

So far, no durable and stable commercial resistant varieties have been applied to breeding programs and are available for agriculture yet. However, biotechnology became a feasible and practical approach to generate genetically modified crops (GM) to cope with diverse CMV isolates and many attempts have been published about pathogen derived resistance in plants generated via biotechnology (Goldbach *et al.*, 2003; Palukaitis and Garcia-Arenal, 2003).

The CMV genome is of positive-sense, single-stranded and distributed on three RNA segments. The three genomic RNAs (gRNAs) were designated RNA 1, RNA 2 and RNA 3. In addition two subgenomic RNAs (sgRNAs) are transcribed, known as RNA 4 and RNA 4A, respectively. Each genome segment is encapsidated separately in an isometric particle (Lot and Kaper, 1976).

On RNA 1 is one open reading frame (ORF) which encodes protein 1a, functioning as part of the viral RNA-dependent RNA polymerase (RDRP). On RNA 2 two partially overlapping ORFs are located, encoding protein 2a, which is part of the RDRP (Hayes and Buck, 1990) and protein 2b, translated from the second ORF of RNA 2 via the subgenomic mRNA 4A. The two ORFs from RNA2 are overlapping partially with 242 nucleotides (nt) (Ding *et al*., 1994). The 2b protein is a multiple function protein and has been ascribed the following functions: host range determinant (Shi *et al*., 2002), determinant of pathogenicity and controlling symptom expression (Ding *et al*.,

1995, 1996; Du *et al*., 2007), suppressor of post-transcriptional gene silencing (PTGS) of the host plants (Brigneti *et al*., 1998; Lucy *et al*., 2000; Baulcombe, 2002; Guo & Ding, 2002; Qi *et al*., 2004) and is a determinant of long-distance movement (Ding *et al*, 1995a; Soards *et al*., 2002).

On RNA 3 the movement protein (MP) and coat protein (CP) are encoded on two ORFs, which are separated by a non-translated intergenic region. The MP is translated directly from 5' terminus of the RNA 3 and is solely responsible for long-distance movement (Canto *et al*., 1997; Li *et al*., 2001). The CP is translated from the subgenomic mRNA 4 transcribed downstream of the MP-ORF of RNA 3. The CP is responsible for the encapsidation of the viral RNAs and enables the vector transmission by aphids. The MP plus CP are essential for the short-distance cell-to-cell movement (Canto *et al*., 1997).

CMV is the type species of the Genus *Cucumovirus* which comprises two additional species, peanut stunt virus (PSV) and tomato aspermy virus (TAV). The genus is a member of the family *Bromoviridae*, which also contains the genera *Bromovirus*, *Alfamovirus*, *Ilarvirus* and *Oleovirus* (Hull, 2001). Based on serological relationship and sequence criteria, all reported CMV species can be divided into two serogroups, I and II, which can be differentiated by specific monoclonal antibodies (Roossinck *et al*., 1999). When looking at nucleic acid sequence data, serogroup I isolates are more heterogeneous than those of serogroup II, therefore serogroup I strains are further divided into subgroup IA and IB according to nucleotide differences of their CP and 5' non-translated region (Roossinck *et al*., 1999).

The development of detection technology, like enzyme-linked immunosorbent assay (ELISA), reverse transcription and polymerase chain reaction (RT-PCR), real time RT-PCR, RT-PCR restriction fragment length polymorphism (RT-PCR-RFLP), immuno-capture RT-PCR (IC-RT-PCR) and oligonucleotide-microarrays made instruments available worldwide to detect and differentiate CMV isolates (Palukaitis *et al*., 1992; Rizos *et al*., 1992; Boonham *et al*., 2003; Yu *et al*, 2005; Zhang *et al*., 2005). Thus many new CMV isolates were reported consecutively in the world. The isolates were grouped serologically in IA, IB and II (Roossinck, 2002)

Many studies have shown that strains of serogroup I are more virulent (Wahyuni *et al*., 1992; Zhang *et al*., 1994; Du *et al*., 2007) and differ in their host range from serogroup II strains

(Daniels & Campbell, 1992; Wahyuni *et al*., 1992). Recent detailed studies of CMV isolates from infected chili plants in Asia have revealed that all isolates belonged to subgroup IB (Zhang, 2005). Du *et al* (2007) described that four subgroup IB isolates, derived from China, showed different virulence on *Nicotiana* species, and may be due to differences in their 2b proteins.

Genetic exchange by recombination or by reassortment of genomic segments, has been shown to be the important process in CMV virus evolution, resulting in new phenotypic changes affecting host range and virulence (Roossinck, 2002; Palukaitis and Garcia-Arenal, 2003; Zhang, 2005; Du *et al*., 2007). A reassortment from subgroup IB and serogroup II isolates developed symptoms on *Nicotiana tabaccum* cv. Xanthi differed from the parents' isolates, which may be due to a segment of 1100 bp on CMV RNA2 that was exchanged (Zhang, 2005). In addition, it has been described that mutation and recombination as well as reassortment modify the replication rate of CMV isolates (Roossnick, 1991) and the transmission specificity by aphids (Ng and Perry, 1999). Based on the high genetic variability among CMV isolates, artificial and natural reassortants were obtained although at low frequency of recombination (Fraile *et al*., 1997; Zhang, 2005; Pierrugues *et al.,* 2007). It could not be ruled out if these observations were due to the fitness of the reassortants or the type of host plants used for selection. However, research with artificially made reassortants contributed a lot to assign specific phenotypes and functions to viral proteins. This was further improved by the development of full-length infectious cDNA clones for all segments of CMV-Fny by Rizzo & Palukaitis (1990). This was the break-through for experimental studies of the effects of biodiversity of CMV and correlation of genetic variation with functions.

The extreme variability of CMV makes it difficult to obtain durable virus resistant plants either generated by conventional breeding or by biotechnological means.

All microbial plant pathogens, viruses, bacteria and fungi, still contributed to significant losses in yields and reduced quality in the production of many vegetable and ornamental crops worldwide (Oerke *et al*. 1994). These pathogens can been controlled using different measures like crop rotation, other cultivation techniques, chemical plant protection, control of their vectors, pathogen-free seed or planting material and breeding for resistance (Hull, 2001; Goldbach *et al*.,

2003). Unfortunately, conventional measures failed especially in modern agroindustrial production with its monocultural production. This facilitates the rapid evolution of these pathogens in nature. Furthermore, other effects are of concern, like global production and shipment resulting in worldwide distribution of pathogens, new mass propagation by in vitro-methods, increasing of ecological farming connected with reduced application of chemicals.

Plants free of viruses and bacteria can be produced from meristem tissue for some crops, but this is difficult for recalcitrant plants like chili and ornamentals. Virus and bacterial diseases of plants are impossible to control like fungi, since no plant protection chemicals are available and the only means to combat them are healthy seed or planting material and of course resistant varieties.

Over the years, many resistance genes from wild species have been the main sources for resistance breeding programs (Stamova *&* Chetelat, 2000). Numerous publications describe different resistance genes (R gene) from germplasms that have been identified and used successfully to breed virus resistant varieties of crop plants contributing to save agricultural production (Saito *et al*., 1987; Meshi *et al*., 1988; Meshi *et al*., 1989; Weber *et al*., 1993, Santa Cruz & Baulcombe, 1993; Nicolas *et al*., 1997; Keller *et al*., 1998; Bendahamane *et al*., 2000; Mestre *et al*., 2000; Johansen *et al*., 2001). Unfortunately such types of R genes are not known to be available against CMV in the solanaceous family in which chili belongs to. Only resistance based on quantitative trait loci (QTL) has been reported (Caranta *et al*., 1997). Resistance breeding with a QTL-based genetic background is very complicated as compared with a single gene based resistance.

Although a promising resistant chili variety, breeding line VC246, is available at World Vegetable Research and Development Center (WVRDC, Taiwan, China), a screening with different CMV isolates from Asian countries revealed that already isolates of CMV exist that could overcome its resistance (Zhang, 2005). Therefore a biotechnological approach for the generation of CMV resistance in chili might be a solution for growers.

The concept of pathogen-derived resistance (PDR) by Sanford and Johnston (1985) was experimentally confirmed by the coat protein-mediated resistance against *Tobacco mosaic virus*

(TMV) in transgenic tobacco plants expressing constitutively the CP of TMV (Powell-Abel *et al*., 1986). Subsequently, expression of different viral sequences from many different plant viruses has extended this first proof of principle. It was only logically that the lack of natural resistance against CMV has led to a large number of attempts to achieve resistant plants by genetic engineering based on CP-mediated approaches (Cuozzo *et al*., 1988; Gonsalves *et al*., 1992, 1994; Yie *et al*., 1992; Nakajima *et al*., 1993; Okuno *et al*., 1993a, b; Rizos *et al*., 1996; Kaniewwski *et al*., 1999; Jacquemond *et al*., 2001; Chen *et al*., 2004; Srivastava & Raj, 2008) and replicase-mediated resistance (Anderson *et al*., 1992; Carr *et al*., 1994; Wintermantel *et al*., 1997; Wintermantel and Zaitlin, 2000). Also extensive studies to induce resistance against CMV with truncated CP or 2a protein expressed in transgenic plants have been reported (Wintermantel and Zaitlin, 2000). Only CMV resistance by transgenic MP has not been reported.

Recently, successful attempts have been described with CMV-based RNA 2 or RNA 3 constructs leading to post-transcriptional gene silencing (PTGS) against the donor CMV isolate (Kalantidis *et al*., 2002; Chen *et al*., 2004; Knierim, 2006). This is a newly described pathway common to many organisms described as PTGS in plants, RNA interference (RNAi) in animals and quelling in fungi (Ding, 2000; Voinnet, 2005; Ding and Voinnet, 2007).

In plants, PTGS is divided into two different pathways: small interfering RNAs (siRNAs) and microRNAs (miRNAs)-mediated gene silencing which are triggered in the cytoplasm. In this process, (I) double-stranded RNAs (dsRNAs)/precursors miRNAs are processed by RNase III-like enzymes (Dicer-like, DCL) (Hamilton and Baulcomb, 1999) into 21-26 nt siRNAs duplexes; (II) the resulting siRNAs duplexes were unwinded and then incorporated into an RNA-induced silencing complex (RISC); (III) the RISC-siRNA duplexes target homologous mRNAs with sequence-specific for degradation based on complementary base pairing; (IV) single-stranded siRNAs were also used as primers for RNA-depended RNA polymerase (RdRP) to generate more dsRNAs, which lead to generate more siRNAs; (V) recruitment of siRNAs makes gene silencing stably maintenance throughout the plant (Waterhouse *et al*., 2001; Mlotshwa *et al*., 2002).

Since it is the dsRNA that finally leads to virus induced gene silencing, currently the approach to generate transgenic resistant plants via the expression of dsRNA derived from inverted repeat constructs of viral sequences. The efficiency of this strategy has already been proven against several different viruses like: *Cucumber green mild mottle mosaic virus* (CGMMV, Shinichiro *et al*., 2007, Kamachi *et al*., 2007); *Tomato yellow leaf curl virus* (TYLCV, Fuentes *et al*., 2006; Zrachya *et al*., 2007); *Plum pox virus*(PPV, Di Nicola-Negri *et al*., 2005); *Potato virus* Y (PVY, Mitter *et al*., 2003; Missiou *et al*., 2004); *Barley yellow dwarf virus* (BYDV, Wang *et al*., 2000); *Capsicum chlorosis virus* (CaCV), *Tomato mosaic virus* (ToMV) (Knierim, 2006); *Tomato spotted wilt virus* (TSWV, Knierim, 2006; Bucher *et al*., 2006); *Soybean dwarf virus* (SbDV, Tougou *et al*., 2006) and others (Waterhouse *et al*., 1998; Smith *et al*., 2000; Helliwell and Waterhouse, 2003; Nomura *et al*., 2004; Hily *et al*., 2005; Riberio *et al*., 2007).

It is well known that viral proteins from plant viruses can interfere with the innate PTGS defense system to allow the establishment of infections. Examples of such silencing suppressors are: 2b protein of CMV, HC-Pro of *Potato virus* Y, the p19 of tombusviruses, the p21 of *Beet yellows virus*, AC4 of *African cassava mosaic virus* and p122 subunit of TMV (Llave *et al*., 2000; Mallory *et al*., 2001; Silhavy *et al*., 2002; Ye *et al*., 2003; Roth *et al*., 2004; Chapman *et al*., 2004; Chellappan *et al*., 2005; Shiboleth *et al*., 2007; Csorba *et al*., 2007). Extensive studies have revealed the detailed modes of the function for these suppressors. The CMV 2b protein interacts directly with Argonaute 1 protein (AGO 1), a component of the RNA-induced silencing complex (RISC) and attenuated its cleaving activity (Zhang *et al.*, 2006), which inhibits the production of silencing signals of small RNAs. As mentioned above, the viral suppressor 2b encoded by CMV_{CM95R} and CMV_{CM95} showed different abilities of binding small siRNA because they differed with one mutated aa (Goto *et al*., 2007). Furthermore, a recent report showed that 2b could suppress PTGS even at the single cell level (Qi *et al*., 2004). Biosafety of transgenic plants derived from protein-mediated and RNA-mediated resistance is of increasing social concern, particularly in Europe (Tepfer, 2002; Fuchs and Gonsalves, 2007). Current argumentations focus on: horizontal gene flow from transgenic plants to non-transgenic plants; generation of new pathogens in transgenic plants by recombination and reassortment leading to resistance breaking and new virus isolates (Feráandez-Cuartero *et al*., 1994); or expansion of host range (Friess *et al*., 1996,

1997); allergic proteins produced in transgenic plants that are dangerous to humans and animals and for vector transmitted viruses transcapsidation, leading to change in vector specificity (Chen and Francki, 1990). Furthermore, an interspecific recombination between CMV and TAV on transgenic plants has been demonstrated (Aaziz and Tepfer, 1999 a, b). These risks, however, are not present or reduced in PTGS-mediated resistant plants (Niu *et al*., 2006). In addition, inverted repeat constructs of CP gene (CPIR) encoded by CMV have been proven to induce high level of resistance in tobacco plants (Kalantidis *et al*., 2002; Chen *et al*., 2004; Knierim, 2006), but inverted repeat constructs of 2b gene and part of 2a gene encoded by CMV have shown to be more efficient in inducing resistance than that of CPIR in *N. benthamiana* plants (Chen *et al*., 2004). However, it could not be excluded that the observed resistance mechanism were both, protein- and RNA-mediated, because the expression of 2b protein and CP protein could not be ruled out (Chen *et al*., 2004).

Since the resistance efficiency cannot be ruled out from the published data due to different screening systems, due to different modified plant species and to the variability of the CMV isolates used for transformation and testing. General suggestion for the use of a specific CMV fragment for the generation of resistance in chili is not possible from the published data.

Based on aforementioned reason, the present work was to design several constructs for a comparative study of the efficiency of different constructs: (I) the start codons (ATG) from CP ($^{\triangle}$CP) and 2b ($^{\triangle}$2b) genes from CMV_{AN} were deleted; (II) three single constructs ($^{\triangle}$CP, $^{\triangle}$2a+$^{\triangle}$2b and $^{\triangle}$2a+2b) and two invert repeated constructs (2bIR and CPIR) were generated to target the region of CP gene and 2b gene encoded by CMV, respectively. All constructs were driven by cauliflower mosaic virus (CaMV) 35S promoter, and furthermore hold the same order between plant selective gene and inserts in T-DNA region in pLH6000 and pBIN19 binary vectors; (III) A comparative resistance testing was carried out on transgenic *N. benthamiana* and *N. tabaccum Samsun* NN plants, which were derived from a series of constructs in pLH6000 and pBIN19 binary vectors. It should provide the basic information to compare the resistance variation on different host plants when transferring the same constructs into target host plants; (IV) attempt to address the resistance variation raised from different binary vector; (V) in addition, the extensive

studies on resistance variation in transgenic *N. benthamiana* and *N. tabaccum Samsun* NN have been carried out by using a chimeric gene construct [GFP+2bIR] in both binary vectors; (VI) the resistant transgenic *N. benthamiana* plants derived from 2bIR and [GFP+2bIR] were challenged with different subgroup CMV isolates as described.

2 Material and Methods

2.1 Material

2.1.1 Plant material

Nicotiana benthamiana and *Nicotiana tabaccum Samsun* NN were used for plant transformation. *Nicotiana glutinosa* was used for virus maintainance. *Vigna unguiculata* and *Chenopodium quinoa* were used for infectivity testing of purified virus.

2.1.2 CMV isolates

Five CMV isolates were used in this study.

(I) CMV_{AN}, isolated from India in 2002 belong to subgroup IB. A 1100 bp region on the genome segment RNA2 including overlapping regions of 2a and 2b had been mapped for resistance-breaking on resistance chili line VC246.

(II) CMV_{P3613} from Taiwan

(III) CMV_{KS44} from Thailand and a reassortment of $CMV_{\square AN}$ are also belong to subgroup IB.

(IV) CMV_{RT52} belong to subgroup IA.

(V) CMV_{PV0420} belong to subgroup II.

All isolates are described in detail in Zhang (2005).

2.1.3 Chemicals

All chemicals and enzymes were purchased from the following companies:

Duchefa (Haarlem, Netherland)
MBI Fermentas (St.Leon-Rot, Germany)
Promega (Mannheim, Germany)
Merck (Darmstadt, Germany)
New England Biolab (Frankfurt am Main, Germany)
Roth (Karlsruhe, Germany)
Sigma (Munich, Germany)
Serva (Heidelberg, Germany)

All chemicals were of *p.a.* grade if not indicated otherwise. All enzymes were used according to manufacturer's specification. All solutions and reagents were prepared with water prepared by a Millipore Q Plus water plant, if not indicated otherwise.

2.1.4 Oligonucleotides (primers)

The primers for PCR or RT-PCR in this study were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The sequences of primers are shown in Table 1.

Table 1 The sequences of primers for PCR or RT-PCR

name	sequences (from 5' to 3')	annealing temperature
KpnI-GFP (forward)	GGTACC[1]AATAACCATAATGGGTAAAGGAGAAGA	56°
XhoI-GFP (reverse)	CTCGAGATTATTTGTATAGTTCAT	
5'-CP(forward)[2]	ATGGACAAATCTGRATCWMCC	59°
3'-CP(reverse)[2]	CTGGATGGACAACCCGTTC	
CMV- CP-NcoI(forward)	CTAGAGCCATGGTGGACAAATCTGGAT	59°
CMV-CP-BamHI(reverse)	GACGTCGGATCCCTGGATGGACAACCC	
P1353-CMVCP-F(forward)	TCGACTAGATAAGGTTCCCGCTCCGCTC	59°
P1353-CMV-CP-REV(reverse)	TACTTTCTCATGTCACCAATA	
5'-RNA2(forward)[2]	GATGAATTCYTGTTTGCTCAC	52°
3'-RNA2(reverse)[2]	GGATGGACAACCCGTTC	
Interval up	GCTCGCCTGTTGAAGTC	
2b-MS-FOR(forward)	GAAGAAAGTGGAATTGAACGAAGGCGC	
2b-MS-REV(reverse)	CGTTCAATTCCACTTTCTTCTTTCGCTGC	
CMV-2b-NcoI(forward)	CTAGAGCCATGGTGAATTCTTGTTTGC	59°
CMV-2b-BamHI(reverse)	GACGTCGGATCCGGATGGACAACCCGT	
35SPRO-FOR(forward)	CAACATGGTGGAGCACGACACTCTCGTC	59°
INTRON-REV(reverse)	CGCGCTCGCCTGCACATCAACAAATTTTG	
Intron_XbaI (forward)	CCCTCTAGATAAGTTTCTGC	55°
Intron_PstI (reverse)	TATACGATCTGCAGGCGCTCGCC	
2b_AN_SacI-NcoI(forward)	ATACAGAGCTCCATGGGCCGAGGCTGC	55°
2b_AN-XbaI(reverse)	GACAGTCTAGAGCAATACTGCC	
2bAN_PstI(forward)	AATACTGCAGACTCAGCCC	55°
2bAN_BamHI (reverse)	TACAGGATCCCAGGATCCGAGGCTG	
T3	ATTAACCCTCACTAAAG	55°
T7	AATACGACTCACTAT	
P1353-Tem-REV(reverse)	GCATGCCTGCAGGTCACTGGATTTTGGTT	
P1353-KpnI-SphI (forward)	GGCATGCGGTACCAAGCTTTCCCTATAG	58°
P1353-CaiI (reverse)	CCTGTTACCAGTGGCTGCTGCC	
Actin1-675	AGTTGCTGACTATACCATGC	56°
Actin2-676	GACAATGGAACTGGAATGGT	
NAD5 sense[3]	GATGCTTCTTGGGGCTTCTTGTT	56°
NAD5 antisense[3]	CTCCAGTCACCAACATTGGCATAA	
C1[4]	ATCATTTGTAGCGACT	60°
C2[4]	AGCTCAAACCTGCTTC	

[1]Underlined sequences contain restriction enzyme recognition sites.
[2] Zhang, 2005, [3]Menzel *et al*., 2002, [4]Sawada *et al*., 1995

2.1.5 Antibodies and antisera

Polyclonal antibody AS-0475 was purchased from DSMZ, it cannot differentiate between serogroups and was used for ELISA, tissue print immunoblots and westernblot assay.

2.1.6 Bacteria strains

Two different *E.coli* strains, NM522 (Pharmacia) and XL-1 Blue (Stratagene), were used for DNA cloning. Two different *Agrobacterium tumefaciens* strains, GV3101 and LBA4404 (Hoekema *et al*., 1983), were used for plant transformation and agroinfiltration.

2.1.7 Plasmids and Vectors

Plasmid pBluescript SK- (Stratagene) was used as a common vector and as T-vector preparation in this study. Plasmid pCKGFPS65C (Reichel *et al*., 1996) contained the GFP gene driven by the constitutive 2x35S promoter from cauliflower mosaic virus. Plasmid P1353dsCMVIR (pLX-CMV, Knierim, 2006) consisted of invert-repeated of CP gene from CMV-Pv0506 separated by intron ST-LS1 IV2 from potato, which was also under control of a constitutive 2x35S promoter. The pLH6000 (Accession No. AY234328) binary vector (DNA Cloning Service, Hamburg Germany) and pBIN19 (Accession No.U09365) binary vector contained the selection marker genes hpt and *nptII* under control of the constitutive 35S promoter, respectively (Bevan, 1984). Detailed characteristics are described in Table 2.

Table 2. Relative plasmids and vectors for all gene constructs

Plasmids / Vectors	Application in this study	Selection in *E.coli*	Selection in plants	promoter
pBluescript SK-	DNA sequencing; cloning	Amp†	-	T3†, T7†
pCKGFPS65C	Cloning	Amp†	-	T3†, T7†, 2x35S†
P1353dsCMVIR	Cloning	Amp†	-	T3†, T7†; 2x35S
pLH6000 binary vector	Cloning	Strep†, Spect†	Hygro†	35S†
pBIN19 binary vector	Cloning	Kan†	Kan†	35S†

† Amp: Ampicillin. Strep: streptomycin sulfate. Spect: spectinomycin. Kan: kanamycin monosulfate. Hygro: Hygromycin B. 35S: 35S promoter from Cauliflower Mosaic virus.

2.1.8 Media

All media for microorganisms were prepared according to Sambrook *et al.* (2001).

SOB-Medium (per liter)	20 g	Tryptone
	5 g	Yeast extract
pH 7,5	0.5 g	NaCl
	0.2 g	KCl
SOC-Medium	20 mM	Glucose
	20 mM	MgCl2
in SOB-Medium		
LB-Medium (per liter)	10 g	Tryptone
	5 g	Yeast extract
	10 g	NaCl
LB-Agar (per liter)	15 g	Micro-agar
in LB-Medium		
Kan-Agar (per liter)	50 mg	Kanamycin
in LB-Agar		

SS-Agar (per liter)	100 mg	Spectinomycin
	300 mg	Streptomycin
in LB-Agar		
AIX-Agar (per liter)	150 mg	Ampicillin
	47 mg	IPTG
	40 mg	X-Gal dissolved in 1 ml Dimethylformamid
in LB-Agar		
RKG-Agar (per liter)	100 mg	Rifampicin dissolved in 1 ml DMSO
	50mg	Kanamycin
only for GV3101 transformation	50mg	Gentamycin
RKGSS-Agar (per liter)	100 mg	Spectinomycin
	300 mg	Streptomycin
in RKG-Agar only for GV3101 transformation		
RS-Agar (per liter)	25mg	Rifampicin dissolved in 1 ml DMSO
	200mg	Streptomycin
in LB-Agar only for LBA4404 transformation		
RSK-Agar (per liter)	50mg	Kanamycin
in RS-Agar only for LBA4404 transformation		
YEP-Medium (per liter)	10 g	Tryptone
	10 g	Yeast extract
	5 g	NaCl
pH 7.4		

All media were autoclaved for 20 min at 121 °C. Glucose, $MgCl_2$, Ampicillin, IPTG, X-Gal, Rifampicin, Gentamycin, Spectinomycin, Streptomycin and Kanamycin were added after the media had reached a temperature of about 50 °C.

Plant transformation media were prepared as following:

Solid Basal MS medium (per liter)	4.705 g	Murashige & Skoog medium
	30.0 g	Sucrose
	8.0 g	Plant agar
pH adjusted to 5.7~5.9 with 1M KOH		
Liquid Basal MS medium (per liter)	4.705 g	Murashige & Skoog medium
	30.0 g	Sucrose
pH adjusted to 5.7~5.9 with 1M KOH		
T1 medium (per liter)	0.2 mg	NAA
	2.0 mg	BAP
	500 mg	Cefotaxime Sodium
in Solid Basal MS medium	20 mg	Hygromycin B[1] or 50mg Kanamycin[2]
[1] for pLH6000 and [2] for pBIN19		
T0 medium (per liter)	500 mg	Cefotaxime Sodium
	20 mg	Hygromycin B[1] or 50mg Kanamycin[2]
in Solid Basal MS medium		
[1] for pLH6000 and [2] for pBIN19		

All plant transformation media were autoclaved for 20 min at 121 °C. Hygromycin B, Cefotaxime Sodium, Kanamycin as well as plant hormone NAA and BAP were added after the media temperature had cooled to about 50 °C.

2.2 Methods

2.2.1 Plant cultivation

Vigna unguiculata, Chenopodium quinoa, *Nicotiana glutinosa* L., *N. benthamiana* and *Nicotiana tabaccum Samsun* NN were grown in the greenhouse at 25±1°C with a photoperiod of 16 hr light/ 8hr dark.

2.2.2 Purification of CMV particles

CMV particles were purified following the procedure originally described by Lot *et al.* (1972).

Extraction buffer	500 mM	Sodium citrate
pH 6.5	5 mM	EDTA
	0.5% (v/v)	Thioglycolic acid
Virus buffer	5 mM	Boric acid
pH 9.0	0.5 mM	EDTA
	2% (v/v)	Triton X-100

Infected leaves were homogenized in an equal volume of extraction buffer (w/v) and filtered through cheesecloth. The filtrate was clarified by addition of one volume pre-cooled chloroform at 4°C and centrifuged (3000 rpm, 4°C, 20 min, rotor HB-4, Sorvall). Virus was precipitated from the aqueous phase with 10% (w/v) PEG (MW 6000) under gentle stirring for 30-45 min at 0-4 °C and sedimented (11000 rpm, 4°C, 15 min, rotor SS34, Sorvall). The pellets were resuspended in 50 ml virus buffer, stirred for 30 min at 4°C and centrifuged (14500 rpm, 4°C, 15 min, rotor SS34). The supernatant was centrifuged at high speed (33800 rpm, 4°C, 3 hours, rotor Ti 70, Beckman) and the virus pellet was dissolved in H_2O. After a final low speed centrifugation (14500 rpm, 4°C, 15 min, rotor SS34, Sorvall) the virus concentration was estimated by photometry (Pharmacia Biotechlology, England).

2.2.3 Plant inoculation with virus particles or viral RNA

Particle inoculation buffer (PIB)	0.02 M	NaH_2PO4 / Na_2HPO_4
pH 7.0	2 % (w/v)	PVP 15
	0.2 % (w/v)	Na_2SO_3
	10 mM	DIECA
PBS	137.0 mM	NaCl
pH 7.4	2.7 mM	KCl
	8.1 mM	Na_2HPO_4
	1.5 mM	KH_2PO_4
RNA inoculation buffer (RIB)	5 % (w/v)	Carborundum (600 mesh)
in PBS		
autoclaved		

For plant inoculation with infected plant material, infected plant material was placed in a precooled mortar and homogenized in PIB at 1:50 (w/v) for dried and 1:10 (w/v) for fresh leaf material, respectively. This suspension was rubbed with glove-covered fingers onto plant leaves which had previously been dusted with carborundum (600 mesh). Following inoculation, the inoculated leaves were rinsed with tap water and the plants were incubated in the greenhouse.

For plant inoculation with virus particles (2.2.2), virus particles were diluted to 75 µg/ml in PBS including 5% (w/v) carborundum (600 mesh) and rubbed with glove-covered fingers onto plant leaves (10µl per leaf, two leaves per plant).
For plant inoculation with viral RNA derived from purified viral particles (2.2.2, 2.2.5), the inoculum was diluted to 0.5µg/µl in RIB and rubbed with glove-covered fingers onto plant leaves (10µl inoculum per leaf).

2.2.4 Silica-based plant RNA extraction

Total plant RNA was extracted according to Rott and Jelkmann (2001).

Grinding buffer (GB)	4.0 M	Guanadine thiocyanate
	0.2 M	Na-Acetate, pH 5.2
	25 mM	EDTA
	1.0 M	K-Acetate
	2.5 % (w/v)	PVP 40
store at 4°C		
Washing buffer (WB)	10 mM	Tris-HCl, pH 7.5
	0.5 mM	EDTA
	5 mM	NaCl
	50 % (v/v)	Ethanol
store at 4°C		
NaI	0.15 M	Na_2SO_3
	6 M	NaI
store at 4°C in a dark bottle		

Preparation of silica: 60 g silica particles (Sigma S5631) were suspended in 500 ml water. The suspended particles were allowed to settle for 24 h. The upper 470 ml of the supernatant were sucked off, and the procedure was repeated by resuspending the sediment in 500 ml water and settling for another 5 h. The upper 440 ml of the supernatant was removed and the remaining 60 ml slurry was adjusted to pH 2.0 with HCl, autoclaved and stored at 4° C in 200µl aliquots.

Leaf tissue (300 mg) was homogenized in a plastic bag (Bioreba, Reinach, Switzerland) with 3 ml GB. 500µl of the homogenate was incubated with 100µl 10% (w/v) N-Laurylsarcosyl at 70°C for 10 min with intermittent shaking and subsequently placed on ice for 5 min. After centrifugation (13000 rpm, 10 min, rt, Sigma) 300µl of the supernatant was mixed with 150µl Ethanol, 300µl NaI and 25µl of resuspended silica. The mixture was incubated at rt for 10 min with intermittent shaking before the silica was sedimented (6000 rpm, 1 min, rt, Sigma). After discarding the supernatant, the silica pellet was resuspended in 500µl WB and sedimented again. The washing step was repeated once, and the pellet was finally allowed to dry for several minutes at room

temperature before it was resuspended in 150µl water. Following incubation at 70° C for 4 min, the silica was sedimented (13000 rpm, 3 min, rt, Sigma), the supernatant was transferred to a fresh reaction tube for storage at -20° C.

2.2.5 Phenol extraction for DNA/RNA purification

An equal volume of phenol (TE-saturated, pH 7.5-8.0, Roth, warmed up to rt) was added to an aqueous DNA/RNA sample, vigorously mixed and centrifuged for phase separation (13000 rpm, 5 min, rt, Sigma). The upper aqueous phase was transferred to a new reaction tube and extracted twice with an equal volume of Chloroform/Isoamylalcohol (24:1, v/v), following centrifugation to allow phase separation (13000 rpm, 5 min, rt, Sigma). The DNA/RNA from the upper aqueous layer was concentrated by Ethanol precipitation (2.2.6).

2.2.6 Ethanol precipitation of DNA/RNA

TE buffer	10 mM	Tris-HCl
pH 8.0	1 mM	EDTA

The DNA/RNA solution was mixed with 2.5 - 3 volumes cold Ethanol, one tenth volume of 3M sodium acetate (pH 4.8) and incubated at -80°C for at least 30 min or at -20°C overnight. The precipitated NA was recovered by centrifugation at 15300 rpm for 30 min at 4°C (rotor 12145, Sigma). The supernatant was discarded and the nucleic acid pellet was washed with cold 70% (v/v) Ethanol for 5 min on ice with intermittent shaking. After centrifugation at 15300 rpm for 15 min (rotor 12145, Sigma), the supernatant was discarded and the washing step repeated once. The DNA/RNA pellet was dried in a Speed-Vac concentrator (Savant Instruments Inc., USA) and resuspended in water or TE buffer.

2.2.7 Determination of DNA and RNA concentration

DNA or RNA concentration was determined by photometry. The DNA or RNA sample was diluted 1:100 with H_2O. The absorbance of solution was measured at 260 and 280nm, using water as blank. An OD_{260nm} of 1 corresponds to a DNA or RNA concentration of 50 (DNA) or 40 (RNA) µg/ml. The ratio 260/280 provides an indication of the purity of the DNA/RNA. The value should be between 2.0 and 2.2.

2.2.8 Agarose-gel electrophoresis

TAE-buffer	0.04 M	Tris-Acetate
pH 8.3	1 mM	EDTA
Loading buffer	50 % (v/v)	Glycerol
	0.1 % (w/v)	Bromphenol Blue

DNA was separated using 0.8 to 2.0 % (w/v) agarose gels in TAE buffer containing Ethidium bromide (0.2µg/ml) with 4 V/cm and examined by UV light at 254 nm, using a transilluminator (Kappa-Messtechnik, Germany). Gels were photographed to record results.

2.2.9 Reverse transcription (RT) and Polymerase chain reaction (PCR)

2.2.9.1 cDNA synthesis (RT)

5 × M-MuLV buffer	250 mM	Tris-HCl
	250 mM	KCl
	20 mM	$MgCl_2$
pH 8.3 at 25 °C	50 mM	DTT

Total-RNA (0.05-0.5µg, 2.2.4) was denatured at 95° C for 5 min in presence of 10µM reverse primer (2.1.4) in a total volume of 10µl and subsequently cooled down on ice to avoid renaturation. cDNA was synthesized by incubation at 42°C for 45-60 min with the following reagents:

5.0 µl	5 × MMLV-buffer
2.0 µl	dNTPs (2mM)
0.1 µl	M-MuLV Reverse transcriptase (200 U/µl)
7.9 µl	H_2O

2.2.9.2 Polymerase chain reaction (PCR)

10 × PCR buffer	200 mM	Tris-HCl
	100 mM	KCl
	100 mM	$(NH4)_2SO_4$
pH 8.75 at 25 C°	1% (v/v)	Triton X-100

The standard PCR reaction was assembled according to the following conditions unless indicated otherwise:

1-2 µl	cDNA or any other template
2.5 µl	10 × PCR buffer
1.5 µl	$MgCl_2$ (25 mM)
2.0 µl	dNTPs (2 mM)
2.0 µl	primer forward (10 µM)
2.0 µl	primer reverse (10 µM)
0.5 µl	Taq DNA-Polymerase (5 U/µl)
Add to 25 µl	H_2O

The PCR was carried out in a Personal Cycler 48 (Biometra, Germany) with the appropriate primers (2.1.4, Table 2) using the following conditions:

1	initial denaturation	5 min	95 °C
2	denaturation	30 sec	95 °C
3	annealing	45 sec	50-65 °C
4	elongation	1min	72 °C
5	final elongation	5 min	72 °C

The steps 2-4 were repeated 29 times.

An aliquot of the PCR products was analyzed by agarose gel electrophoresis (2.2.8).

2.2.9.3 Single-tube PCR

10 × IC-PCR buffer	100 mM	Tris-HCl
	500 mM	KCl
	15 mM	$MgCl_2$
pH 8.8 at 25 °C	1% (v/v)	Triton X-100

RT-PCR was carried out in one reaction tube with:

1-1.5µl	total RNA (2.2.6)
2.5 µl	10 × IC-PCR buffer
3.0 µl	1.7 % (v/v) Triton X-100
2.0 µl	dNTPs (2 mM)
1.0 µl	primer forward (10 µM)
1.0 µl	primer reverse (10 µM)
0.2 µl	Taq DNA-Polymerase (5 U/µl)
0.5 µl	M-MuLV Reverse transcriptase (200 U/µl)
Add to 25 µl	H_2O

Synthesis was carried out according to the conditions indicated below:

1	reverse transcription	45 min	42 °C
2	initial denaturation	2 min	92 °C
3	denaturation	30 sec	92 °C
4	annealing	45 sec	59 °C
5	elongation	1 min	72 °C
6	final elongation	5 min	72 °C

Steps 3 to 5 were repeated 39 times.

An aliquot of the PCR product was analyzed by agarose gel electrophoresis (2.2.8).

2.2.10 Clone screening by PCR

After transformation (2.2.17) bacteria were plated on selection agar (2.1.8) to obtain single colonies. A single colony was picked up by a toothpick and dissolved in 50µl water and 1µl was used as a template for the PCR reaction (2.2.9.2) to verify the insert.

2.2.11 PCR-based site-directed mutagenesis

To introduce point mutations into the cloned CP and 2b gene derived from CMV-AN (2.1.2), a PCR-based, site-directed mutagenesis was carried out according to the procedure of Higuchi *et al*. (1988).

The first PCR was carried out with primer pairs A and C/Reverse or primer pairs B and C/Forward, respectively (Fig 1). Primer C/Reverse and primer C/Forward are two complementary primers, which contained a single nucleic acid mutation. The two PCR fragments were purified (2.2.14) by agarose gel electrophoresis (2.2.8) to remove the template and primers from the first PCR. In a final PCR the mutated fragment was amplified from a mixture (1:1) of both purified fragments using primer pairs A and B (Fig. 1).

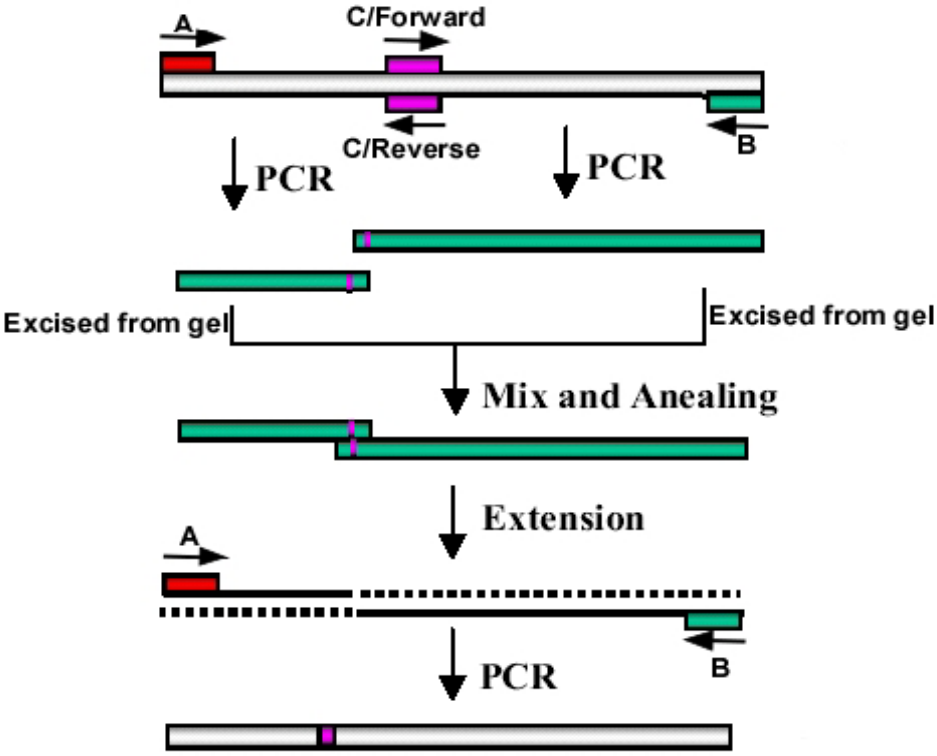

Fig. 1: Scheme of PCR-based site-directed mutagenesis (Zhang, 2005).
The first PCR was carried out with primer pairs A and C/Reverse or primer pairs B and C/Forward, respectively. Primer C/Reverse and primer C/Forward are two complementary primer, which contained a single nucleic acid mutation. The two PCR fragments were purified with agarose gel electrophoresis and excised from the gel. In a final PCR the mutated fragment was amplified from a mixture (1:1) from both fragments using primer pairs A and B.

2.2.12 PCR product purification

PCR products were purified using a E.Z.N.A. Cycle-pure Kit (PEQLAB Biotechnologie GMBH, Erlangen, Germany). DNA was eluted from the column with 40µl H_2O.

2.2.13 Restriction enzyme digestion

Purified DNA fragment or plasmid was digested with the appropriate restriction enzyme according to manufacturer's recommendation.

2.2.14 DNA fragment purification from agarose gel

Digested DNA fragments (2.2.13) or PCR products were separated on agarose gel (2.2.8). The fragment of interest was excised from the gel with a razor blade under UV light and purified with the E.Z.N.A. Gel Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). DNA was eluted from the column with 40µl H_2O.

2.2.15 Preparation of cloning vector

2.2.15.1 Preparation of T-vector

The T-vector for cloning of PCR products (2.2.12) was prepared according to the procedure of Marchuk *et al*. (1991). The pBluescript SK- plasmid (2.1.7) was linearized with EcoRV (2.2.13), followed by phenol/chloroform extraction (2.2.5) and ethanol precipitation (2.2.6). The linearized vector (5µg) was resuspended in 8µl water. A T-overhang was added to the termini by the Terminal deoxynucleotidyl Transferase (TdT), using the following reagents:

15.0µl	linearized pBluescript SK- (5 µg)
8.0 µl	5×Tailing buffer (MBI Fermentas)
1.0 µl	1mM ddTTP
3.0 µl	5mM $CoCl_2$
5.0 µl	TdT (25U/µl, MBI Fermentas)

This was followed by incubation for 1h at 37°C.
The vector was extracted with phenol/chloroform (2.2.5), followed by ethanol precipitation (2.2.6) and diluted in water to a 20 ng/µl concentration for the ligation reaction (2.2.16).

2.2.15.2 Preparation of binary vector or cloning vector

Plasmid of binary vector or cloning vector (2.1.2) was digested with appropriate enzymes (2.2.13), followed by phenol/chloroform extraction (2.2.5) and ethanol precipitation (2.2.6). The linearized vector was resuspended in water to 50ng/µl (2.2.7) and stored at -20°C until use.

2.2.15.3 Preparation of dephosphorylated binary vector or cloning vector

5~10µg linearized plasmid of binary vectors or cloning vector (2.2.13) was directly precipitated by Ethanol (2.2.6), subsequently resuspended in 20µl water. The dephosphorylation was carried out the following procedures:

20.0 µl	5~10µg linearized plasmid
3.0 µl	1u/µl calf intestinal alkaline phosphatase(CIAP) (MBI Fermentas)
3.0 µl	10x CIAP buffer (MBI Fermentas)
Add to 30 µl	H_2O

This was followed by incubation 30 min at 37°C, additional 3µl CIAP and 3µl CIAP buffer was added and incubation another 30min at 37°C. CIAP was inactivated by incubation 15 min at 65°C, before the vectors were extracted with phenol/chloroform (2.2.5), followed by ethanol precipitation (2.2.6) and diluted in water to a 50 ng/µl concentration for the ligation reaction (2.2.16).

2.2.15.4 Fill-in recessed 3'-termini of binary vector or cloning vector

5µg linearized plasmid of binary vectors or cloning vector (2.2.13) was directly precipitated by ethanol (2.2.6), then fill-in was performed as following:

5.0µg	linearized plasmid of binary vector or cloning vector
2.0µl	dNTPs (2mM)
5 U	Klenow fragment (exo-) 5 U/µl (MBI, Fermentas)
3.0µl	Klenow fragment buffer (MBI, Fermentas)
25.0µl	H_2O

The mixture was incubated 20 min at 37°C. 1µl EDTA (0.5M) was added and the mixture was incubated for 15 min at 65°C to inactivate the enzyme. Phenol/chloroform extraction and ethanol precipitation were performed as described in 2.2.5 and 2.2.6.

2.2.16 Ligation

A 1:2 to 1:4 ratio of vector: DNA fragment (2.2.13 to 2.2.15) was generally used for the ligation reaction.

1~2 µl	pBluescript SK--T vector (2.2.15.1) or other linearized vector (50 ng/µl)
2-4 µl	purified DNA fragment (～150 ng)
1 µl	10×Ligation buffer (MBI Fermentas)
1-2 µl	T4-DNA Ligase (1U/ µl, MBI Fermentas)
add to 15µl	H_2O

The mixture was incubated overnight at 15°C or 2hr at 22°C.

For self-ligation or blunt end ligation 1µl of 50% (w/v) PEG (MW 4000) solution was added.

2.2.17 Preparation of competent cells and chemical transformation

Preparation of competent cells and chemical transformation with *E.coli* were prepared according to Sambrook *et al.* (2001).

TFB	45 mM	$MnCl_2.4H_2O$
	100mM	RbCl
	10 mM	$CaCl_2.2H_2O$
	3mM	$Co(NH_3)_6Cl_3$
	10mM	MES-KOH pH 6.3
	TFB solution was sterilized by filtration (0.22 µm, Millipore) and stored at 4 °C	

DND	1 M	Dithiothreitol
10 ml	90 % (v/v)	DMSO
	10 mM	K-acetate pH 7.5

make 280 µl aliquots and store at -20°C

All steps were performed on ice with chilled solutions.

Several single colonies of *E. coli* (NM522) were picked by toothpicks resuspended in 30 ml SOB-medium (2.1.8) and propagated to a density of OD_{550nm} = 0.48~0.52. The bacteria were sedimented by centrifugation (2000 rpm, 10 min, 4°C, rotor12139, Sigma) and the sediment was incubated on ice for 10 min. The bacteria were resuspended very gently in 10 ml of ice-cold TFB buffer and left on ice for 10 min. After the cells were sedimented again, the pellets were

resuspended immediately by swirling in 4 ml of ice-cold TFB buffer and incubated another 10 min on ice. Thereafter 140µl DND buffer was added to resuspend the cells with very gently swirling and incubated on ice 15min; this step was repeated once.

For transformation, 200µl cells were added to the ligation product (2.2.16). The mixture was incubated on ice for 30 min. The cells were shocked in a 42°C circulating water bath for exactly 90 sec and cooled down on ice for 1-2 min. SOC medium (2.1.8) (600µl, pre-warmed to 37°C) was added, and the reaction tubes were incubated with shaking (about 220 rpm) for approximately 1h at 37°C. Cells were plated at different volumes on LB-plates (2.1.8) with appropriate antibiotics and incubated at 37°C for 12-14 h.

2.2.18 Preparation of competent cells of *agrobacterium tumefaciens* strain GV3101 and transformation

All steps are done as described in the protocol from http://www.dna-cloning-service.de with small modification;

$CaCl_2$ buffer	20mM $CaCl_2$ Sterilized by filtration and stored at 4°C

Agrobacterium strain GV3101 was grown under shaking in the present of Rifampicin 100mg/l, Gentamycin 50mg/l and Kanamycin 50mg/l in 20ml YEP medium (2.1.8) overnight at 28°C, 250rpm. 2ml of the overnight culture was added to 50ml YEP medium (2.1.8) and incubated at 28°C until an OD_{600nm} of 0.5 to 1.0, and the culture was chilled on ice for 15 min. The cells were sedimented at 3000g for 5 min at 4°C (Rotor SS34, Sorvall) and resuspended in 1ml of ice-cold 20mM $CaCl_2$ with gently swirling. Aliquots of 100µl were taken and frozen in liquid N_2 and stored at -80°C.

For transformation, 0.5µg plasmid derived from pLH6000 binary vector was added to the frozen competent *agrobacterium* cells and cells were incubated for 5 min at 37°C. 200µl pre-warmed SOC medium (2.1.8) was added after incubation the cells on ice for 30 min. Aliquots of the cells were spread to LB plates containing appropriate antibiotics. Colonies will appear after 2 days of incubation at 28°C.

2.2.19 Preparation of electrocompetent cells of *agrobacterium tumefaciens* strain LBA4404 and transformation

Hepes buffer	1mM	Hepes pH adjusted to 7.0 with 1M KOH before autoclaving stored at 4°C
storage buffer	10%(v/v)	glycerol store at 4°C after autoclaving

Agrobacterium tumefaciens strain LBA4404 was grown in 20ml YEP medium (2.1.8) with

Rifampicin 100mg/l and streptomycin 200mg/l overnight at 28°C, 250rpm. 10ml of the overnight culture was added to 500ml YEP medium (2.1.8) and incubated at 28°C until an OD_{600nm} of 0.5 to 0.8. The culture was chilled on ice for 20 min and the cell suspension was sedimented at 4000g for 15 min at 4°C (Rotor SLA-1500, Sorvall). The suspension was discharged and the cells was resuspended in 100ml ice-cold 1mM Hepes solution and centrifuged again for 4000g for 15 min at 4°C. The pellets were washed in 100ml ice-cold 0.1mM Hepes and centrifuged again. The supernatant was discharged and the pellet was resuspended in ice-cold water or 10% (v/v) ice-cold glycerol, aliquoted to 40µl and snap frozen in liquid N_2 and stored at -80°C.

For transformation, frozen cells were thawed on ice before 3µl plasmid derived from pBIN19 binary vector (2.1.7) was added and incubated on ice for 1 min. The mixture was transferred to a ice-cooled electroporation cuvette (1mm, EQIUBIO, UK) and the dry cuvette was placed into a electroporation chamber (Eppendorf, Germany) and a voltage of 1500 Volt was applied. Immediately, 1 ml ice cold SOC medium (2.1.8) was added with gentle up and down pipetting and transferred to a reaction tube for incubation at 3~4h at 28°C with moderate shaking. Aliquots of the transformed cells were spread on LB plates containing appropriate antibiotics, colonies will appear after 2 days of incubation at 28°C.

2.2.20 Plasmid isolation from bacteria

A bacterial culture grown overnight in the presence of the appropriate antibiotic was used for the purification of plasmid DNA. For further processing or manipulation of plasmid DNA, the miniprep method (2.2.20.1) was chosen (Birnboim and Doly, 1979); for sequencing, the plasmid was isolated with the E.Z.N.A. Plasmid Miniprep Kit I (2.2.20.2).

2.2.20.1 Minipreps

Solution A	25 mM	Tris-HCl
pH 8.0	50 mM	Glucose
	10 mM	EDTA
Solution B	200 mM	NaOH
	1 % (w/v)	SDS
Solution C	3 M	Na-Acetate
pH 4.8		
Solution D	50 mM	Tris-HCl
pH 8.3	100 mM	Na-Acetat

Cells from an overnight culture (1.5 ml) were sedimented (12000 rpm, 5 min, rt), resuspended in 200µl solution A, and incubated for 5 min at rt before 400µl of solution B and 300µl solution C were added. After incubation for at least 15 min on ice, the suspension was centrifuged for 10 min at 12000 rpm at rt. The supernatant was transferred to a fresh tube and centrifuged again.

Plasmids were precipitated from the supernatant by adding 600µl isopropanol and sedimented (14000 rpm, 10 min, rt). The pellet was dissolved in 200µl solution D, precipated again with 400µl 100 % (v/v) ethanol and sedimented (14000 rpm, 10 min, rt). The pellet was dried in a Speed-Vac concentrator (Savant Instruments Inc., USA) and resuspended in 50µl H_2O containing RNase A (1mg/ml).

2.2.20.2 Plasmid preparation for sequencing

For sequencing, plasmids were isolated with the E.Z.N.A. Plasmid Miniprep Kit I (PEQLAB Biotechnologie GMBH, Erlangen, Germany). After elution from the columns with water, 2 - 2.5µg plasmid was precipitated (2.2.6) and dried on a heating block at 50-55°C before being sent to MWG Biotech (Ebersberg, Germany) for sequencing. The plasmid was sequenced from both directions.

2.2.21 *Agrobacterium*-mediated plant transformation

2.2.21.1 Preparation of sterilized plant seedlings

Seeds of *N.benthamiana* and *N.tabaccum Samsun* NN were sterilized for 2 min in 70%(v/v) Ethanol, and soaked into 7%(v/v) NaOCl solution for about 3~5 min. Seeds were washed three times with sterilized water, for 3 min each. Dry sterilized seeds were placed on MS medium (2.1.8) for germination at 25℃ with a photoperiod of 16hr light/8hr dark for about 2 weeks.

2.2.21.2 Preparation of plant explants

Leaf discs in size of 0.5 cmx0.5 cm without the margins and midrib, were excised from the full expanded leaves of 30-45 days seedlings (2.2.21.1).

2.2.21.3 Preparation of recombinant *Agrobacterium tumefaciens*

Recombinant *Agrobacterium tumefaciens* for plant transformation (2.2.18 and 2.2.19), which were cultured in YEP medium (2.1.8) with appropriate antibiotic (strain GV3101: 100mg/l Rifampicin+50mg/l Kanaymcin+50mg/l Gentamycin+100mg/l Spectinomycin+300mg/l Streptomycin; strain LBA4404: 100mg/l Rifampicin+200mg/l streptomycin+50mg/l Kanaymcin+0.4g/l $MgSO_4$) for 48hr at 28°C, and shaking at 250 rpm. The *Agrobacterium* was collected by centrifugation 8000rpm 2 min at rt (Rotor12139, Sigma), The supernatant was discharged and the pellets washed twice with MS medium (2.1.8) (Rotor 12139, Sigma), In the last step the pellet was diluted with MS medium at a working concentration of OD_{550nm} of 0.6 to 1.0.

2.2.21.4 Co-culture of explants and *agrobacterium*

20~30ml recombinant *Agrobacteria tumefaciens* suspension (2.2.21.2) supplement with 100μM Acetosyringone (final concentration) were placed into plastic petri dish and leave for 2min. Leaf discs (2.2.21.1) were placed and submerged into the *Agrobacterium tumefaciens* suspension for 10 min with intermittent gently shaking. Superfluous suspensions from these explants were removed with sterilized whatman paper. Leaf discs were transferred to petri dishes with MS solid medium (2.1.8) and sealed by parafilm for incubation at 25±1°C for 48hr in the dark.

After two days, the leaf discs were transferred to T1 medium (2.1.8) with appropriate antibiotic (plasmids derived from pLH6000 binary vector are supplemented with Hygromycin B 20mg/l, Cefotaxime Sodium 500mg/l; plasmids derived from pBIN19 binary vector are supplemented with Kanaymycin 50mg/l, Cefotaxime Sodium 500mg/l) and plant growth regulators auxin 0.2mg/l NAA and cytocin 2mg/l Kinetin.. Around 10 leaf discs were cultured in each petri dish sealed by parafilm.

2.2.21.5 Selection and Regeneration

Explants were incubated at 25±1°C with a photoperiod of 16hr light/8hr dark. The medium were changed every two weeks to keep continuous selection pressures and to prevent false positive transformants to grow. Callus formation on solid T1 medium (2.1.8) was about 2~3 weeks, while adventitious shoots formed from the callus on the T1 medium (2.1.8) need another 2 weeks. Shoots with a size of 1-1.5 cm were cut with a sterile knife and rooted on solid T0 medium with appropriate antibiotic for 2~3 weeks.

2.2.21.6 Transplant of plantlets

The young plantlets were acclimated for 3~4 days with opening covers before they transplanted to pots with matrix in the greenhouse. The roots of those plantlets were washed gently with tap water to remove plant agar completely. The plantlets are transferred into pots with sterilized matrix and covered with transparent plastic covers to keep higher moisture. Everyday they were acclimated to the grow condition of the greenhouse for few hours without plastic covers. As normal, growth condition of plantlets was a photoperiod of 16hr light/8hr dark at 25±1°C used.

2.2.22 DNA extraction from transgenic plants

Extraction buffer	100 mM	Tris-HC
pH 8.0	700 mM	NaCl
	50 mM	EDTA

50~100mg leaves from transgenic plants were grinded in liquid N_2. 1330μl of prewarmed (65°C) extraction buffer was added and the mixture vortexed for 1 min. Subsequently the mixture was incubated 15min at 65°C with intermittent shaking. Cooled down for 1 min at rt before 650μl

chloroform/isoamyalcohol (24:1 v/v) was added with intensive shaking for 5 min at rt. The mixture was centrifuged (14000rpm, 2 min at rt, Sigma) and the supernatant transferred into new reaction tubes. 10µl RNase A (10mg/ml) was added and incubated for 10 min at 37°C before 700µl isopropanol was added. The mixture was mixed before centrifugation (14000rpm, 10 min at rt, Sigma) and the pellets were washed with 500µl 70% (v/v) cold Ethanol and sedimented again (14000rpm, 5 min at 4 °C), this step was repeated once. Dried pellets were dissolved in 50µl water and stored at -20°C. The concentration of DNA was determined as described in 2.2.7.

2.2.23 RNA extraction from transgenic plants

RNA extraction was preformed following the procedures of Spiegel et al (1993):

Extraction Buffer	200mM	Tris-HCl, pH 8.5
	1% (w/v)	Lithium Dodecylsulfonate
	375 mM	LiCl
	1% (w/v)	SDS
	1%(v/v)	Triton X-100
	10mM	EDTA pH8.0

100~300mg plant tissues derived from transgenic plants were grinded in liquid N_2 and the fine powder was transferred to reaction tubes containing 900µl extraction buffer and vortexed for 30 seconds. Subsequently, 500µl of then suspension was mixed with 750µl 5M KOAC (pH 6.5) in a fresh reaction tubes and incubated on ice for 10 min. The supernatant was clarified by centrifugation (14000rpm for 10 min at 4°C, Rotor 12145, Sigma), and 600µl of the supernatant was transferred to a new reaction tubes and mixed with 500µl isopropanol. The mixture was incubated on ice for 5 min before centrifugation 14000rpm for 20 min at 4°C (Rotor 12145, Sigma). The supernatant was decanted and the pellets washed with 1ml 70% (V/V) ethanol, this step was repeated again. Dried pellets were resuspended in 50µl water and stored at -20°C. The concentration of RNA was determined as described in 2.2.7.

2.2.24 PCR screening of transgenic plants

PCR screening on transgenic plants was performed with approx. 150 ng total DNA as template (2.2.21) For the RT-PCR approx. 80-120ng total RNA was used (2.2.22).

2.2.25 Double Antibody Sandwich (DAS) Enzyme-Linked Immunosorbent Assay (ELISA)

Coating buffer	15 mM	Na_2CO_3
	35 mM	$NaHCO_3$
pH 9,6		
PBS-T	PBS (2.2.3)	with 0.05 % (v/v) Tween-20
Sample buffer	2 % (w/v)	PVP 15 in PBS-T
Conjugate buffer	0.2 % (w/v)	Ovalbumin in Sample Buffer

Substrate buffer	9.7 % (v/v)	Diethanolamine
pH 9.8 with HCl		

For DAS-ELISA microtiter plates (Greiner, Germany) were coated with 100µl IgG (AS-0475, 1 mg/ml diluted 1:1000 in coating buffer) at 37°C for 4 h. The plates were washed three times with PBS-T before 100 µl leafsap after homogenization 1:30 in sample buffer was added. After incubation overnight at 4° C, plates were washed again with PBS-T and incubated at 37°C for 4 h with 100 µl anti-CMV IgG conjugated with alkaline phosphatase (1 mg/ml diluted 1:1000 in conjugate buffer). After a final washing step, p-Nitrophenylphosphate (1 mg/ml dissolved in substrate buffer) was added to the wells and colour development was measured photometrically (Dynatech MR5000, USA) at 405 nm and 630 nm as reference, against buffer as blank.

2.2.26 Tissue print immunoblots assay

Tissue print immunoblots were performed as described by Lin *et al.* (1990) with some modifications. The leaves of transgenic plants were detached, rolled into a tight roll and cut with a new razor blade for each sample. The newly cut surface was pressed onto nitrocellulose membrane (Protran®, Schleicher & Schuell GmbH, Dassel, Germany) to obtain tissue-print. The membranes were dried, and incubated in blocking buffer (5% (w/v) fat-free milk powder in PBS-T, 2.2.25) for 30 min at rt. The membrane was then incubated with anti-CMV polyclonal antibody from rabbit (AS-0475, diluted 1:500 in PBS-T with 1% (w/v) fat-free milk powder) for 1-2 h. Unbound antibody was removed by washing with PBS-T. This was followed by incubation for 1-2 h with goat-anti-rabbit alkaline phosphatase-conjugated IgG (Sigma A-3687, 1:30,000 in PBS-T with 1 % (w/v) fat-free milk powder). The membrane was washed with PBS-T and detection of virus was accomplished by Fast-red staining substrate (2.2.27).

2.2.27 Chemical detection (Fast-red)

Fast Red-buffer	0.2 M	Tris-HCl
pH 8.0	2 mM	$MgCl_2$
Fast Red staining solution 1	6 mg	Naphtol AS-MX-Phosphat-disodium salt
in 15 ml		H_2O
Fast Red staining solution 2	90 mg	Fast Red TR salt
in 15 ml		Fast Red buffer

Fast Red staining solution 1 and 2 were mixed immediately before staining. The membrane was developed at rt or overnight at 4° C.

2.2.28 Transient gene expression by agroinfiltration on tobacco plants

MES buffer	100mM	MES pH adjusted to 5.7 with 1M KOH, sterilization by filtration stored at 4 °C
Acetosyringone solution	2mM	3,5-dimethoxy-4-hydroxy-acetophenone

		Sterilization by filtration and stored at 4 °C
$MgCl_2$ solution	2M	$MgCl_2$ Sterilization by filtration and stored at 4 °C

Transient gene expression was used to check the gene constructs. As a positive control agrobacterium with a GFP gene construct expressing GFP (2.2.18 and 2.2.19) was used.

Recombinant *agrobacterium* were grown in 10ml YEP medium (2.1.8) overnight at 28°C with appropriate antibiotics. 50µl of the overnight culture was added to fresh YEP medium (10ml) supplement with 10mM MES buffer (final concentration), 150µM acetosyringone (final concentration) and appropriate antibiotics, which were incubated overnight at 28°C again. The cells were collected by centrifugation (3000g, 10min at 4°C) and resuspended to a final concentration of OD_{600nm} of 1.0 in a solution containing 10mM $MgCl_2$, 10mM MES and 150µM acetosyringone. The mixture was incubated 3h at room temperature before agroinfiltration.

Six-leaf-stage *N. Benthamiana* (2.1.1) was used for agroinfiltration. The mixture was delivered on the back side of the leaf by pressing the syringe directly the leaf. Each leaf was treated twice in two different locations. Each gene construct was applied in three plants. For mock infiltration buffer only was used as control. These plants were grown at 25±1°C with a photoperiod of 16hr light/8hr dark. After 24hr, fluorescence of GFP protein was observed and photographed by a LAS 3000 camera (Fujifilm, Japan).

2.2.29 Sequences analysis and alignments

Sequence analysis and alignments were done with the program DNAMAN (Version 5.2.2) with default parameters. Secondary RNA-folding of the inverted repeat constructs CPIR and 2bIR was done by web program Mfold (http://www.bioinfo.rpi.edu, Zuker, 2003) with default parameters. All maps of gene constructs in the Appendix were drawn by the program Gene construction Kit (Version 2.5).

3 RESULTS

3.1 Gene constructs in pLH6000 binary vector

3.1.1 Preparation of the pLH6000

Since chili plants revealed a natural resistance against the antibiotic kanamycin, all gene constructs were introduced into the binary vector pLH6000 (2.1.4) containing the plant selection marker gene hygromycin phosphotransferase (hpt) conferring resistance against hygromycin B. For cloning the inserts GFP (3.1.2), ΔCP (3.1.3) and Δ2a+2b (3.1.4), pLH6000 was digested with SpeI and KpnI (2.2.13 and 2.2.15.2), purified by phenol extraction (2.2.5 and 2.2.6) and named [pLH6000-SpeI/KpnI]. For cloning the inserts Δ2a+Δ2b (3.1.5) and 2bIR (3.1.7), pLH6000 was digested with HindIII (2.2.13 and 2.2.15.2), dephosphorylated (2.2.15.3), purified by phenol extraction (2.2.5 and 2.2.6) and named [pLH6000-HindIII]. For cloning the inserts CPIR (3.1.6), pLH6000 was digested with SalI and KpnI (2.2.13 and 2.2.15.2), purified by phenol extraction (2.2.5 and 2.2.6) and named [pLH6000-SalI/KpnI].

3.1.2 Construction of [pLH6000-GFP] in which GFP is translatable

For further cloning the BamHI recognition site of the MCS of pBluescriptSK- (2.2.15.1) has to be removed, pBluescriptSK- was digested with BamHI (2.2.13) and overhangs were filled in (2.1.7 and 2.2.15.4). The resulting plasmid was named [SK-(-BamHI)].

A fragment of 1867 bp, containing the GFP-gene was driven by a double 35S promoter and with a Nos terminator [2x35S/GFP/Nos], was released by HindIII digestion (2.2.13) from the plasmid pCKGFPS65C (2.1.7), and isolated by preparative gel electrophoresis (2.2.8 and 2.2.14). The fragment was ligated into the HindIII linearized [SK-(-BamHI)] vector (2.2.13), which had been dephosphorylated (2.2.15.3) and transformed into *E.coli* NM522 (2.2.17). White colonies were screened by PCR (2.2.10) with primers T3 and T7 (2.1.4). The correct orientation (see Fig. 2) of recombinants (SK-(-BamHI)-[2x35S/GFP/Nos]) was identified by PCR (2.2.10) with primers T3 and GFP-XhoI (2.1.4). In a correct orientation the SpeI site from SK-(-BamHI) is located upstream of the 35S promoter (see Fig. 2). Sequencing confirmed that no mutation had been

generated during construction (2.2.29).

The cassette of (-BamHI)-[2x35S/GFP/Nos] was isolated (2.2.5 and 2.2.6) from clone [SK-(-BamHI)-[2x35S/GFP/Nos] by SpeI and KpnI digestion (2.2.13) and cloned into [pLH6000-SpeI/KpnI] (3.1.1). The resulting plasmid was named [pLH6000-GFP] (Fig 5. a).

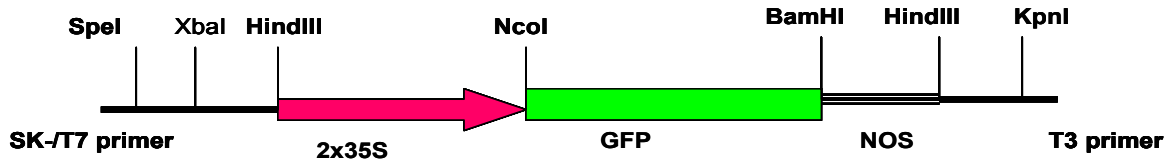

Figure 2 Part of the map of SK-(-BamHI)-[2x35S/GFP/Nos]
SK-: pBluescript SK-; T3 primer and T7 primer;
2x35S: double 35S promoter; GFP: green fluorescence protein; Nos: Nos terminator;
Positions of restriction enzymes HindIII, NcoI, BamHI, XbaI, PstI, SpeI and KpnI are shown.

3.1.3 Construction of pLH6000-ΔCP in which CP is not translatable

The CP gene (Fig. 4, a) was modified into a non-translatable construct called ΔCP by removing the start codon by deleting adenine of the ATG (~~A~~TG) by RT-PCR using the primer CMV-CP-NcoI. Additionally, for cloning purposes the restriction sites NcoI and BamHI were generated.

The RNA of CMV_{AN} infected leaf material (2.1.1) was extracted (2.2.4) and cDNA was synthesized (2.2.9.1) with primer 3'-CP (2.1.4). The cDNA was amplified by PCR (2.2.9) with primers CMV-CP-NcoI and CMV-CP-BamHI (2.1.4) and subcloned into a T-vector (2.2.15.1). The resulting construct was named [SK-ΔCP] .Sequencing confirmed that the start codon had been deleted successfully (2.2.29 and Fig.3 B) Eleven single nucleotide exchanges were found when compared with original CMV_{AN} (Appendix 7.2.1). Although the ORF of the CP had been deleted, three other ORFs are found with sizes of 4.1 to 8.5 KDa. The proteins translated from these ORFs showed no homologies with the CP from CMV_{AN}.

To join the ΔCP fragment with the 2x35S promoter and NOS terminator, the GFP gene from the clone [SK-(-BamHI)-[2x35S/GFP/Nos] (3.1.2) was removed by NcoI/BamHI digestion (2.2.13) and the remaining vector, containing 2x35 S promoter and NOS terminator, was excised and purified (2.2.14) from a preparative agarose gel (2.2.8).

The [SK-ΔCP] was digested by NcoI and BamHI (2.2.13) and the ΔCP fragment was isolated (2.2.14), before it was ligated into the NcoI/BamHI linearized plasmid [SK-(BamHI)-[2x35S/GFP/Nos] (3.1.2). Positive colonies were checked with PCR (2.2.10) using primers CMV-CP-NcoI and CMV-CP-BamHI (2.1.4) and the resulting clone was named [SK-(BamHI)-[2x35S/ΔCP/Nos]. From this clone the [2x35S/ΔCP/Nos] cassette was isolated SpeI/KpnI (2.2.13 and 2.2.14) and ligated into [pLH6000-SpeI/KpnI] (3.1.1). After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) using primers CMV-CP-NcoI and CMV-CP-BamHI (2.1.4) and digested with HindIII (2.2.13). The resulting plasmid was named [pLH6000-ΔCP] (Fig.5, d).

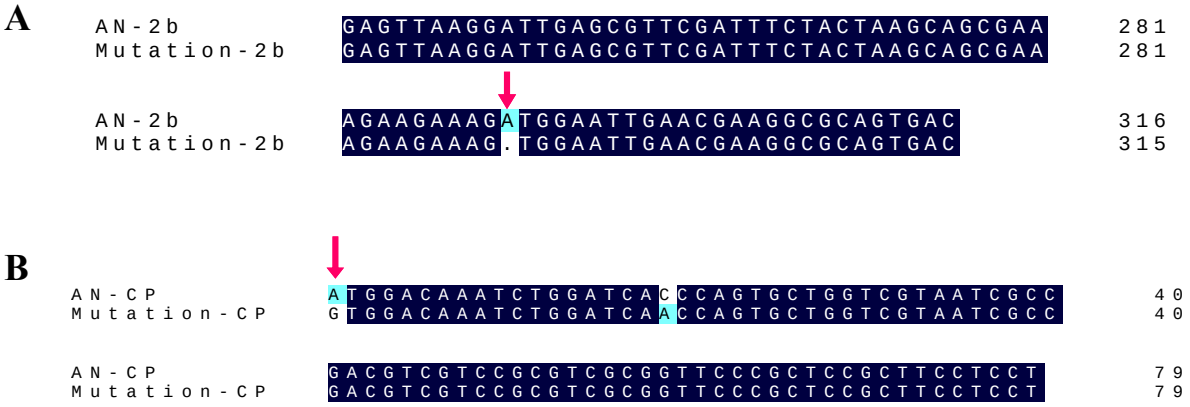

Fig.3 Alignment the partial of AN-2b andΔ2b (Mutation-2b), AN-CP and ΔCP (Mutation-CP), respectively. (**A**) Alignment of AN-2b and Δ2b, red arrow indicates mutation of start codon. (**B**) Alignment of AN-CP and ΔCP, red arrow indicates mutation of start codon. (AN-CP: Accession No.AJ810260 in EMBL)

3.1.4 Construction of pLH6000-Δ2a+2b in which 2b is translatable

A 735 bp fragment containing 641bp of the 3' part of 2a and the complete 336 bp of the 2b gene from CMV_{AN} (2.1.2), with a 242 bp overlap of 2a and 2b, located between nucleotide position 2130 and 2864 on the CMV_{AN} RNA 2 (Fig.4), was amplified by RT-PCR (2.2.9.1 and 2.2.9.2) with primers 5'-RNA2 and 3'-RNA2 (2.1.4), subcloned into a T-vector (2.2.15.1) and named [SK-Δ2a+2b].

Two new restriction sites for further subcloning, NcoI and BamHI, were introduced by PCR (2.2.9.2) with the primers CMV-2b-NcoI and CMV-2b-BamHI (2.1.4) using plasmid [SK-2b] as template. The use of the primers CMV-2b-NcoI and CMV-2b-BamHI for RT-PCR failed due to

their high annealing temperatures, however using the [SK-Δ2a+2b] as a template for PCR was successful. Sequence analysis revealed no mutation in the Δ2a+2b fragment (2.2.29 and Appendix 7.2.4) and confirmed the translatability of the 2b gene.

The [SK-Δ2a+2b] PCR fragment was digested with NcoI and BamHI (2.2.13) and purified (2.2.14), then it was ligated into the NcoI/BamHI linearized plasmid [SK-(BamHI)-[2x35S/GFP/Nos] (3.1.2) and transformed into *E. coli* NM522 (2.2.17). Positive colonies were checked with PCR (2.2.9.2) using primers 5'-RNA2 and 3'-RNA2 (2.1.4) and named [SK-(BamHI)-[2x35S/2b/Nos]. The cassette [–(-BamHI)-[2x35S/Δ2a+2b/Nos] was isolated by SpeI/KpnI (2.2.13, 2.2.14) and ligated into the linearized [pLH6000-SpeI/KpnI]. After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) using primers 5'-RNA2 and 3'-RNA2 (2.1.4), the recombinant plasmids were digested with NcoI and BamHI. The resulting plasmid was named [pLH6000- Δ2a+2b] (Fig.5, b).

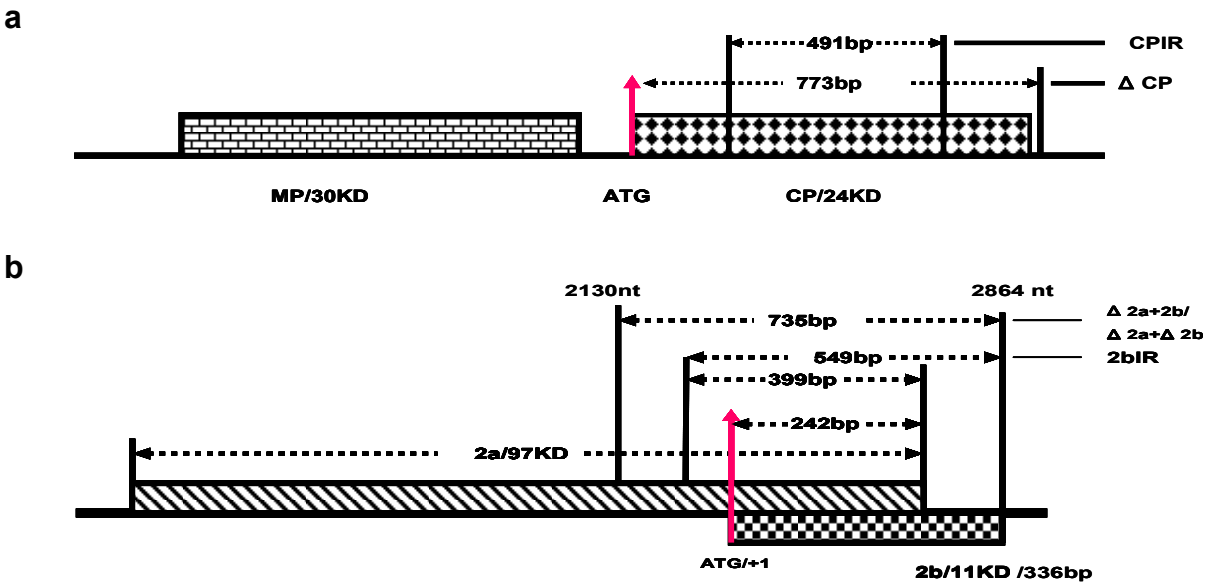

Figure 4 Genome organizations of CMV RNA2 and RNA3.

a: structure of CMV RNA3, which encodes movement protein (MP/30KD) and coat protein (CP/24KD). A 491 bp fragment from the middle of CP gene from CMV-PV0506 was used for the CPIR (3.1.6) construct; a 773 bp fragment without A of start codon (~~A~~TG) of CP gene (ΔCP) from CMV_{AN} was used to construct ΔCP, red arrow indicates the position of start codon (ATG).

b: structure of CMV RNA2, which encodes 2a protein (2a//97KD) and 2b protein (2b/11KD). A 735 bp fragment containing 400bp of the 3' part of 2a gene and 336 bp of 2b gene was used to construct Δ2a+2b and Δ2a+Δ2b. A 549 bp fragment containing 336 bp of 2b gene and 399 bp of 3' part of 2a gene was used to construct 2bIR. A 242 bp fragment is an overlapping region of 2a gene and 2b gene; red arrow indicates the start codon of 2b gene and the position of start codon mutated by site-directed mutagenesis.

3.1.5 Construction of pLH6000- Δ2a+Δ2b in which 2b is not translatable

The 2b gene was modified into a non-translatable construct called [Δ2a+Δ2b] by removing the start codon by deleting the A. Using the plasmid [SK-2b] (3.1.4) as a template, the start codon of 2b gene was removed by site-directed mutagenesis (2.2.11) with the two primer pairs 2b-MS-FOR/CMV-2b-BamHI and CMV-2b-NcoI/2b-MS-REV (2.1.4) to generate the Δ2a+Δ2b fragment. The Δ2a+Δ2b fragment was reamplified (2.2.9.2) by primers CMV-2b-NcoI and CMV-2b-BamHI (2.1.4) with the Δ2a+Δ2b fragment as template and cloned into a T-vector (2.2.15.1), and transformed into *E.coli* NM522 (2.2.17). Positive colonies were verified by PCR (2.2.10) with primers 5'-RNA2 and 3'-RNA2 (2.1.4), the recombinant plasmids were digested by NcoI and BamHI and named [SK-Δ2a+Δ2b]. Sequence analysis confirmed that the start codon was deleted successfully (Fig.3 A). Nine additional single nucleotide exchanges were found after an alignment with the sequence of RNA2 of CMV_{AN} (2.2.29 and Appendix 7.2.2). The fragment Δ2a+Δ2b from plasmid [SK-Δ2a+Δ2b] was excised from gel (2.2.14) after digestion with NcoI and BamHI (2.2.13), and then subcloned into the NcoI/BamHI linearized plasmid [SK-(-BamHI)-[2x35S/GFP/Nos] (3.1.2). Positive colonies were screened by PCR (2.2.10) with primers 5'-RNA2 and 3'-RNA2 (2.1.4), the recombinant plasmid was digested by NcoI and BamHI (2.2.13) and named SK-(-BamHI)-[2x35S/Δ2a+Δ2b/Nos]. The fragment of [(-BamHI)-[2x35S/Δ2a+Δ2b/Nos] from recombinants SK-(-BamHI)-[2x35S/Δ2a+Δ2b/Nos] was digested by HindIII and cloned into the dephosphorylated [pLH6000-HindIII] (3.1.1). The resulting clone was named [pLH6000-Δ2a+Δ2b] (Fig.5, c).

3.1.6 Construction of CP with an inverted repeat [pLH6000-CPIR]

The plasmid [p1353dsCMVIR] (2.1.7) contains two arms of sense stranded CP and antisense stranded CP forming an inverted repeat of the CP gene (CP/IR/Nos) from CMV_{PV0506} (2.1.7), separated by the intron ST-LS1 IV2 derived from potato (2.1.7). The transcription is driven by the 2x35S promoter (2.1.7, Fig.2 b). The plasmid [p1353dsCMVIR] served as starting material for the construct of the [pLH6000-CPIR]. For introduction of a KpnI site in the clone [p1353dsCMVIR], a 1000 bp fragment located downstream of the terminator in the plasmid [p1353dsCMVIR] was amplified by PCR (2.2.9.2) with primers p1353-KpnI-SphI and p1353-CalI

(2.1.4 and 2.2.14) and subcloned into the T-vector (SK-ΔKpnI, 2.2.15.1). The 1000 bp cassette from [SK-ΔKpnI] was excised from gel (2.2.8) after digestion by SphI and CalI (2.2.13 and 2.2.14) and purified (2.2.14). The plasmid [p1353dsCMVIR] was digested by SphI and CalI (2.2.13), the 3500 bp vector fragment was excised from gel (2.2.8) and purified (2.2.14). Then the vector was ligated with the SphI and CalI isolated cassette of [SK-ΔKpnI] and transformed into *E. coli* NM522 (2.2.17). Positive colonies with introduced KpnI recognition site were verified by PCR (2.2.10) with primers p1353-KpnI-SphI and p1353-CalI (2.1.4) and digestion with KpnI and CalI (2.2.13). The resulting clone was named [p1353ΔKpnI]. The fragment CP/IR/Nos from plasmid [p1353ΔKpnI] containing sense CP, antisense CP, intron and Nos terminator but not the 2x35 S promoter, was first generated by digestion with SalI and KpnI (2.2.13), and excised from gel (2.2.8) and purified (2.2.14). The fragment CP/IR/Nos was ligated (2.2.15.1) with [pLH6000-SalI/KpnI] (2.2.15.2, 3.1.1) and transformed into *E. coli* NM522 (2.2.17). The positive colonies were verified by PCR (2.2.10) with primers P1353-CMVCP-F and P1353-CMVCP-REV (2.1.4). The recombinant plasmid was further verified by digestion with SalI and KpnI (2.2.13) and named [pLH6000- CP/IR/Nos].

To introduce the 2x35S promoter, the fragment containing the 2x35S promoter was excised and isolated (2.2.8 and 2.2.14) after digestion the plasmid [p1353ΔKpnI] with EcoRI and SalI (2.2.13), before it was ligated into the EcoRI/SalI linearized pBluescript SK- (2.1.7). After transformation into *E. coli* NM522 (2.2.17), positive colonies were verified by PCR (2.2.10) with primers 35SPRO-FOR and T3 (2.1.4). Recombinant plasmids were digested by EcoRI and SalI (2.2.13) and named [SK-2x35S]. In this [SK-2x35S] the required SpeI site for further cloning is located in the MCS upstream of the 2x35S promoter insert. The 2x35S promoter from plasmid [SK-2x35S] was excised from a gel (2.2.8, 2.2.14) after digestion by SpeI and SalI (2.2.13), ligated with SpeI/SalI linearized [pLH6000-CP/IR/Nos] (2.2.13) and transformed into *E.coli* NM522 (2.2.17). Positive colonies were verified by PCR (2.2.10) with primers 35SPRO-FOR and INTRON-REV (2.1.4) and by digestion with SpeI and SalI (2.2.13). The resulting clone was named [pLH6000-CPIR] (Fig.5, e).

3.1.7 Construction of 2b with an inverted repeat [pLH6000-2bIR]

For construction of the [pLH6000-2bIR], all functional elements were generated separately while introducing restriction sites and subcloned consecutively.

First the intron (3.1.6) was cloned into the T-vector (2.2.15.1) and named [SK-Intron]. Using plasmid [Sk-Δ2a+Δ2b] (3.1.6) as template, the ORF sense 2b and antisense 2b were cloned into the T-vector (2.2.15.1), named [SK-sense2b] and [SK-anti2b], respectively. Antisense 2b from [SK-anti2b] was cloned into the [Sk-Intron], this plasmid was named [SK-Intron-anti2b]; sense 2b from [SK-sense2b] was cloned into the [Sk-Intron-anti2b], and the combination with both fragments together was named [SK-ds2bIR]. while the 2x35S promoter and Nos terminator from plasmid [SK-(-BamHI)-[2x35S/Δ2b/Nos] (3.1.6) were assembled into the SK-ds2bIR, the new recombined plasmid was named [SK-(-BamHI)-[2x35S/2bIR/Nos]. Finally, the cassette of [2x35S/2bIR/Nos] was cloned into dephosphorylated (2.2.15.3) [pLH6000-HindIII] (3.1.1) binary vector.

The 198 bp intron from plasmid [p1353dsCMVIR] (2.1.7) was amplified (2.2.9.2) by primers Intron_PstI and Intron_XbaI (2.1.4), and then subcloned into the T-vector [SK-Intron].

Antisense and sense strand of the 2b gene, a fragment of 549 bp (containing 335 bp from 2b gene and 455bp from 3' part of 2a gene but with a 242 bp overlapping region) from position 2253 nt to 2802 nt of CMV-AN RNA 2 (2.1.2 and Fig.4), were amplified with primers 2bAN_PstI and 2bAN_BamHI_XhoI for antisense, 2b_AN_SacI_NcoI and 2b_AN_XbaI for sense using plasmid [SK-Δ2a+Δ2b] (3.1.5) as template (2.1.4). The two fragments were subcloned into T-vectors ([SK-anti2b] and [SK-sense2b], respectively). The anti2b fragment from plasmid [SK-anti2b] was generated by PstI and XhoI digestion, isolated (2.2.14) and ligated (2.2.15.1) with a PstI and XhoI linearized (2.2.13) SK-Intron. After transformation into *E. coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers 2bAN_PstI and 2bAN_BamHI_XhoI (2.1.4). The recombinant plasmids were digested with PstI and XhoI and were named [SK-Intron-anti2b]. The sense2b fragment was isolated from plasmid [SK-sense2b] by XbaI digestion (2.2.13), and ligated (2.2.15.1) with XbaI linearized (2.2.13) and dephosphorylated (2.2.15.3) [SK-Intron-anti2b] vector. After transformation into *E.coli* NM522 (2.2.17) positive colonies were verified with PCR

(2.2.10) using primers 2b_AN_SacI_NcoI and 2b_AN_XbaI (2.1.4).

The orientation of the recombinant was identified by BamHI digestion (2.2.13) and named [SK-ds2bIR]. Sequencing confirmed that no base was exchanged in the new construct (2.2.29).

The DNA fragments of 2x35S promoter and Nos terminator from plasmid [SK-(-BamHI)-[2x35S/Δ2b/Nos] (3.1.2) were digested by SpeI and NcoI (35S promoter) as well as BamHI and KpnI (Nos terminator) (2.2.13, 2.2.14), respectively. The 2x35S promoter fragment was excised from a gel (2.2.14) and then ligated with the SpeI/NcoI linearized plasmid [SK-ds2bIR]. After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers 35SPRO-FOR and INTRON-REV (2.1.4), the recombinant plasmids were digested by SpeI/NcoI and named [SK-2x35S/2bIR]. The Nos terminator fragment was excised from a gel (2.2.14), and then ligated with BamHI/KpnI linearized plasmid [SK-2x35S/ds2bIR]. After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers 2bAN_PstI and T3 (2.1.4), the recombinant plasmids were digested by BamHI/KpnI and named [SK-2x35S/2bIR/Nos]. Then the cassette [2x35S/2bIR/Nos] from plasmid [SK-2x35S/2bIR/Nos] was digested by HindIII (2.2.13), ligated with HindIII linearized and dephosphorylated (2.2.15.3) [pLH6000-HindIII] (3.1.1). After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers 35SPRO-FOR and INTRON-REV (2.1.4), the recombinant plasmids were digested by HindIII and SpeI/NcoI, respectively. It was named [pLH6000-2bIR] (Fig.5, f).

3.1.8 Chimeric gene construct of [pLH6000-GFP+2bIR]

To join 2bIR (3.1.7) with GFP (3.1.2), a fragment of 2bIR from plasmids of [SK-2bIR] (3.1.7) was digested with BamHI (2.2.13), isolated (2.2.8) and purified (2.2.14). This cassette was ligated (2.2.15.1) with a BamHI linearized (2.2.13) and dephosphorylated (2.2.15.3) [SK-(-BamHI)-[2x35S/GFP/Nos] vector (3.1.2). After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers KpnI-GFP and XhoI-GFP, 2bAN_PstI and T3 (2.1.4), respectively. The correct orientation of the recombinant plasmid, GFP:sense2b:intron:antisense2b, was determined by NcoI digestion (2.2.13). The resulting recombinants were named [Sk-(-BamHI)-[2x35S/GFP+2bIR/Nos]. The fragment [GFP+2bIR]

from plasmid [Sk-(-BamHI)-[2x35S/GFP+2bIR/Nos] was digested by SpeI and KpnI, isolated (2.2.8) and purified (2.2.14) and was ligated (2.2.15.1) with [pLH6000-SpeI/KpnI] (3.1.1). After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers KpnI-GFP and XhoI-GFP, 2bAN_PstI and T3 (2.1.4), respectively. The recombinant plasmids were further verified by SpeI and KpnI as well as BamHI and KpnI digestion (2.2.13), respectively. The new recombinant was named pLH6000-[GFP+2bIR] (Fig 5. g). In this construct GFP is translatable.

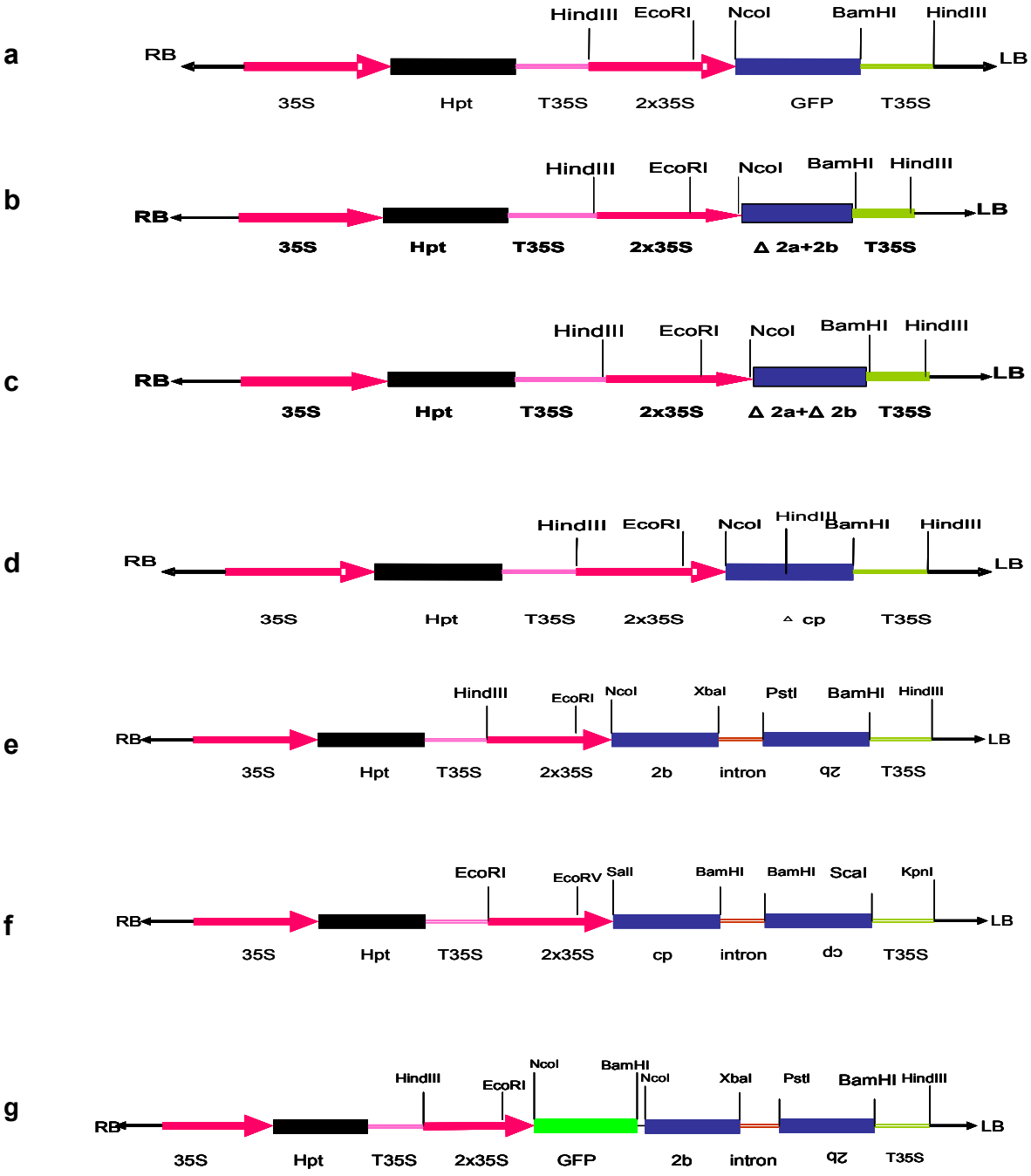

Figure.5 Schematic organizations of T-DNA region of each gene construct in pLH6000 binary vector.

a: pLH6000-GFP; b: pLH6000-Δ2a+2b; c: pLH6000-Δ2a+Δ2b; d: pLH6000-ΔCP;
e: pLH6000-2bIR; f: pLH6000-CPIR; g: pLH6000-GFP+2bIR. RB: right border;
LB: left border; Hpt; hygromycin phosphotransferase gene;
35S: promoter from cauliflower mosaic virus (CaMV 35S); 2x35S: double 35S promoter;
intron: intron ST-LS1 IV2 from potato; T35S: 35S terminator;
Positions of restriction enzymes HindIII, EcoRI, NcoI, BamHI, XbaI, PstI, SalI, ScaI and KpnI are indicated.

The arrangement of all construct in pBIN19 binary vector corresponds to the pLH6000 with the exception of plant selective marker gene.

3.2 Gene constructs in pBIN19 binary vector

3.2.1 Preparation of pBIN19

To compare the possibility of resistance variation in transgenic plants due to a binary vector different from pLH6000, all gene constructs mentioned in chapters (3.1.1 to 3.1.8) were introduced also into pBIN19 binary vector. For cloning the inserts GFP (3.2.2) and ΔCP (3.2.2), pBIN19 was digested with XbaI and KpnI (2.2.13 and 2.2.15.2), purified by phenol extraction (2.2.5 and 2.2.6) and named [pBIN19-XbaI/KpnI]. For cloning the inserts Δ2a+2b (3.2.3), Δ2a+Δ2b (3.2.3) and 2bIR (3.2.4) pBIN19 was digested with HindIII (2.2.13 and 2.2.15.2) and dephosphorylated (2.2.15.3), purified (2.2.5 and 2.2.6) and named [pBIN19-HindIII]. For cloning the inserts CPIR (3.2.4) pBIN19 was digested with EcoRI and KpnI (2.2.13 and 2.2.15.2), purified (2.2.5 and 2.2.6) and named [pBIN19-EcoRI/KpnI].

3.2.2 Construction of [pBIN19-GFP] in which GFP is translatable and [pBIN19-ΔCP] in which CP is untranslatable

The cassette [2x35S/GFP/Nos] was obtained from plasmid [SK-(-BamHI)-[2x35S/GFP/Nos] (3.1.2) by digestion with SpeI and KpnI (2.2.13), isolated (2.2.14) and ligated (2.2.15.1) with [pBIN19-XbaI/KpnI] (3.2.1), XbaI and SpeI creates compatible cohesive ends. After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers KpnI-GFP and XhoI-GFP (2.1.4), the recombinant plasmids were further verified by digestion with KpnI and XbaI, NcoI and BamHI (2.2.13). The resulting recombinant was designated [pBIN19-GFP]. An identical strategy was used for the ΔCP (3.1.3) construction, using plasmid [SK-(-BamHI)-[2x35S/ΔCP/Nos] (3.1.3) as a source of the cassette [2x35S/ΔCP/Nos], the resulting clone was designated [pBIN19-ΔCP].

3.2.3 Construction of [pBIN19-Δ2a+2b] in which 2b is translatable and [pBIN19-Δ2a+Δ2b] in which 2b is untranslatable

The cassette [2x35S/▵2a+2b/Nos] from plasmid [SK-(-BamHI)-[2x35S/▵2a+2b/Nos] (3.1.4) was obtained by digestion with HindIII (2.2.13), isolated (2.2.14) and ligated (2.2.15.1) with [pBIN19-HindIII] (3.2.1). After transformation into *E. coli* NM522 (2.2.17), positive colonies were

screened by PCR (2.2.10) with primers 5'-RNA2 and 3'-RNA2 (2.1.4), the correct orientation recombinant plasmid was further verified by digestion with HindIII (2.2.13) and named [pBIN-Δ2a+2b]. A similar strategy was used for the Δ2a+Δ2b construct, using plasmid [SK-(-BamHI)-[2x35S/Δ2a+Δ2b /Nos] (3.1.3) as a source of the cassette [2x35S/Δ2a+Δ2b/Nos], the recombinant plasmid was named [pBIN-Δ2a+Δ2b].

3.2.4 Construction of [pBIN19-CPIR] and [pBIN19-2bIR]

The cassette [2x35S/CPIR/Nos] from plasmid P1353ΔKpnI (3.1.6) was isolated (2.2.14) by digestion with EcoRI and KpnI (2.2.13) and ligated (2.2.15.1) with [pBIN19-EcoRI/KpnI] (3.2.1). After transformation into *E.coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers 35SPRO-FOR and INTRON-REV (2.1.4). The recombinant plasmids were further verified by digestion with EcoRI and KpnI as well as SalI and KpnI. The recombinant plasmids were named [pBIN19-CPIR].

To construct [pBIN19-2bIR], the cassette [2x35S/2bIR/Nos] from plasmid [SK-[2x35S/2bIR/Nos] (3.1.7) was digested with HindIII (2.2.13), isolated (2.2.14) and ligated (2.2.15.1) with [pBIN19-HindIII] (3.2.1). After transformation into *E. coli* NM522 (2.2.17), positive colonies were screened by PCR (2.2.10) with primers 35SPRO-FOR and INTRON-REV (2.1.4), the recombinant plasmids were further verified by digestion HindIII, XbaI and BamHI (2.2.13). The orientation of inserts was verified by BamHI digestion (2.2.13). The resulting clone was named [pBIN19-2bIR].

3.2.5 Construction of [pBIN19-GFP+2bIR]

The cassette [GFP+2bIR] from plasmid [Sk-(-BamHI)-[2x35S/GFP+2bIR/Nos] (3.1.8) was digested by SpeI and KpnI, then cloned into [pBIN19-XbaI/KpnI]. The other procedures were similar to pLH6000-[GFP+2bIR], using plasmid [SK-(-BamHI)-[2x35S/ GFP+2bIR/Nos] (3.1.3) as a source of the cassette [2x35S/ GFP+2bIR/Nos], the resulting clone was designated [pBIN19-GFP+2bIR].

3.3 Prediction on stability of RNA secondary structure of CPIR and 2bIR

Prediction on stability of RNA secondary structure of CPIR and 2bIR was performed with the Mfold program (http://www.bioinfo.rpi.edu, 2.2.29). The predicted difference of minimum free energy between folded and unfolded state under folding conditions at 37°C and 1M NaCl, were determined as -1188.49kcal/mol for 2bIR and -1086.25kcal/mol for CPIR.

3.4 Analysis of transgenic plants

All plasmids cloned into the pLH6000 (3.1.1 to 3.1.8) vector were transformed into the *Agrobacterium tumefaciens* strain GV3101 (2.2.18), whereas all the gene constructs in the pBIN19 binary vector (3.2.1 to 3.2.4) were transformed into *Agrobacterium tumefaciens* strain LBA4404 (2.2.19). In order to determine the possibility of resistance variation due to the plant species *N. benthamiana* and *N. tabaccum* cv. *Samsun* NN were transformed with the same gene constructs in parallel. All plants were genetically modified by agrobacterium-mediated leaf disc transformation (2.2.21).

From all gene constructs 286 lines were selected from independent calli. By PCR (2.2.9) 224 out of 286 transgenic lines were identified as positive with the corresponding primers (2.1.4), non-transformants serving as PCR negative control. In addition, an agrobacterium- specific PCR was performed to ascertain that the positive PCR signals did not originate from the bacteria used for transformation (data not shown). In all lines listed in Table 3 no agrobacterium had been identified. All positive lines from Table 3 of F0 generation were planted in the soil for seeds production.

Table 3. Transgenic lines of F0 generation from different gene constructs

Gene constructs	***N. benthamiana***			***N. tabaccum Samsun*** NN.		
	No. of screened lines	**No. of transgenic lines**	**Transformation[2] efficiency (%)**	**No. of screened lines**	**No. of transgenic lines**	**Transformation efficiency (%)**
pLH[1]-CPIR	17	13	76.47	14	12	85.71
pLH-△2a+△2b	13	7	53.85	11	9	81.82
pLH-2bIR	14	8	57.14	11	10	90.91
pLH-△2a+2b	11	8	72.73	11	11	100.00

Gene constructs	*N. benthamiana*			*N. tabaccum Samsun* NN.		
	No. of screened lines	No. of transgenic lines	Transformation[2] efficiency (%)	No. of screened lines	No. of transgenic lines	Transformation efficiency (%)
pLH-△CP	15	14	93.33	10	10	100.00
pLH-GFP	8	8	100.00	8	5	62.50
pLH	-	2	-	-	2	-
pBin19-CPIR	8	5	62.50	13	11	84.62
pBin19-2bIR	13	7	53.85	21	9	42.86
pBin19-△2a+2b	18	10	55.56	12	12	100.00
pBin19-△CP	10	10	100.00	13	12	92.31
pBin19-△2a+△2b	7	7	100.00	12	12	100.00
pBin19-GFP	6	4	66.67	10	10	100.00
pBin19	-	2	-	-	2	-

[1]pLH: pLH6000. [2]Transformation efficiency (%): No. of transgenic lines/No. of screened lines

The transformation efficiency between the two tobacco species varied slightly with most constructs showing a better efficiency in *N. tabaccum Samsun* NN (Fig. 6 and 7). However, the constructs pBIN19-2bIR and pBIN19-△CP showed a reverse order of efficiency with *N. tabaccum Samsun* NN slightly lower than *N. benthamiana* (Fig. 7).

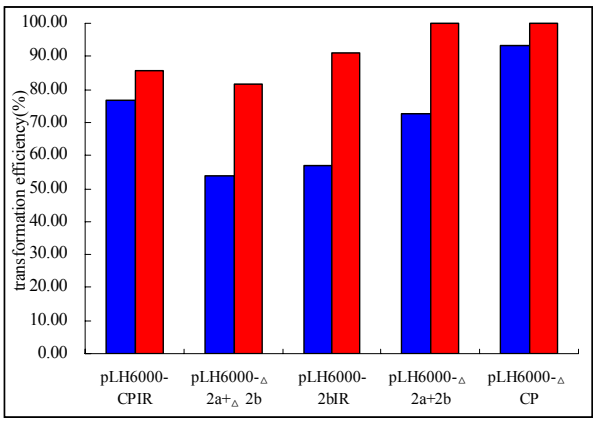

Fig. 6 Comparison of transformation efficiency between different tobacco species for the same gene constructs in pLH6000 binary vector.

: *N. benthamiana* : *N. tabaccum Samsun* NN.

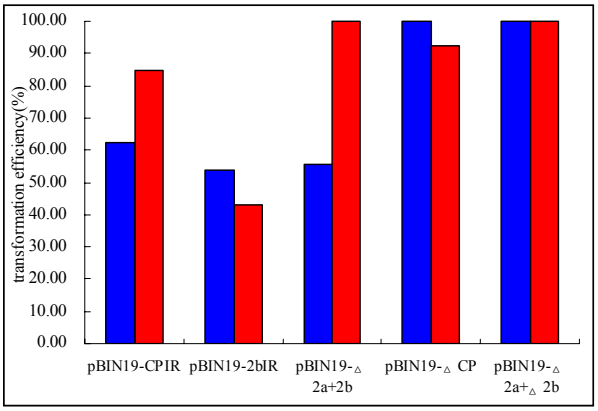

Fig.7 Comparison of transformation efficiency between different tobacco species for the same gene constructs in in pBIN19 binary vector.

: *N. benthamiana* : *N. tabaccum Samsun* NN

All positive lines listed in Table 3 were used for seed production after self-pollination under paper-bag covers.

For transformants of *N. benthamiana*: 5 out of 13 lines of pLH6000-CPIR, 1 out of 8 lines pLH6000-2bIR, 1 out of 10 lines pLH6000-ΔCP, and 2 out of 8 lines of pLH6000-Δ2a+2b, derived from each gene construct in pLH6000 and pBIN19 binary vector were sterile. The sterile transformants of *N. tabaccum Samsun* NN were also observed (data not shown). An high percentage of sterile transformants of *N.benthamiana* up to 50%, were observed for the transgene pBIN19-Δ2a+2b.

To get interpretable results when screening transformed plants for virus resistance, F1 generation seeds of 4 to 6 lines from each gene construct, were subjected to selection by germination on MS medium supplemented with the appropriate selective antibiotics (100 mg/l Hygromycin B for gene constructs in pLH6000 binary vector, 150mg/l Kanamycin for gene constructs in pBIN19 binary vector).

The segregation patterns of the selective marker gene for the F1 generation were evaluated and the number of selection marker resistant seedlings was determined. The results are summarized in Table 4. Forty-six tested lines followed a segregation pattern of 3:1 and confirmed F1 generation to be heterozygous, containing probably one integration site. Considering one independent segregating gene leads to a 3:1 segregation; a double insertion with independent segregation should result in a pattern of 15:1, which was observed for five lines (Table 4). Among the tested lines, eight out of fifty-four lines violated the law of independent segregation (Table 4).

Transformants of *N. benthamiana* plants harboring pBIN19-Δ2a+2b and pBIN-Δ2a+Δ2b, respectively, exhibited phenotypes that clearly distinguished them from non-transformants: plants were stunted, twisted petioles and upturning of leaf borders. Since both, translatable and untranslatable constructs behaved similar, it suggested that the new phenotype didn't correlate with the expression of the 2b protein in plants.

Table 4. Segregation patterns of marker gene for F1 generation of *N. benthamiana* and *N. tabacum Samsun* NN derived from pLH6000 binary vector evaluated by seed germination on selective medium.

N.benthaminana				*N. tabaccum Samsun* NN.			
Transgenic lines	Segregation of marker gene	χ^2-test for segregation	[b]$\chi^2_{1,0.05}\leq 3.84$	Transgenic lines	Segregation of marker gene	χ^2-test for segregation	$\chi^2_{1,0.05}\leq 3.84$
[a]pLH-CPIR line17	50:17	3:1	< 0.01	pLH-CPIR line 9	127:37	3:1	0.52
pLH-CPIR line 5	56:17	3:1	0.11	pLH-CPIR line14	148:49	3:1	<0.01
pLH-CPIR line 2	66:23	3:1	0.03	pLH-CPIR line 7	116:38	3:1	<0.01
pLH-CPIR line 1	45:16	3:1	0.05	pLH-CPIR line 6	127:45	3:1	0.09
pLH-CPIR line14	59:22	3:1	0.20	pLH-CPIR line13	83:27	3:1	0.01
pLH-CPIR line6	95:32	3:1	< 0.01	pLH-CPIR line 2	67:28	3:1	1.01
pLH-2bIR line 8	40:16	3:1	0.38	pLH-2bIR line 1	124:6	15:1	0.59
pLH-2bIR line11	54:21	3:1	0.36	pLH-2bIR line10	153:6	15:1	1.66
pLH-2bIR line1	78:24	3:1	0.12	pLH-2bIR line 7	133:8	15:1	0.08
pLH-2bIR line 3	67:26	3:1	0.43	pLH-2bIR line 3	147:6	15:1	1.42
pLH-2bIR line 7	88:20	3:1	2.42	pLH-Δ2a+2bline8	91:28	3:1	0.14
pLH-2bIR line9	75:22	3:1	0.31	pLH-Δ2a+2bline6	98:31	3:1	0.06
pLH-Δ2a+2bline 6	143:45	3:1	0.11	pLH-Δ2a+2bline10	120:41	3:1	0.02
pLH-Δ2a+2bline 8	150:52	3:1	0.06	pLH-Δ2a+2b line 2	124:43	3:1	0.05
pLH-Δ2a+2bline 5	152:57	3:1	0.58	pLH-Δ2a+Δ2bline7	137:22	3:1	10.57
pLH-Δ2a+2bline 7	162:44	3:1	1.46	pLH-Δ2a+Δ2bline11	112:38	3:1	<0.01
pLH-Δ2a+2bline11	209:49	-	-	pLH-Δ2a+Δ2b line 9	122:45	3:1	0.34
pLH-Δ2a+Δ2bline4	148:28	-	-	pLH-ΔCP line 1	88:31	3:1	0.07
pLH-Δ2a+Δ2bline1	157:30	-	-	pLH-ΔCP line10	98:29	3:1	0.32
pLH-Δ2a+Δ2bline2	126:40	3:1	0.43	pLH-ΔCP line 2	60:21	3:1	0.04
pLH-Δ2a+Δ2bline5	134:45	3:1	< 0.01	pLH-ΔCP line 6	41:16	3:1	0.29
pLH-Δ2a+Δ2bline13	163:36	-	-	pLH-ΔCP line 9	57:26	3:1	1.77
pLH-ΔCP line1	140:47	3:1	< 0.01	pLH-ΔCP line 7	64:31	3:1	2.95
pLH-ΔCP line2	152:52	3:1	0.03	pLH-GFP line 1	106:27	3:1	1.57
pLH-ΔCP line13	215:30	-	-	pLH-GFP line 7	128:45	3:1	0.09
pLH-ΔCP line 6	205:68	3:1	< 0.01	pLH6000 line 2	76:28	3:1	0.21
pLH-ΔCP line14	155:30	-	-				
pLH-ΔCP line 5	184:23	-	-				
pLH-ΔCP line15	136:18	-	-				
pLH-GFP line 7	116:37	3:1	0.05				
pLH-GFP line 2	132:4	15:1	2.54				
pLH-GFP line5	114:39	3:1	0.02				
pLH6000 line1	111:37	3:1	<0.01				

[a]pLH: pLH6000. b: these data were calculated by Chi-square test (χ^2) with a confidence value of $P_{0.05}\leq 3.48$.

3.5 Resistance variation of F1 generation challenged with CMV_{AN}

To evaluate if resistance variability observed in transgenic *N. benthamiana* and *N. tabaccum Samsun* NN plants depends on their different genotypes, the resistance screening was carried out in parallel on both plant species harboring the same transgenes delivered by pLH6000 and pBIN19 binary vectors, respectively.

Here the results for F1 generation seedlings containing single gene constructs (Δ2a+2b, Δ2a+Δ2b, ΔCP) as well as plants containing CPIR or 2bIR constructs, obtained with the vector pLH6000 and pBIN19, are presented. Non-transformants as well as transgenic plants containing GFP single gene construct or empty binary vectors served as susceptible controls.

Typical systemic CMV disease symptoms are developed at 10 days post-inoculation (d.p.i) at 25 ±1° C. Crinkling, mosaic with curling down on newly emerging leaves were observed in *N. benthamiana*, whereas vein yellowing and mosaic on upper non-inoculated leaves happened in *N. tabaccum Samsun* NN.

3.5.1 Establishment of the resistance screening system

To ensure consistent, effective and comparable results for all resistance screening tests, infectivity of inocula was estimated using the local lesion host plant *C. quinoa*. For each test the inoculum was adjusted to induced 30~60 local lesions per leaf, when using 10µl/per leaf. The experiment was evaluated when all of the non-transformants (wild type) became infected (100%) by the challenging CMV_{AN} (2.1.2).

At least four till up to six lines of transgenic plants derived from the same gene construct were subjected to screening test in the greenhouse on both tobacco species, where eight plants at four to five leaf-stage were inoculated (2.2.3) at their two lower leaves or two lower fully expanded leaves. Development of visual symptoms was documented and infection was verified by tissue print immunoblot assays (2.2.26).

Three different resistance phenotype classes were determined as following:

- Immunity = no symptoms were observed during screening and no virus was detected by tissue print immunoblot assays.
- Tolerant = no symptoms / mild symptoms or symptoms were delayed sometimes and virus was detected in plants or only on inoculated leaves by tissue print immunoblots.
- Recovery = symptoms were observed at early stage and virus was only detectable in the inoculated leaves at later stage. Upper leaves were free of symptoms and virus-free when tested with tissue print immunoblots.

Susceptible = symptoms similar to non transformed controls were observed without delay.

3.5.2 Resistance evaluation of transgenic lines harboring Δ2a+Δ2b derived from pLH6000 and pBIN19 binary vector in *N. benthamiana* and *N. tabaccum* Samsun NN

The 2b protein encoded on CMV RNA2 served, among other functions, for the suppression of plant-based RNA silencing. Based on the experimental results described in the introduction, constructs targeting the 2b protein either as translatable construct or untranslatable were used. Two out of five tested lines of pLH6000-Δ2a+Δ2b in *N. benthamiana* (lines 2 and line 7, Table 5) were immune against infection when challenged with the homologous CMV_{AN}. In these two immune lines, in seven out of 8 tested plants no virus was detected in inoculated and upper emerging leaves by tissue print immunoblot assay 21 d.p.i. The resistant efficiency of the other three lines was 12.5%, 25% and 50%, i.e. one out of eight plants from line5,two out of eight plants from line1 and three out of eight plants from line 4 was immune to CMV_{AN}. One out of eight plants from line 4 recovered at 35 d.p.i. (Table 5). In contrast, almost all of tested plants from five different transgenic lines of *N. tabaccum Samsun* NN harboring the same pLH6000-$^{\triangle}$2a+$^{\triangle}$2b construct remained susceptible and developed typical CMV disease symptoms 10 d.p.i. Three plants out of eight from line 3 recovered and virus could not be detected on emerging leaves after 35 d.p.i. The same results were obtained in two independent experiments.

All four tested *N. benthamiana* lines derived from pBIN19-$^{\triangle}$2a+$^{\triangle}$2b revealed no resistance phenotype at all in two independent experiments. All tested plants were susceptible and

developed typical CMV disease symptoms at the same time as the controls. The same results were also observed with three out of four tested *N. tabaccum* lines. However, three plants out of eight from line 6 showed the tolerant phenotype of resistance, which was confirmed by tissue print immunoblot assays 14 d.p.i. and 21 d.p.i., respectively (Table 5).

In summary, the construct pLH6000-Δ2a+Δ2b induced higher resistance efficiency in transgenic *N. benthamiana* plants, but failed to do so in *N. tabaccum Samsun* NN. For the construct pBIN19-Δ2a+Δ2b, there is no difference in resistant efficiency for the two tobacco species.

In contrast, in *N. benthamiana* the resistance efficiency of pLH6000-Δ2a+Δ2b was higher than that of pBIN19-Δ2a+Δ2b, while there is no difference in *N. tabaccum Samsun* NN for both constructs.

All results are summarized in Table 5.

Table 5. Summary of resistance phenotypes obtained in *N. benthamiana* and *N. tabaccum Samsun* NN harboring Δ2a+Δ2b derived from pLH6000 and pBIN19 binary vectors

Transgenic lines	**No. of different resistance phenotype in *N.benthamiana***					**Transgenic lines**	**No. of different resistance phenotype in *N. tabaccum Samsun***				
	Immu-nity	tolerant	recovery	Suscepti-ble	Resistance efficiency%		Immu-nity	tolerant	recovery	Suscepti-ble	[3]Resistance efficiency%
[1]pLH-Δ2a+Δ2bline1	2	-	-	6	25.00	pLH-△2a+△2bline 3	-	-	3	5	37.50
pLH-△2a+△2bline 2	7	-	-	1	87.50	pLH-△2a+△2b line 4	-	-	-	8	-
pLH-△2a+△2b line 4	3	-	1	4	50.00	pLH-△2a+△2b line 5	-	-	-	8	-
pLH-△2a+△2b line 5	1	-	-	7	12.50	pLH-△2a+△2b line 8	-	-	-	8	-
pLH-△2a+△2b line 7	7	-	-	1	87.50	pLH-△2a+△2bline10	-	-	-	8	-
[2]pBin-△2a+△2bline 1	-	-	-	8	-	pBin-△2a+△2bline 5	-	-	-	8	-
pBin-△2a+△2bline 3	-	-	-	8	-	pBin-△2a+△2bline 6	-	3	-	5	37.50
pBin-△2a+△2bline 4	-	-	-	8	-	pBin-△2a+△2bline 7	-	-	-	8	-
pBin-△2a+△2bline 7	-	-	-	8	-	pBin-△2a+△2bline 10	-	-	-	8	-

[1]pLH: pLH6000. [2]pBin: pBin19. "-": not found. [3]Resistance efficiency (%) of each tested line was calculated: No. of resistance plants (containing immunity, tolerant and recovery)/ No. of screened plants.

3.5.3 Resistance evaluation of transgenic lines harboring ∆ 2a+2b derived from pLH6000 and pBIN19 binary vector in *N. benthamiana* and *N. tabaccum* Samsun NN

In *N. benthamiana,* transformed with the translatable construct pLH6000- ∆ 2a+2b, the resistant efficiency varied from 25% to 37.5% according to tissue print immunoblot assay 14 d.p.i and 28 d.p.i, respectively. Two plants from line 5, 3 plants from line 6, 3 plants from line 7, 3 plants from line8 and 2 plants from line11 were immune to the infection of CMV_{AN} (Table 6). Subsequently, an increasing level of resistance was observed since plants recovered 35 d.p.i. (Table 6) and the level of resistance increased from 62.5% to 87.5%. Three plants from line 5, 3 plants from line 6, 4 plants from line 7, 2 plants from line 8 and 3 plants from line 11 exhibited tolerance to CMV_{AN} (Table 6).

Four tested transgenic *N. tabaccum* Samsun NN lines harboring pLH6000- ∆2a+2b were susceptible to CMV_{AN} 28 d.p.i. However, two plants from line 2 and one plant from line 8 recovered 35 d.p.i. The observed phenotype of resistance in the two tobacco species was similar to that of the untranslatable construct pLH6000- ∆2a+ ∆2b.

Three tested *N. benthamiana* lines from pBIN19- ∆2a+ ∆2b were completely susceptible to the infection of CMV_{AN} (Table 6). In six tested *N. tabaccum* lines from pBIN19- ∆2a+ ∆2b, one plant from line 8 and one plant from line 10 were of the tolerant phenotype. Seven plants (four plants from line 8, two plants from line 1 and one plant from line 10) recovered 35 d.p.i, which showing no visible symptoms on upper emerging leaves. The *N. tabaccum* lines 4 and line7 were fully susceptible to CMV_{AN} (Table 6).

In summary, the construct pLH6000- ∆2a+2b induced higher resistance efficiency in transgenic *N. benthamiana* plants, but failed to do so in *N. tabaccum Samsun* NN. Compared with pLH6000- ∆2a+ ∆2b, a similar behavior was observed in the two tobacco species. For the construct pBIN19- ∆2a+2b, no immune plants had been observed in the two tobacco species, but tolerant and recovery phenotypes were observed in *N. tabaccum Samsun* NN plants, only.

In contrast, in *N. benthamiana* the induced resistance efficiency of pLH6000- ∆2a+2b was higher than that of pBIN19- ∆2a+2b, while in *N. tabaccum Samsun* NN the induced resistance efficiency of pBIN19- ∆2a+2b and pLH6000- ∆2a+2b was not different (Table 6).

All results are summarized in Table 6.

Table 6. Summary of resistance phenotypes obtained in *N. benthamiana* and *N. tabaccum Samsun* NN harboring ∆2a+2b derived from pLH6000 and pBIN19 binary vectors

Transgenic lines	No. of different resistance phenotype in *N.benthamiana*					Transgenic lines	No. of different resistance phenotype in *N. tabaccum Samsun*				
	Immun -ity	tolerant	recovery	Suscept -ible	Resistance efficiency%		Immun -ity	tolerant	recovery	Suscept -ible	[3]Resistance efficiency%
[1]pLH-△2a+2bline5	2	-	3	3	62.50	pLH-△2a+2bline2	-	-	2	6	25.00
pLH-△2a+2bline 6	3	-	3	2	75.00	pLH-△2a+2bline6	-	-	-	8	-
pLH-△2a+2bline 7	3	-	4	1	87.50	pLH-△2a+2bline8	-	-	1	7	12.500
pLH-△2a+2bline 8	3	-	2	3	62.50	pLH-△2a+2bline10	-	-	-	8	-
pLH-△2a+2bline11	2	-	3	3	62.50						
[2]pBin△2a+2bline 7	-	-	-	8	-	pBin-△2a+2bline 1	-	-	2	6	25.00
pBin-△2a+2bline 11	-	-	-	8	-	pBin-△2a+2bline 4	-	-	-	8	-
pBin-△2a+2bline 13	-	-	-	8	-	pBin-△2a+2bline 5	-	-	-	8	-
						pBin-△2a+2bline 7	-	-	-	8	-
						pBin-△2a+2bline 8		1	4	3	62.50
						pBin-△2a+2bline 10	-	1	1	6	25.00

[1]pLH: pLH6000. [2]pBin: pBin19. "-": not found. [3]Resistance efficiency (%) of each tested line was calculated: No. of resistance plants (containing immunity, tolerant and recovery)/ No. of screened plants.

3.5.4 Resistance evaluation of transgenic lines harboring ∆CP derived from pLH6000 and pBIN19 binary vector in *N. benthamiana* and *N. tabaccum* Samsun NN

Tested transgenic *N. benthamiana* lines containing pLH6000- ∆CP transgene revealed immunity type resistance between 25% to 50% when tested by tissue print immunoblot assay 21 d.p.i. (i.e. 2 out of 8 plants from line 1, 3 out of 8 plants from line14, 2 out of 8 plants from line 5 and 4 out of 8 plants from line 2 were immune to the infection CMV_{AN}. Tolerant and recovery resistance phenotypes were not observed during six weeks after inoculation (Table 7).

In contrast, immune *N. tabaccum Samsun* NN plants in five tested lines were not obtained during screening. Only a total of four plants from five tested lines (1 plant from line 2, 1 plant from line 6 and 2 plants from line 9) showed recovery 35 d.p.i. (Table 7). The other plants remained susceptible and developed typical disease symptoms, compared with nontransgenic plants infected with CMV_{AN}.

All four tested pBIN19- ΔCP *N. benthamiana* lines were susceptible to CMV_{AN}. In four tested *N. tabaccum* lines, four plants (two plants from line 3 and two plants from line 9) recovered 35 d.p.i., while the other tested plants were susceptible (Table 7).

In summary, transgenic *N. benthamiana* and *N. tabaccum Samsun* NN plants harboring pLH6000-ΔCP are significant different in resistance variation. The resistance efficiency in *N. benthamiana* was higher than in *N. tabaccum Samsun* NN. For the construct pBIN19- ΔCP, there is no clear difference in two tobacco species, although recovery plants in *N. tabaccum Samsun* NN were observed.

In contrast, constructs pLH6000-ΔCP and pBIN19-ΔCP induced different resistance variation in *N. benthamiana*. Transgenic *N. benthamiana* plants harboring pLH6000-ΔCP induced higher resistance efficiency; whereas pLH6000-ΔCP and pBIN19-ΔCP did not induce different resistance in *N. tabaccum Samsun* NN plants.

All results are summarized in Table 7.

Table 7. Summary of resistance phenotypes obtained in *N. benthamiana* and *N. tabaccum Samsun* NN harboring ΔCP derived from pLH6000 and pBIN19 binary vectors

Transgenic lines	**No. of different resistance phenotype in *N.benthamiana***					**Transgenic lines**	**No. of different resistance phenotype in *N. tabaccum Samsun***				
	Immun-ity	tolerant	recovery	Suscept-ible	Reistance efficiency%		Immun-ity	tolerant	recovery	Suscept-ible	[1]Resistance efficiency%
pLH6000-ΔCP line 1	2	-	-	6	25.00	pLH6000-ΔCP line 2	-	-	1	7	12.50
pLH6000-ΔCP line 2	4	-	-	4	50.00	pLH6000-ΔCP line 6	-	-	1	7	12.50
pLH6000-ΔCP line 5	2	-	-	6	25.00	pLH6000-ΔCP line 7	-	-	-	8	-
pLH6000-ΔCPline14	3	-	-	5	37.50	pLH6000-ΔCP line 9	-	-	2	6	25.00
						pLH6000-ΔCP line10	-	-	-	8	-
pBin19-ΔCP line 2	-	-	-	8	-	pBin19-ΔCP line3	-	-	2	6	25.00
pBin19-ΔCP line 3	-	-	-	8	-	pBin19-ΔCP line8	-	-	-	8	-
pBin19-ΔCP line 4	-	-	-	8	-	pBin19-ΔCP line9	-	-	2	6	25.00
pBin19-ΔCP line 7	-	-	-	8	-	pBin19-ΔCP line10	-	-	-	8	-

"-": not found. [1]Resistance efficiency (%) of each tested line was calculated: No. of resistance plants (containing immunity, tolerant and recovery)/ No. of screened plants.

3.5.5 Resistance evaluation of transgenic lines harboring CPIR derived from pLH6000 and pBIN19 binary vector in *N. benthamiana* and *N. tabaccum* Samsun NN

The CPIR construct contained a non-translatable CP gene from CMV isolate PV0506. The nucleotide identity of the CP genes from CMV-PV0506 and CMV_{AN} was determined to be 94% (Appendix 7.2.3).

Four out of six tested pLH6000-CPIR *N. benthamiana* lines showed 12.5% to 25% immune plants (2 plants from line 1, one plant from line 2, one plant from line 13 and one plant from line 14), while all tested plants from line 5 and line 17 were susceptible to the infection of CMV_{AN} (Table 8).

Four out of six tested *N. tabaccum Samsun* NN lines exhibited the resistance frequency from 12.5 to 25%, furthermore three different resistance phenotypes were observed. One plant from line 2 was immune, two plants (one plant from line 2 and one plant from line 7) were tolerant, six plants (two plants from line 6, one plant from line 7 and one plant from line 2) recovered 35 d.p.i. All other plants remained susceptible and developed typical CMV symptoms (Table 8).

In three tested pBIN19-CPIR *N. benthamiana* lines, four out of eight plants from line 8 displayed the immune phenotype associated with no visual disease symptoms. However, only three out of eight plants from line 8 delayed symptom development of about 7-10 days in the repetition of the experiment (data not shown). Other tested plants from line 4 and line 7 were susceptible (Table 8).

In the tested six pBIN19-CPIR *N. tabaccum* lines, two plants from line 8 were immune, one plant from line 3 and one plant from line 8 were tolerant, whereas the other plants showed vein yellowing and mosaic on upper emerging leaves as typical CMV symptoms 10 d.p.i. (Table 8).

In summary, the construct pLH6000-CPIR induced the same low resistance efficiency in two tobacco species when challenged with heterologous virus. For pBIN19-CPIR, no difference in induced resistance efficiency for two tobacco species was observed.

In contrast, the constructs pLH6000-CPIR and pBIN19-CPIR induced the same resistance efficiency in *N. benthamiana* and *N. tabaccum Samsun* NN plants, respectively.

All results are summarized in Table 8.

Table 8. Summary of resistance phenotypes obtained in *N. benthamiana* and *N. tabaccum Samsun* NN harboring CPIR derived from pLH6000 and pBIN19 binary vectors

Transgenic lines	No. of different resistance phenotype in *N.benthamiana*					Transgenic lines	No. of different resistance phenotype in *N. tabaccum Samsun*				
	Immun-ity	tolerant	recovery	Suscept-ible	Resistance efficiency%		Immun-ity	tolerant	recovery	Suscepti-ble	[1]Resistance efficiency%
pLH6000-CPIR line 1	2	-	-	6	25.00	pLH6000-CPIR line 2	1	1	1	5	37.50
pLH6000-CPIR line 2	1	-	-	7	12.50	pLH6000-CPIR line 6	-	-	2	6	25.00
pLH6000-CPIR line 5	-	-	-	8	-	pLH6000-CPIR line 7	-	1	1	6	25.00
pLH6000-CPIRline13	1	-	-	7	12.50	pLH6000-CPIRline13	-	-	-	8	-
pLH6000-CPIRline14	1	-	-	7	12.50	pLH6000-CPIRline14	-	-	-	8	-
pLH6000-CPIRline17	-	-	-	8	-						
pBin19-CPIR line 4	-	-	-	8	-	pBin19-CPIR line1	-	-	-	8	-
pBin19-CPIR line 7	-	-	-	8	-	pBin19-CPIR line3	-	1	-	7	12.50
pBin19-CPIR line 8	4	-	-	4	50.00	pBin19-CPIR line4	-	-	-	8	-
						pBin19-CPIR line5	-	-	-	8	-
						pBin19-CPIR line6	-	-	-	8	-
						pBin19-CPIR line8	2	1	-	5	37.50

"-": not found. [1]Resistance efficiency (%) of each tested line was calculated: No. of resistance plants (containing immunity, tolerant and recovery)/ No. of screened plants.

3.5.6 Resistance evaluation of transgenic lines harboring 2bIR derived from pLH6000 and pBIN19 binary vector in *N. benthamiana* and *N. tabaccum* Samsun NN

Plants (seven out of eight plants from line 9) of one of five tested transgenic pLH6000-2bIR *N. benthamiana* lines were immune to the infection of CMV_{AN}. Virus was not detected on inoculated leaves and upper non-inoculated leaves in these plants by tissue print immunoblot assays 14 and 28 d.p.i., respectively. The other four tested lines showed resistance efficiency from 12.5% to 37.5% (3 plants from line 1, one plant from line 3, 3 plants from line 7 and 3 plants from line 11 were also immune to the infection of CMV_{AN}). All resistant plants remained symptomless in their lifetime, but none of the susceptible plants recovered (Fig. 6 and Table 9).

All seven tested pLH6000-2bIR *N. tabaccum Samsun* NN lines did not show immunity and tolerant plants 14 and 28 d.p.i. Seven plants from four lines (one plant from line 6, 3 plants from line 9, one plant from line 3 and 2 plants from line 7) recovered 35 d.p.i. (Table 9). All plants from line 1, line 4 and line 10 were susceptible to CMV_{AN}.

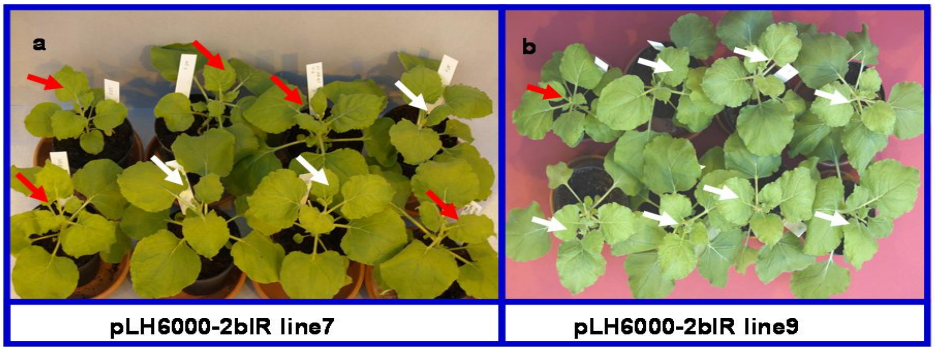

Figure 6. Phenotypes of resistance in transgenic *N. benthamiana* from line 7 and line 9 of pLH6000-2bIR after challenging with the homologous isolate of CMV_{AN} 14 d.p.i.
Red arrows indicate typical CMV disease symptoms of curling on upper non-inoculated leaves and dwarfing of the plant, which are susceptible to $CMV_{AN.}$ White arrows show symptomless leaves on upper non-inoculated leaves, which are immune to CMV_{AN}.

In four tested pBIN19-2bIR *N. benthamiana* lines, plants from line 5, line 7 and line 12 (7 out of 8) were susceptible to the challenging CMV_{AN}. However, in one line, all eight tested plants from line 11 exhibited the immunity phenotype and remained symptomless during the full time of experiments (Fig. 7). No virus could be detected in these plants by tissue print immunoblot assays 14 and 21 d.p.i. The results were confirmed by single tube RT-PCR (2.2.9.3, Fig. 8) and back inoculation experiments (2.2.3). The same results were obtained in two independent experiments. In addition, one plant from line 12 was also immune to the challenging virus.

In six tested pBIN19-2bIR *N. tabaccum* lines, only four plants were resistant against the challenging virus: one plant from line 11 was immune to CMV_{AN}, one plant from line 7 was tolerant and two plants from line 7 recovered 35 d.p.i.(Table 9).

Figure 7. Comparison between line 11 of pBIN19-2bIR and line 4 of pBIN19-GFP after challenging with CMV_{AN} 21d.p.i. Right: plants indicate typical CMV disease symptoms of curling down and blistering on upper non-inoculated leaves. (a): plants show no symptoms on upper non-inoculated leaves, which are immune to CMV_{AN}; (b) plants are susceptible to CMV_{AN}.

In summary, the construct pLH6000-2bIR induced higher resistance efficiency in *N. benthamiana* than in *N. tabaccum Samsun* NN. Furthermore these resistant plants from tested *N. benthamiana lines* were immune to the challenging virus. Thus, there is significant resistance variability for pLH6000-2bIR in the two tobacco species. For construct pBIN19-2bIR, resistance efficiency in *N. benthamiana* lines was higher than that in *N. tabaccum Samsun* NN. Moreover 100% tested *N. benthamiana* plants from line11 were immune to the challenging virus.

In contrast, in pLH6000-2bIR *N. benthamiana* plants a higher resistance efficiency than pBIN19-2bIR was observerd. However, plants of one pLH6000-2bIR line and one pBIN19-2bIR line were immune to CMV_{AN}. In *N. tabaccum Samsun* NN plants, there is no difference between pLH6000-2bIR and pBIN19-2bIR in induced resistance efficiency.

All results are summarized in Table 9.

Table 9. Summary of resistance phenotypes obtained in *N. benthamiana* and *N. tabaccum Samsun* NN harboring 2bIR derived from pLH6000 and pBIN19 binary vectors

Transgenic lines	**No. of different resistance phenotype in *N.benthamiana***					**Transgenic lines**	**No. of different resistance phenotype in *N. tabaccum Samsun***				
	immun-ity	tolerant	recovery	Suscept-ible	Resistance efficiency%		immun-ity	tolerant	recovery	Suscept-ible	[1]Resistance efficiency%
pLH6000-2bIRline1	3	-	-	5	37.50	pLH6000-2bIR line 1	-	-	-	8	-
pLH6000-2bIRline3	1	-	-	7	12.50	pLH6000-2bIR line 3	-	-	1	7	12.50
pLH6000-2bIRline7	3	-	-	5	37.50	pLH6000-2bIR line 4	-	-	-	8	-
pLH6000-2bIRline9	7	-	-	1	87.50	pLH6000-2bIR line 6	-	-	1	7	12.50
pLH6000-2bIRline11	3	-	-	5	37.50	pLH6000-2bIR line 7	-	-	2	6	25.00

Transgenic lines	No. of different resistance phenotype in *N.benthamiana*					Transgenic lines	No. of different resistance phenotype in *N. tabaccum Samsun*				
	immun-ity	tolerant	recovery	Suscept-ible	Resistance efficiency%		immun-ity	tolerant	recovery	Suscept-ible	[1]Resistance efficiency%
						pLH6000-2bIR line 9	-	-	3	5	37.50
						pLH6000-2bIRline10	-	-	-	8	-
pBin19-2bIR line 5	-	-	-	8	-	pBin19-2bIR line1	-	-	-	8	-
pBin19-2bIR line 7	-	-	-	8	-	pBin19-2bIR line5	-	-	-	8	-
pBin19-2bIR line11	8	-	-	0	100.00	pBin19-2bIR line6	-	-	-	8	-
pBin19-2bIR line12	1	-	-	7	12.50	pBin19-2bIR line7	-	1	2	5	37.50
						pBin19-2bIR line11	1	-	-	7	12.50
						pBin19-2bIR line21	-	-	-	8	-

"-": not found. The red highlighted frame line will be screened with different CMV isolates (3.7) [1]Resistance efficiency (%) of each tested line was calculated: No. of resistance plants (containing immunity, tolerant and recovery)/ No. of screened plants.

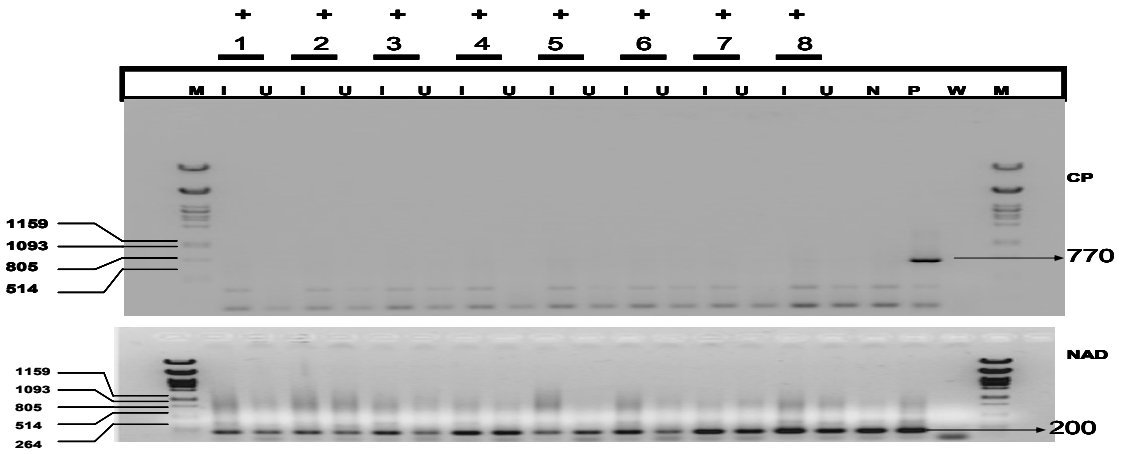

Figure 8. Agarose gel showing the results of single tube RT-PCR from *N. benthamiana* plants total RNA of pBIN19-2bIR line11 at 21d.p.i. after challenging with CMV_{AN}.

Total RNA from 8 *N. benthamiana* plants of pBIn19-2bIR was extracted (2.2.4). RT-PCR was performed with CP primers and NAD primers (2.1.4). I= inoculated leaves; U=upper noninoculated leaves; N=Negative control; P=positive control; M=λDNA/PstI molecular weight markers; W=water. CP=single tube RT-PCR pattern after amplification with CP primers; NAD=single tube RT-PCR pattern after amplification with NAD primers; Number= No. of plant; "+"=the phenotype of resistance is immunity.

3.5.7 Comparison of resistance in *N. benthamiana* and *N. tabaccum Samsun* NN plants harboring different gene construct derived from pLH6000

Comparing resistance affected in the two tested tobacco species with the different constructs as summarized in Table 10. A higher efficiency of resistance was obtained with *N. benthaminana*. Especially the immune phenotype occurred mostly in plants from *N. benthamiana* and was observed with each gene construct, whereas in only one plant a transgenic *N.tabaccum Samsun* NN line developed it. Also tolerant phenotype was not observed in *N. tabaccum* plants, in contrast to the transgenic *N. benthamiana* plants. However, the recovery phenotype was observed in *N. tabaccum* for each gene construct and this phenotype occurred in *N. benthaminana* plants only with the pLH6000-Δ2a+2b construct. Not only the phenotype of resistance differed between the two species used for transformation, also the *N. benthaminana* lines from pLH6000-2bIR, pLH6000-Δ2a+2b and pLH6000-Δ2a+Δ2b revealed more efficiency in inducing resistant plants. As summarized in Table 10, the results indicated that both, the type of resistance induced by each gene construct and their overall efficiency depend on the plant species.

Table 10. Summary of resistance types obtained in *N. benthamiana* and *N. tabaccum Samsun* NN plants derived with different constructs in pLH6000 binary vector

Gene constructs	No. of different resistance phenotype in *N. benthamiana*				Gene constructs	No. of different resistance phenotype in *N. tabaccum Samsun* NN			
	Immunity	tolerant	recovery	Susceptible		Immunity	tolerant	recovery	susceptible
pLH6000-2bIR	17	-	-	23	pLH6000-2bIR	-	-	7	49
pLH6000-Δ2a+2b	13	-	15	12	pLH6000-Δ2a+2b	-	-	3	29
pLH6000-Δ2a+Δ2b	20	-	1	19	pLH6000-Δ2a+Δ2b	-	-	3	37
pLH6000-CPIR	5	-	-	35	pLH6000-CPIR	1	2	6	39
pLH6000-ΔCP	11	-	-	29	pLH6000-ΔCP	-	-	4	36

"-": not found.

3.5.8 Comparison of resistance in *N. benthamiana* and *N. tabaccum Samsun* NN plants harboring different gene construct derived from pBIN19

When using the pBIN19 binary vector only the inverted repeat constructs CPIR and 2bIR led to resistant plants, but this in both species, *N. benthamiana* and *N. tabaccum* NN. No any resistant *N. benthamiana* plant was obtained from pBIN19-Δ2a+2b, pBIN19-Δ2a+Δ2b and pBIN19-ΔCP.

In contrast, all three different resistance phenotypes were observed in *N. tabaccum* plants, although with few plants from each gene construct. Again, it became evident, that the resistant phenotypes of *N. tabaccum* plants were predominantly of tolerance or recovery. However, compared with resistance variation in the two tobacco species derived from the same gene construct, pBIN19-2bIR line is the line with the highest number of resistant plants (Table 11).

Table 11. Summary of resistance types in *N. benthamiana* and *N. tabaccum Samsun* NN plants derived from different constructs in pBIN19 binary vector

Gene constructs	No. of different resistance phenotype in *N. benthamiana*				Gene constructs	No. of different resistance phenotype in *N. tabaccum Samsun* NN			
	Immunity	tolerant	recovery	Susceptible		Immunity	tolerant	recovery	susceptible
pBIN19-2bIR	9	-	-	23	pBIN19-2bIR	1	1	2	44
pBIN19-Δ2a+2b	-	-	-	24	pBIN19-Δ2a+2b	-	2	7	31
pBIN19-Δ2a+Δ2b	-	-	-	32	pBIN19-Δ2a+Δ2b	-	3	-	29
pBIN19-CPIR	4	-	-	20	pBIN19-CPIR	2	2	-	44
pBIN19-ΔCP	-	-	-	32	pBIN19-ΔCP	-	-	4	28

"-": not found.

3.6 Chimeric construct GFP+2bIR containing GFP gene as flanking sequence of 2bIR could enhance/influence resistance against the challenge CMV_{AN} in transgenic *N. benthamiana* and *N. tabaccum Samsun* NN

All tested plants from transgenic Line 11 of pBIN19-2bIR (Table 9) were immune against CMV_{AN} and the virus could neither be detected in the inoculated nor in newly developing leaves in two independent experiments. In order to explore whether flanking sequence enhance or reduce the efficiency of the 2bIR construct, the available reporter gene GFP (3.1.2) as flanking sequence to generate construct GFP+2bIR (3.1.8 and 3.2.5) was used and tested.

All transformants harboring GFP+2bIR were identified by PCR (2.2.24) with specific primers for GFP and 2b (2.1.4) before being used for production of F1 seed (data not shown). A total of nine lines in *N. benthamiana* and ten lines in *N. tabaccum Samsun* NN were derived from pBIN19-GFP+2bIR (3.2.5).

Each, 3 lines in *N. benthamiana* and *N. tabaccum Samsun* NN were derived from pLH6000-GFP+2bIR (3.1.8). All lines were further subjected selection screening on media

containing antibiotic (3.4) to determine the segregation pattern for marker resistance variation on F1 generation level. Antibiotic resistant seedlings were used for resistance screening in the greenhouse with CMV_{AN} (Table 12).

In *N. benthamiana,* six out of nine (67%) *N. benthamiana* lines from pBIN19-GFP+2bIR were immune against the infection of CMV_{AN} (Table 12). Furthermore, all tested plants from four lines (line 1, line 3, line 2 and line 7) were immune to the infection of CMV_{AN}, while 4 out of eight plants from line 4 and 7 out of eight plants from line 5 were immune to CMV_{AN}. Tested plants from line 6, line 8 and line 9 exhibited susceptibility to CMV_{AN}. This was further confirmed by detecting inoculated and upper non-inoculated leaves with tissue print immunoblot assays (2.2.26) 14d.p.i. and 21d.p.i, respectively. With *N. benthamiana* line 1 and line 3 additional testing was carried out twice. In this repetition all tested plants from the two lines were also immune to CMV_{AN}. Subsequently, inoculated leaves and upper emerging leaves were further analyzed by single tube RT-PCR (2.2.9.3) as well as back inoculation (2.2.3). In experiments cases no PCR product or infectious virus was obtained.

In *N. tabaccum Samsun* NN from pBIN19-[GFP+2bIR], four out of 10 lines were immune. All tested plants from line 5 and line 7 were immune against CMV_{AN}, while 4 out of eight plants from line 9 and 7 out of 8 plants from line 8 were immune to CMV_{AN} (Table 12, Fig. 11). This immunity was confirmed by tissue print immunoblot assays (2.2.26) 14 d.p.i. and 21 d.p.i., respectively.

Both, in *N. Benthamiana* and *N. tabaccum Samsun* NN transformed with pBIN19-GFP+2bIR, no any tolerant and recovered plant was observed. All resistant plants remained symptomless in their life time and were able to produce seeds.

In *N. tabaccum Samsun* NN transformed with pLH6000-[GFP+2bIR], plants from three tested lines behaved tolerant or immune to the challenging virus CMV_{AN}. Eight tested plants from line 6 were immune to the challenged virus CMV_{AN} as well as three plants from line 2 and four plants from line 3. This was confirmed by tissue print immunoblot assays 10d.p.i. and 21d.p.i. (Table12 and Fig.12). In addition, five plants from line 2 and four plants from line 3 remained symptomless, but virus was detectable in inoculated and upper non-inoculated leaves by tissue print immunoblot assay (2.2.26) 10d.p.i and 21d.p.i. Furthermore, all tolerant as well as immune plants remained symptomless in their lifetime and were able to produce seeds.

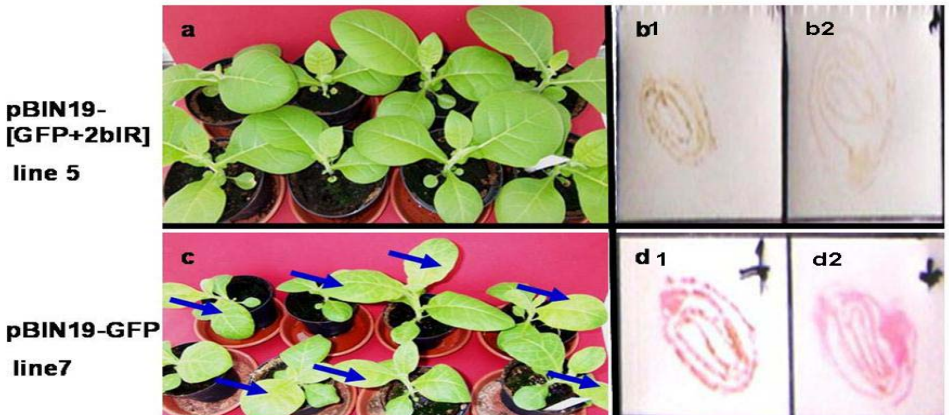

Figure11. Pattern of symptom expression in transgenic *N. tabaccum Samsun* NN plants of pBIN19-[GFP+2bIR] when challenging with CMV_{AN} at 14 d.p.i..
(**a**): no symptom of pBIN19-[GFP+2bIR] transgenic plants; (**b**): tissue print immunoblots assay of upper noninoculated (**b1**) and inoculated leaves (**b2**) of pBIN19-[GFP+2bIR] transgenic plants, virus could not be detected; (**c**): CMV disease symptoms of pBIN19-GFP transgenic plants, blue arrows indicate typical symptoms on upper leaves; (**d**): tissue print immunoblots assay of upper noninoculated (**d1**) and inoculated leaves (**d2**) of pBIN19-GFP transgenic plants, virus was detected.

In *N. benthamiana* transformed with pLH6000-[GFP+2bIR], gave different results with *N. tabaccum Samsun* NN. Only one plant from three tested lines (one plant from line1) exhibited immunity to CMV_{AN}, the other tested plants were susceptible.

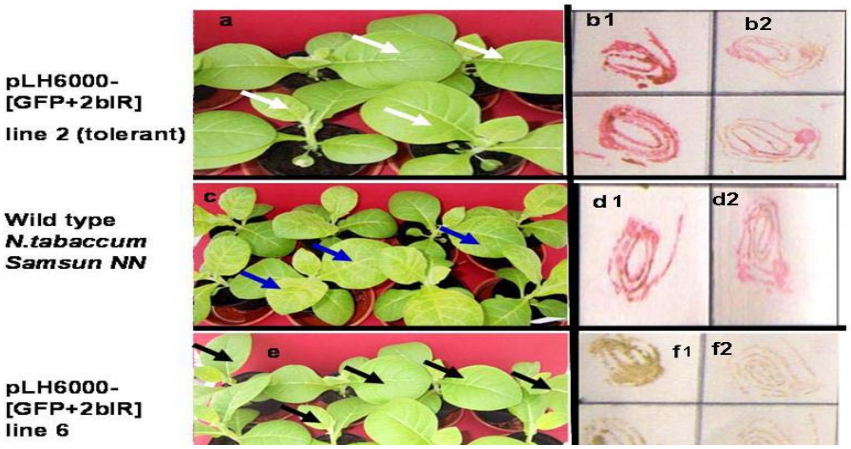

Figure12. Pattern of different resistance phenotypes in transgenic *N. tabaccum Samsun* NN plants derived from pLH6000-[GFP+2bIR] when inoculated with CMV_{AN} 14 d.p.i..
(**a**): symptomless on transgenic plants of pLH6000-[GFP+2bIR] line 2; (**b**): tissue print immunoblot assays of upper noninoculated (**b1**) and inoculated leaves (**b2**) of pLH6000-[GFP+2bIR] line 2, virus was detected; (**c**) CMV disease symptoms on upper

non-inoculated leaves of non-transformants (wild type); (**d**) tissue print immunoblot assays of upper noninoculated (**d1**) and inoculated leaves (**d2**) of non-transformants, virus was detected; (**e**): symptomless in transgenic plants of pLH6000-[GFP+2bIR] line 6 and exhibited immunity to the challenging CMV_{AN}; (**f**): tissue print immunoblot assays of upper noninoculated (**f1**) and inoculated leaves (**f2**) of pLH6000-[GFP+2bIR] line 6, virus could not be detected.

In summary, the construct pBIN19-GFP+2bIR induced higher efficiency of resistance in two different tobacco species, and furthermore all resistant plants were immune. However, the efficiency of resistance in *N. benthamiana* (67%) was higher than in *N. tabaccum Samsun* NN (40%). In addition, immune *N. tabaccum Samsun* NN plants from pBIN19-GFP+2bIR were observed (Table 12), while not in *N. tabaccum Samsun* NN transformed with pBIN19-2bIR (3.5.6, Table 9). In *N. benthamiana*, a higher efficiency of resistance was obtained in plants transformed with pBIN19-GFP+2bIR but not from pBIN19-2bIR (Table 12 and Table 9).

For the construct pLH6000-GFP+2bIR, a higher efficiency of resistance (100%) was observed in *N. tabaccum Samsun* NN but not in *N. benthamiana* (4%) (Table 12). Furthermore, three tested *N. tabaccum Samsun* NN lines exhibited immunity or tolerance to the challenging virus. However, the same results were not observed in *N. benthamiana.* A higher efficiency for resistance was observed in *N. benthamiana* from pLH6000-2bIR but not from pLH6000-[GFP+2bIR], while the reverse order was observed in *N. tabaccum Samsun* NN (Table 12 and Table 9).

In contrast, *N. tabaccum Samsun* NN plants from 2bIR and GFP+2bIR the resistance efficiency was enhanced, irrespective of the use of pLH6000 and pBIN19 as the binary vector. In *N. benthamiana* plants from 2bIR and GFP+2bIR the resistance efficiency was enhanced in pBIN19, whereas the reverse order was observed in pLH6000 (Fig.13). However, the common tendency was a higher efficiency of resistance in GFP+2bIR but not in 2bIR.

Table 12. Resistance variation in transgenic *N. benthamiana* and *N. tabaccum Samsun* NN plants transformed with GFP+2bIR

Transgenic lines	*N. benthamiana*					Transgenic lines	*N. tabaccum* Samsun				
	Immun-ity	tolerant	recovery	Suscep-tible	Resistance efficiency%		Immun-ity	tolerant	recovery	Suscep-tible	[3]Resistance efficiency%
[1]pBin-[GFP+2bIR]line1	**8**	**-**	**-**	**-**	100.00	pBin-[GFP+2bIR]line1	**-**	**-**	**-**	8	-
pBin-[GFP+2bIR]line2	**8**	**-**	**-**	**-**	100.00	pBin-[GFP+2bIR]line2	**-**	**-**	**-**	8	-

Transgenic lines	*N. benthamiana*					Transgenic lines	*N. tabaccum* Samsun				
	Immun -ity	tolerant	recovery	Suscep- tible	Resistance efficiency%		Immun -ity	tolerant	recovery	Suscep -tible	[3]Resistance efficiency%
pBin-[GFP+2bIR]line3	**8**	**-**	**-**	**-**	100.00	pBin-[GFP+2bIR]line3	**-**	**-**	**-**	8	-
pBin-[GFP+2bIR]line4	**4**	**-**	**-**	**4**	50.00	pBin-[GFP+2bIR]line4	**-**	**-**	**-**	8	-
pBin-[GFP+2bIR]line5	**7**	**-**	**-**	**1**	87.50	pBin-[GFP+2bIR]line5	**8**	**-**	**-**	-	100.00
pBin-[GFP+2bIR]line6	**-**	**-**	**-**	**8**	-	pBin-[GFP+2bIR]line6	**-**	**-**	**-**	8	-
pBin-[GFP+2bIR]line7	**8**	**-**	**-**	**-**	100.00	pBin-[GFP+2bIR]line7	**8**	**-**	**-**	-	100.00
pBin-[GFP+2bIR]line8	**-**	**-**	**-**	**8**	-	pBin-[GFP+2bIR]line8	**7**	**-**	**-**	1	87.50
pBin-[GFP+2bIR] line9	**-**	**-**	**-**	**8**	-	pBin-[GFP+2bIR]line9	**4**	**-**	**-**	4	50.00
						pBin-[GFP+2bIR]line10	**-**	**-**	**-**	8	-
[2]pLH-[GFP+2bIR]line1	**1**	**-**	**-**	**7**	12.50	pLH-[GFP+2bIR]line2	**3**	**5**	**-**	-	100.00
pLH-[GFP+2bIR]line5	**-**	**-**	**-**	**8**	**-**	pLH-[GFP+2bIR]line3	**4**	**4**	**-**	-	100.00
pLH-[GFP+2bIR]line6	**-**	**-**	**-**	**8**	**-**	pLH-[GFP+2bIR]line6	**8**	**-**	**-**	-	100.00

[1]pBin: pBin19. [2]pLH: pLH6000. "-": not found. The highlighted frame indicated those two lines were selected to broad-resistance experiments (3.7); [3]Resistance efficiency (%) of each tested line was calculated: No. of resistance plants (containing immunity, tolerant and recovery)/ No. of screened plants.

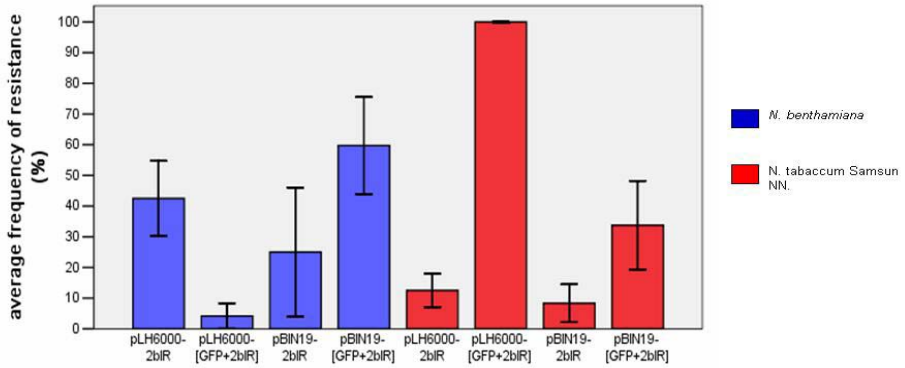

Figure13. Comparison of the frequency of the resistance in transgenic *N. benthamiana* and *N. tabaccum Samsun* NN transformed with 2bIR and GFP+2bIR in different binary vectors

3.7 Broad -resistance against several different CMV isolates in transgenic N. *benthamiana* plants transformed with pBIN19-[GFP+2bIR] and pBIN19-2bIR

Previous results have indicated that immunity or other kind of resistance on several lines of transgenic *N. benthamiana* derived from the construct of pBIN19-[GFP+2bIR] as well as line 11 of pBIN19-2bIR (3.5.6, Table 9 and 12) exists when challenged with a homologous isolate CMV_{AN}. To investigate whether such transgenic [GFP+2bIR] *N. benthamiana* plants have a broad-resistance against different CMV isolates belonging to different sero- and subgroups or known resistance-breaking CMV isolates, therefore the highly resistant transformants were challenged with different purified CMV isolates adjusted to the same specific infectivity (3.5.1).

3.7.1 Sequence comparison of the 2b gene from different subgroup CMV isolates that were used for resistance testing of [GFP+2bIR] harboring plants

Five different CMV isolates were used as challenging viruses for the transgenic plants (Table 13). Subgroup IB P3613 and KS44 are resistance-breaking on chili PBC370 plants. $CMV_{\triangle AN}$ is a reassortant consists of (2.1.1) RNA 1 and RNA 3 from CMV_{Fny}, and a replacement of 1100 bp from nt-position 1841 to 2958 on RNA 2 of CMV_{Fny} with the corresponding fragment from CMV_{AN}, which belongs to subgroup IB. CMV isolate TR52 belongs to subgroup IA and PV0420 to subgroup II, both are originate from USA.

Comparisons were made on nucleotide basis against the sequence used for the construct, CMV_{AN} (2.2.29 and Fig. 14). The 2b gene revealed 92% identity with KS44, 88% identity with P3613, 83% identity with RT52 and 56% identity with PV0420. The alignment revealed, 30 nt in 2b gene from PV0420 and 3 nt in 2b gene from RT52 have been deleted (Fig. 14).

However, the alignment of 2b genes on amino acid (aa) basis revealed two well conserved regions, at position 38 aa to 47 aa and 85 aa to 99 aa. These areas are included in the gene constructs 2bIR (3.1.7 and 3.2.4) and GFP+2bIR (3.1.8 and 3.2.5) (data not shown).

Table 13. Details of CMV isolates used as challenging viruses

CMV isolates	sero- and subgroups	Length of 2b gene	Original host plants	original isolated from
AN	I B	336	chili	India
P3613	I B	336	chili	China
KS44	I B	336	chili	Thailand
ΔAN	I B	336	chili	reassortant
RT52	I A	333	squash	USA
PV0420	II	303	pepper	USA

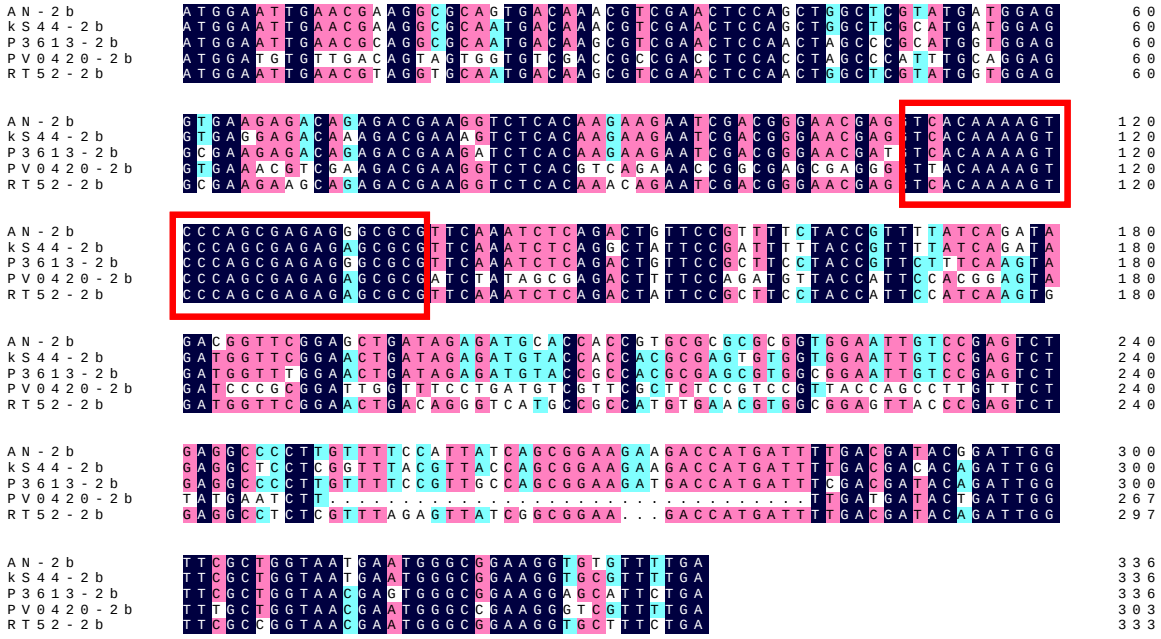

Figure 14. Alignment of 2b gene encoded from different subgroups.
CMV_{AN}, CMV_{KS44} and CMV_{P3613} belong to subgroup IB. CMV_{PV0420} belong to group II. CMV_{RT52} belongs to subgroup IA. The sequence of 2b from reassortant $CMV_{\Delta AN}$ and CMV_{AN} is identical. The red highlighted frame a 23 nt conserved region among the five isolates is indicated.

3.7.2 Resistance testing on the F1 generation of transgenic *N. benthamiana* plants against different CMV isolates

The same screening system setup (3.5.1) was used for challenging the transformed plants with different isolates. Line 11 from pBIN19-2bIR (3.5.6 and Table 9) as well as line 1 and 3 from pBIN19-[GFP+2bIR] (3.6 and Table 12) was used to challenge with purified viruses. Three lines showed higher efficiency of resistance when challenged with the homologous isolate. To

compare the results obtained with the different virus isolates, they were adjusted to the same specific infectivity of 30~60 local lesions per 10 µl inoculum per *C. quinoa* leaves. This led to the following concentration for each isolate: 75µg/ml of CMV_{AN}, 60µg/ml of $CMV_{\triangle AN}$, 60µg/ml of CMV_{P3613}, 50µg/ml of CMV_{KS44}, 150µg/ml of CMV_{PV0420} and 40µg/ml of CMV_{RT52} (Figure 15).

Wild type *N. benthamiana* plants showed typical CMV disease symptom of curling down, mosaic on leaves and dwarfing of plants 10 to 14 d.p.i. when inoculation with p3613, KS44, RT52, △AN and AN. Symptoms of mild mosaic and slightly curling down of leaves were observed when inoculated with PV0420 (Figure 16).

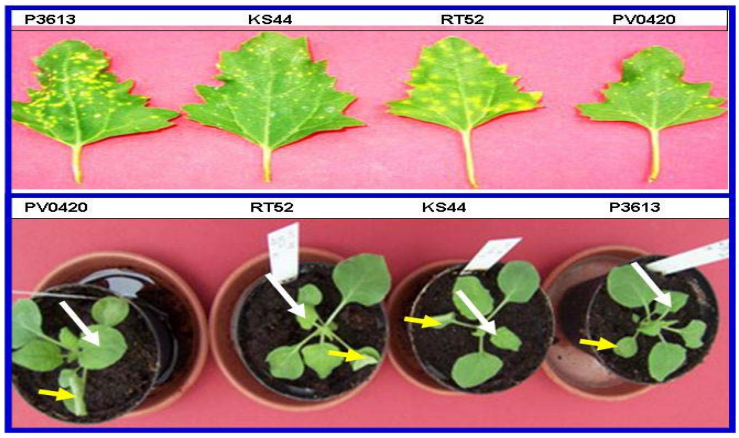

Figure 15. Different pattern of symptom expression on non-transformed *N. benthamiana* plants at 10d.p.i. and infectivity testing on *C. quinoa* inoculated with P3613, KS44, RT52 and PV0420. White arrows indicate typical disease symptoms of each isolate; yellow arrows indicate inoculated leaf of each plant.

Three tested lines (line 11 of pBIN19-2bIR, line 1 and line 3 of pBIN-GFP+2bIR) were resistant against all challenging viruses, although resistance variation was observed.

When RT52 was used as inoculum, one out of eight tested plants from line 1 of pBIN19-[GFP+2bIR] transformed *N. benthamiana* delayed visual symptoms for 7 days with a very mild mosaic on upper newly emerging leaves. Virus was detectable in this plant by tissue print immunoblot assay 14 and 21 d.p.i.(2.2.26). This plant was infected and virus could spread systemically (Figure 16). However, the visual symptoms disappeared on the upper non-inoculated leaves 28 d.p.i. The other seven plants of this line were immune to RT52. This result can deduce that the F1 generation was heterozygous (Table 14).

When the other two resistance-breaking isolates, KS44 and P3613, were used as inoculum, all tested plants from line 1 of pBIN19-[GFP+2bIR] transformed *N.benthamiana* were immune and remained no symptom in their life time. These immune plants were confirmed by tissue print immunoblot assays 14 and 21 d.p.i. (2.2.26). The same results were observed when CMV-$^{\Delta}$AN and PV0420 served as inocula (Table 14, Figure 17).

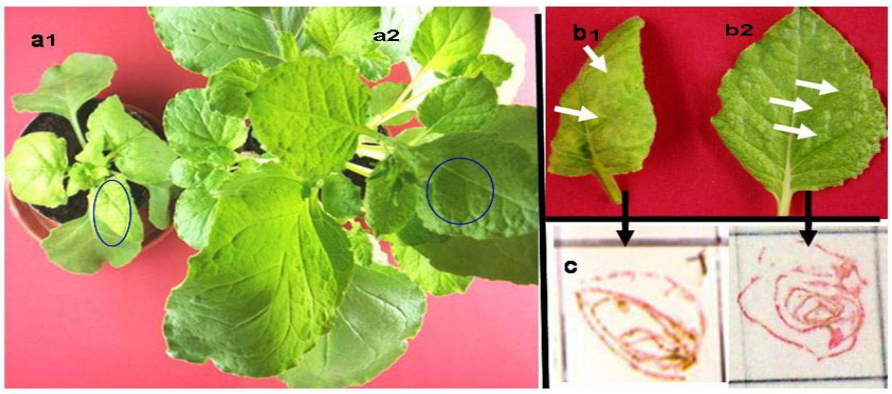

Figure 16. Pattern of symptom expression on upper non-inoculated leaves between susceptible non-transformants and a tolerant transformed plant from line 1 of pBIN19-[GFP+2bIR] in *N. benthamiana* challenged with CMV_{RT52} 14 d.p.i.

(**a**): typical symptoms on upper non-inoculated leaves from susceptible non-transformants (**a1**) and mild mosaic symptoms on upper non-inoculated leaves from line 1 of pBIN19-[GFP+2bIR] (**a2**); **(b)**: magnified symptoms leaves from blue loops in (**a1** to **b1; a2** to **b2**), white arrows indicated symptoms on leaves; (**c**): tissue print immunoblot assays showed virus was detected.

When transgenic *N. benthamiana* plants from line 3 of pBIN19-[GFP+2bIR] were challenged with CMV_{KS44}, one out of eight tested plants displayed typical symptoms of mild mosaic and curling down on upper non-inoculated leaves 10 d.p.i. and the visual symptom attenuated 28d.p.i. (Table 14). All other tested plants were symptomless and were determined to be immune by tissue print immunoblot assays 14 and 21 d.p.i. (Table 14). When P3613, RT52, AN and PV0420 were used as inoculums, all tested plants were immune and able to produce seeds.

When transgenic *N. benthamiana* plants from line 11 of pBIN19-2bIR were challenged with the serogroup II isolate PV0420, three out of eight tested plants developed visual symptoms of mild mosaic and curling down on upper non-inoculated leaves delayed. Virus was detectable in plants by tissue print immunoblot assay 14 d.p.i. All other tested plants showed immune phenotype when P3613, RT52, AN and KS44 were used as inocula (Table 14).

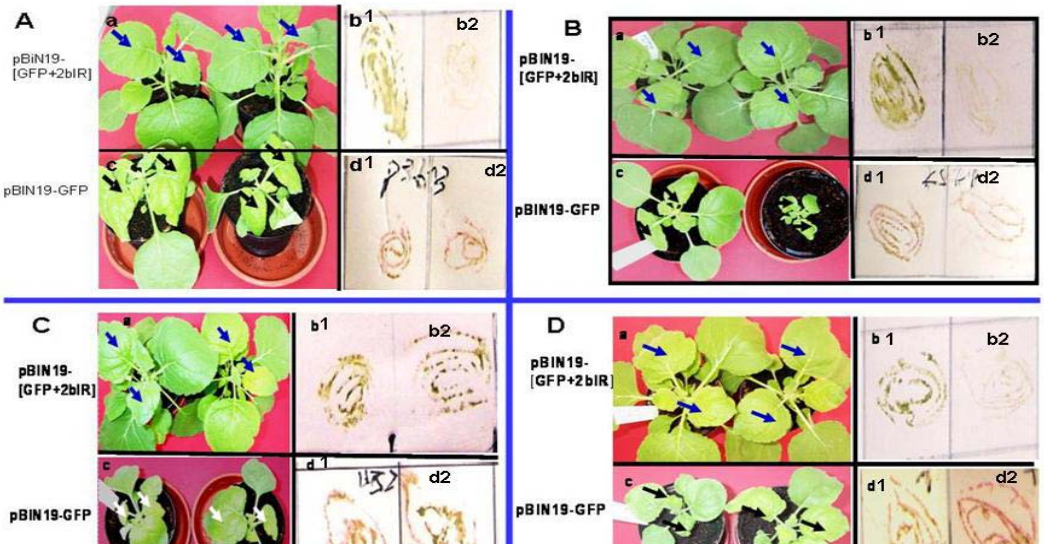

Figure17. Patterns of symptoms expression in transgenic *N. benthamiana* plants derived from pBIN19-GFP and pBIN19-[GFP+2bIR] line1 challenged with p3613, KS44, RT52 and PV0420 at 14 d.p.i.

A: Symptom expression in transgenic *N. benthamiana* plants derived from pBIN19-GFP and pBIN19-[GFP+2bIR] line1 when challenged with p3613 (**a**): no symptom on transgenic plants of pBIN19-[GFP+2bIR], blue arrows indicate no symptom on upper leaves; (**b**): tissue print immunoblot assays of upper noninoculated (**b1**) and inoculated leaves (**b2**) of pBIN19-[GFP+2bIR] transgenic plants, virus could not be detected; (**c**): typical CMV disease symptoms on transgenic plants of pBIN19-GFP, black arrows indicate typical symptom on upper leaves; (**d**): tissue print immunoblot assays of upper noninoculated (**d1**) and inoculated leaves (**d2**) of pBIN19-GFP transgenic plants, virus was detected.

B: Symptom expression in transgenic *N. benthamiana* plants derived from pBIN19-GFP and pBIN19-[GFP+2bIR] when challenged with KS44. (**a**): no symptom on transgenic plants of pBIN19-[GFP+2bIR], blue arrows indicate no symptom on upper leaves; (**b**): tissue print immunoblot assays of upper noninoculated (**b1**) and inoculated leaves (**b2**) of pBIN19-[GFP+2bIR] transgenic plants, virus could not be detected; (**c**): typical CMV disease symptoms on transgenic plants of pBIN19-GFP, black arrows indicate typical symptom on upper leaves; (**d**): tissue print immunoblots assays of upper noninoculated (**d1**) and inoculated leaves (**d2**) of pBIN19-GFP transgenic plants, virus was detected.

C: Symptom expression in transgenic *N. benthamiana* plants derived from pBIN19-GFP and pBIN19-[GFP+2bIR] when challenged with RT52. (**a**): no symptom on transgenic plants of pBIN19-[GFP+2bIR], blue arrows indicate no symptom on upper leaves; (**b**): tissue print immunoblot assays of upper noninoculated (**b1**) and inoculated leaves (**b2**) of pBIN19-[GFP+2bIR] transgenic plants, virus could not be detected; (**c**): typical CMV disease symptoms on transgenic plants of pBIN19-GFP, white arrows indicate typical symptom on upper leaves; (**d**): tissue print immunoblots assays of upper noninoculated (**d1**) and inoculated leaves (**d2**) of pBIN19-GFP transgenic plants, virus was detected.

D: Symptom expression in transgenic *N. benthamiana* plants derived from pBIN19-GFP and pBIN19-[GFP+2bIR] when challenged with PV0420. (**a**): no symptom on transgenic plants of

pBIN19-[GFP+2bIR] 1, blue arrows indicate no symptom on upper leaves; (**b**): tissue print immunoblot assay of upper noninoculated (**b1**) and inoculated leaves (**b2**) of pBIN19-[GFP+2bIR] transgenic plants, virus could not be detected; (**c**): typical CMV disease symptoms on transgenic plants of pBIN19-GFP, black arrows indicate typical symptom on upper leaves; (**d**): tissue print immunoblots assays of upper noninoculated (**d1**) and inoculated leaves (**d2**) of pBIN19-GFP transgenic plants, virus was detected.

Table 14. Resistance testing of transgenic *N. benthamiana* plants from pBIN19-2bIR and pBIN19-[GFP+2bIR] with purified CMV isolates from different sero- and subgroups

Transgenic lines	Different isolates							
	CMV-P3613				CMV-KS44			
	immunity	**tolerant**	**recovery**	**Susceptible**	**immunity**	**tolerant**	**recovery**	**susceptible**
Line11 of 2bIR	**8**	**-**	**-**	**-**	**8**	**-**	**-**	**-**
Line1 of [GFP+2bIR]	**8**	**-**	**-**	**-**	**8**	**-**	**-**	**-**
Line3 of [GFP+2bIR]	**8**	**-**	**-**	**-**	**7**	**-**	**-**	**1**

Transgenic lines	Different isolates							
	CMV-AN				CMV-$^{\triangle}$AN			
	immunity	**tolerant**	**recovery**	**Susceptible**	**immunity**	**tolerant**	**recovery**	**susceptible**
Line11 of 2bIR	8	-	-	-	NT	NT	NT	NT
Line1 of [GFP+2bIR]	8	-	-	-	8	-	-	-
Line3 of [GFP+2bIR]	8	-	-	-	NT	NT	NT	NT

Transgenic lines	Different isolates							
	CMV-RT52				CMV-PV0420			
	immunity	**tolerant**	**recovery**	**Susceptible**	**immunity**	**tolerant**	**recovery**	**susceptible**
Line11 of 2bIR	8	-	-	-	5	-	-	3
Line1 of [GFP+2bIR]	7	1	-	-	8	-	-	-
Line3 of [GFP+2bIR]	8	-	-	-	8	-	-	-

"-" not found. NT: not tested.

In summary, transgenic *N. benthamiana* plants from the three selected lines (line 11 from pBIN-2bIR, line1 and line 3 from pBIN19-GFP+2bIR showed a broad resistance when they are challenged with different CMV isolates from subgroup I (IA and IB) and subgroup II. The non-immune plants, behaved tolerant or susceptible to the invading viruses.

In contrast, when subgroup II PV0420 as inoculum was used, line 1 and line 3 *N. benthamiana* transformed with pBIN19-GFP+2bIR exhibited higher efficiency of resistance than line 11 of pBIN19-2bIR (Table 14). When RT52, KS44, P3613 and AN as inocula, these three lines showed the frequency of resistance up to 100% (one plant from line1 of pBIN19-GFP+2bIR was tolerant to RT52). When ΔAN as inoculum was used, all plants from line 1 of pBIN19-GFP+2bIR were also immune to isolate ΔAN.

4 DISCUSSIONS

Cucumber mosaic virus (CMV) is an important pathogen on many crops in the world (Hull, 2002). So far, no durable and stable commercial resistant varieties have been applied to breeding programs and are available for agriculture. However, biotechnology became a feasible and practical approach to generate genetically modified crops (GM) to cope with diverse CMV isolates and many attempts have been published about pathogen derived resistance in plants generated via biotechnology (Goldbach *et al.*, 2003; Palukaitis and Garcia-Arenal, 2003). However, due to many different possible target plant species, the high variability of CMV isolates and different experimental testing systems, a comprehensive evaluation of the most efficient construct is difficult from the published data.

Thus, in this work several different gene constructs were generated in two different binary vectors pBIN19 and pLH6000, which are based on RNA2 and RNA3 of CMV_{AN}. Resistant transgenic tobacco plants were selected through comparative bioassays in a standardized screening system. Resistance variation was observed, which was independent of the transformed tobacco species and/or binary vectors used for transformation. Resistance screening revealed that constructs aiming for RNA-mediated resistance were more efficient when based on RNA2 rather than on RNA3 in the two tobacco species. The resistance, achieved with the inverted repeat construct of the CP gene (CPIR) from isolate CMV-PV0506, was reduced, when compared with literature data (e.g. Knierim, 2006). This is probably due to the heterologous inoculum CMV_{AN} used for challenging. However, the resistance tests of the inverted repeat construct containing the non translatable 2b ($^{\triangle}$2b) gene from CMV_{AN} (2bIR), showed a good resistance in *N. benthamiana* but not in *N. tabaccum Samsun* NN, regardless of the binary vector used. At the same time transgenic *N. benthamiana* plants of pBIN19-2bIR line 11 further showed broad resistance against five CMV isolates from different sero- and subgroups.

To enhance the resistance efficiency, I use an asymmetric construct in further study, containing a verified non-target DNA (GFP), which could enhance the resistance of 2bIR in the two tobacco species. The obtained transgenic *N. benthamiana* plants of [pBIN19-GFP+2bIR] line1 and line3 showed extreme broad resistance against five different CMV isolates and a reassortant $Fny_{109}Fny_{209\triangle AN}Fny_{309}$. The detailed mechanisms, how the asymmetric addition enhanced the achieved resistance, needs to be explored further. Nevertheless 2bIR and GFP+2bIR were the most promising and worthwhile constructs applicable to some recalcitrant crops, like *Capsicum annuum* and ornamental plants, for transformation.

Based on intensive biological studies of the variability among different CMV strains collected throughout the chili production areas in Asia, it was found, that the CMV_{AN} from India was able to break the resistance of a chili variety, VC246, with good field resistance. Further, investigation mapped a fragment of 1100 bp on RNA2 of CMV_{AN}, which contained 336 bp of the 2b ORF and 764 bp of the 3' part of the 2a ORF (an overlapping region of 242 bp for 2a and 2b) (Zhang, 2005). The 2b protein functions as a viral suppressor of posttranscriptional RNA silencing a virus defence of the host plants (Lakatos *et al*., 2006; Li and Ding, 2006). CMV 2b can also block salicylic acid mediated virus resistance in *Arabidopsis spp*. (Ji and Ding, 2001) and Diaz-Pendon *et al* (2007) showed that it could reduce virus silencing in *Arabidopsis spp*. effected on siRNAs. Recently, new functions of 2b gene like directing interspecies (CMV and TAV) recombination (Shi *et al*., 2008) and interfering with PTGS by binding 19 nt siRNA in a sequence-specific and length-preferred manner (Chen *et al.*, 2008), have been reported. Interestingly, 2b protein encoded by CMV and TAV, suppressed systemic silencing induced by sense-transgenes, but not by inverted repeat transgenes (Li *et al*., 2002; Cao *et al*. 2005; Yaegashi *et al*., 2007). In addition, Praveen *et al* (2008) further reported that the 2b gene can suppress the antiviral silencing at an early stage of viral infection through constitutive expression of sense and antisense 2b in *N. tabaccum* plants. Furthermore, Goto *et al.*(2007) showed 2b gene acts as VSRs with different activities due to differences of their sequences, thus it can be speculated that the 2b protein of the AN-isolate has stronger abilities of inhibiting gene silencing than that of other isolates. It is now generally believed, that in plants the 2b gene-mediated resistance is more desirable. This hypothesis was checked by generating fragments of 2a and 2b, in which the start codons of both

genes were deleted (Δ2a+2b and Δ2a+Δ2b) and incorporated in both binary vectors pBIN19 and pHL6000.

Until the complete understanding of the mechanisms underlying virus induced gene silencing (VIGS), the most widely used method to confer resistance against CMV, as well as other plant viruses, was the introduction of a translatable CP gene (Cuozzo *et al*., 1988; Yie *et al*., 1992; Gonsalves *et al*., 1992, 1994; Nakajima *et al*., 1993; Okuno *et al*., 1993a, b; Rizos *et al*., 1996; Kaniewwski *et al*., 1999; Jacquemond *et al*., 2001; Srivastava *et al*., 2008;). This could be explained by comparing the sequence data from Genebank, where the homology of CMV-CP genes from different isolates is above 90%, indicating a multiple-resistance against CMV is likely to be achieved by CP in a protein-mediated approach (De Haan et al., 1992). Later, also replicase-mediated resistance against CMV has been documented (Wintermantel *et al*., 1997; Wintermantel and Zaitlin, 2000). Furthermore, extensive studies to induce resistance with truncated CP or 2a protein expressed in transgenic plants against CMV have been reported (Wintermantel and Zaitlin, 2000).

Later, when the role of dsRNA for the induction of PTGS and VIGS is well known, inverted repeat gene constructs were designed and transformed into plants, where they demonstrated to be more efficient approach to obtain virus resistance, also against CMV (Kalanditis *et al*., 2002; Chen *et al.,* 2004; Knierim, 2006). For comparative purposes and to exclude a protein-mediated resistance mechanism, the start codon (ATG) from the CP and 2b protein was deleted to render the mRNA untranslatable.

Resistance screening revealed that resistance efficiency in *N. benthamiana* plants harboring single gene constructs in pLH6000 was higher than in pBIN19 (Table 5, Table 6 and Table 7), however, the same variation was not observed in *N. tabaccum Samsun* NN. It indicates that transformation with the binary vector pLH6000 led to a larger number of resistant plants than the binary vector pBIN19 and this was independent of the plant species used for transformation. On the other hand, it indicates that pLH6000 was more effective in inducing resistance in *N. benthamiana* plants than pBIN19.

Dougherty *et al*. (1995) proposed the threshold model of gene silencing, which implies, that gene silencing is triggered when the transcript level of the gene of interest reaches a certain level. However, agroinfiltration experiments with a GFP construct cloned in the two vectors revealed lower expression efficiency for the pLH6000 as compared to the pBIN19 vector. A similar observation has been published by Schwach *et al*. (2004), where the best resistance against TSWV was observed in transgenic tobacco with the lowest expression rate. It might be possible that the transcription level of the two binary vectors differ and therefore cause the differences in resistance level.

In contrast to *N. benthamiana*, all transgenic *N. tabaccum* plants from three single gene constructs in pLH6000 exhibited very low resistance efficiency or did not show resistance at all (Table 5, Table 6 and Table 7). This indicated that resistance variation happened in both tobacco species, although the tobacco species have a relative close relationship in botany. It is speculated, that the level of mRNA in *N. tabaccum* is lower than in *N. benthamiana* (Kaniewski *et al*., 1999). However, it is not clear what caused this phenomenon.

Transgenic *N. tabaccum* plants harboring single gene constructs derived from pBIN19 showed tolerant plants and recovery in a few plants at 35 d.p.i., while transgenic *N. benthamiana* did not show resistance at all (Table 5, Table 6 and Table 7). However, a higher resistance efficiency was observed in transgenic plants harboring the CP gene (Cuozzo *et al*., 1988; Rizos *et al*., 1996; Kaniewski *et al*., 1999). To compare the results from previous reports with this study, the resistance efficiency of transgenic plants derived from pBIN19 was marginal at best, which is in agreement with previous results from Chen *et al*. (2004). In spite of the close relationship between *N. benthamiana* and *N. tabaccum*, their different genetic background could lead to different expression patterns via interactions between gene constructs and host plants. This can be deduced from plant gene expression patterns after virus infection of tobacco plants as determined by microarrays (Senthil *et al*., 2005). It indicates, resistance variation would occur in the chili plants, if chili plants are used to transform with these gene constructs.

In the mechanism of PTGS, the RNA-dependent RNA polymerases (RdRP) from viruses or host plants plays critical roles in amplifying and maintaining gene silencing in plants (Dalmay *et al.,*

2000). Interestingly, Yang *et al*. (2004) discovered two genes encoding RdRPs in *N. benthamiana* plants; one was identical in sequence with SDE1/SGS2 (Accession No. AF268093 in NCBI)(RdRP inducing virus resistance in *Arabidopsis* spp.) required for maintenance of transgene silencing by expressing dsRNA, another was mutated in nature and similar to the salicylic acid (SA)-inducible RdRP from *N. tabaccum* required for defence against virus, but is associated with increased susceptibility to viruses in *N. benthamiana*. In comparison with SDE1/SGS2, nucleic acids alignments revealed identities of 100% with the RdRP from *N. benthamiana* (AAU21242), 72% with another RdRP (natural mutated RDRP) from *N. benthamiana* (AAU21243) and 37% with the RdRP from *N. tabaccum Samsun* NN (AJ011576), respectively. These findings seemed to imply, that resistance variation in transgenic *N. benthamiana* plants might differ in efficiency depended on the competition between two different RdRP's and substrate siRNA (Kalantidis *et al*., 2002; Chen *et al*., 2004; Bucher *et al*., 2006; Kamachi *et al*., 2007), whereas lower resistance efficiency in *N. tabaccum* could be due to the lower activity of its RdRP. Regarding RdRP, this likely explains why *N. benthamiana* is used so frequently as model plant to study gene silencing of crops from the *Solanaceae* family. Recently it was reported, that RdRP is required for gene silencing in plants (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Qu *et al*., 2005; Herr *et al*., 2005, Schwach *et al*., 2005) and in *Neurospora crassa* (Cogoni and Macino, 1999).

The first virus-resistant transgenic plants have been described 1986 (Powell-Abel *et al*.), . Since then, biosafety of transgenic plants and plant products was always of concern. The topics of concern ranged from gene flow and the production of allergic protein towards recombination dependent modification of different plant pathogens in transgenic plants. Recombination between mRNA and viral RNA has been demonstrated in transgenic plants containing viral sequence from different viruses (Tepfer, 2002), *Cowpea chlorotic mottle virus* (CCMV) (Allison *et al*., 1990), PPV and *Tobacco mosaic virus* (TMV) (Beck *et al*., 1990), *Tomato bushy stunt virus* (TBSV) (White *et al*., 1994) and CMV (Turturo *et al*., 2008). So it is necessary to evaluate the potential risk of expressing viral sequence of 2b gene or CP gene in plants. For that, only a non-translation construct of CP and translation as well as non-translation constructs of 2b were included and tested in my study. Since the construct GFP+2bIR is able to express GFP, it should

be modified to a non-translation construct for further testing and finally for commercial use.

Although the 2b protein encoded by CMV is known to suppress silencing by inactivating the silencing signals (Brigneti *et al*., 1998; Lucy *et al*., 2000; Ji and Ding, 2001; Guo and Ding, 2002), transgenic *N.benthamiana* plants with untranslatable 2b from CMV_{Fny} (Ji and Ding, 2001) were challenged by agroinfiltration with (*Potato leaf roll virus*) PLRV-GFP, his result showed untranslatable 2b gene from CMV_{Fny} also exhibited weak gene silencing activity (Taliansky *et al.,* 2004). This could lead to a possible explanation for the effect of the ∆2b constructs in my study. The ∆2b gene used in my study may contain some key functional motif, like dsRNA binding domain of 2b gene, albeit modified (Mayers *et al*., 2000). At the same time, it also indicated that mRNA of 2bIR in line 11 of pBIN19-2bIR was very stable as well as line 1 and line 3 of pBIN19-[GFP+2bIR]. However, the variability of resistance efficiency can always be explained by the position effects in the plant genome.

For the two non-translational constructs, it was extremely evident, that transformation with the vector pLH6000 resulted in good resistance, whereas the plants transformed with the pBIN19 vector, although successful, resulted in very few resistant plants. This is surprising, since agroinfiltrations done with GFP constructs in both vectors revealed a very low translation of GFP when the GFP was in the pLH6000 vector in contrast to the well visible translation of the GFP when it was cloned in pBIN19. The fact that good resistant plants do not depend on the transcription level was already described by Schwach *et al*. (2004), who observed best results for TSWV in transgenic plants associated with very low transcript levels.

N.benthamiana and *N. tabaccum* plants derived from pLH6000-CPIR and pBIN19-CPIR showed resistance at a very low frequency when inoculated with CMV_{AN}, although sequence alignment showed an identity of 94% for the CP genes of CMV_{pv0506} and CMV_{AN}. In comparison with the results from Knierim (2006), he found resistance levels up to 50% in transgenic *N.benthamiana* when inoculated with the homologous isolate CMV_{pv0506}. The results in my study suggested, that the resistance induced by CPIR was highly sequence specific, which is consistent with RNA-mediated resistance (Kalantidis *et al*., 2002; Chen *et al*., 2004) and explains the lower resistance efficiency in my tests when challenging with a virus isolate differencing from the

source of the transgene.

As summarized in Table 9, besides line 11 of pBIN19-2bIR, all tested lines of transgenic *N. benthamiana* plants from pLH6000-2bIR showed a high frequency of resistance. In contrast, all tested lines of *N. tabaccum* derived from both binary vectors did not show the immunity type of resistance, although some plants recovered at 35 d.p.i. It is well known, that inverted repeat constructs have a higher efficiency to induce resistance in plants (Chen *et al*, 2004; Fuentes *et al*., 2006; Zrachya *et al*., 2007).

Chen *et al.* (2004) used a CMV RNA2-based inverted repeat construct containing the 3' part of 2a gene and the 2b gene. The resistance variation of his transgenic *N. benthamiana* plants against CMV revealed that the differences might due to the parts of the two sequences he used (Chen *et al*., 2004). His long inverted repeat (LIR) covers 1534 bp and induced resistance in 75% of the plants, while small inverted repeat (SIR) covers 490 bp and induced only 30% resistant plants. In my study, the resistance frequency ranged between 30-40% in *N. benthamiana* plants derived from pLH6000-2bIR and pBIN19-2bIR (Table 9). The 2bIR insert covers a length of 549 bp. These results are similar to that of Chen's SIR, although a different region of RNA2 was used for my construct. However, in both cases, due to the overlap of the ORF's of 2a and 2b a silencing reaction could be induced against both targets.

The presence of an intron in inverted repeat constructs enhances their silencing efficiency (Smith *et al*., 2000; Wesley *et al*., 2001), which is based on more stable mRNA production via splicing of introns. In plants, introns also can act post-transcriptionally to increase the accumulation of mRNA by stabilizing transcripts. This has been demonstrated in maize (Rethmeier *et al*., 1997) and in *Arabidopsis thaliana* (Wang *et al*., 2002). However, Heilersig *et al*. (2006) and Chen *et al*. (2004) demonstrated that the presence of an intron in inverted repeat constructs did not enhance the efficiency of PTGS. Taken together, introns in inverted repeat constructs could be good for formation of stable duplex of dsRNA and assembling two arms of hairpin constructs during construction, although the role of the intron ST-LS1 IV2 from potato (Zhao *et al.*, 2001) on resistance variation has not been analyzed in my studies. Interestingly, transformants of *N. tabaccum* plants, derived from pBIN19-2bIR and pLH6000-2bIR, did not show resistance at all

on F1 generation level, but transformants of *N. tabaccum* from pBIN19-[GFP+2bIR] and pLH6000-[GFP+2bIR] exhibited extreme resistance against the infection with CMV_{AN}. Thus it is possible to speculate, that the intron ST-LS1 IV2 in 2bIR may be spliced incompletely or spliced not at all in plants from pBIN19-2bIR and pLH6000-2bIR.

When comparing the resistance induced by 2bIR with that of GFP+2bIR, an enhancement for the latter was clearly observed in both transgenic tobacco species. The rank of pBIN19-GFP+2bIR was *N. benthamiana* > *N. tabaccum*; while the observed rank was reversed for pLH6000-GFP+2bIR. The enhancement is consistent with the results obtained by others, who fused GFP with a single fragment or a peptide of the N gene from TSWV and reported enhanced resistance in tobacco plants (Pang *et al*., 1997; Rudolph *et al*., 2003). Considering the efficiency and stability of resistance, GFP+2bIR was the best gene construct in my study in both tobacco plants, while 2bIR ranked second.

Remarkably, transgenic *N. benthamiana* from pLH6000-2bIR line9 and pBIN19-2bIR line11 exhibited over 87.5% immunity as resistance (Table 9). Interestingly, the findings showed higher frequency of resistance of up to 100% in tested *N. benthamiana* lines derived from pBIN19-[GFP+2bIR], while the same results were also observed in *N. tabaccum* derived from pBIN19-[GFP+2bIR] and pLH6000-[GFP+2bIR] (Table 12). In addition, these resistant plants were confirmed by symptomatology and tissue print immunoblots assay at 14 and 21 d.p.i. as well as back inoculation. Moreover, the resistance efficiency of GFP+2bIR was obviously higher than that of 2bIR in both tobacco plants (Table 9 and Table 12). It seemed that the flanking sequence played an important role for the induction of resistance in the plants. Pang *et al.* (1997) and Rudolph *et al.* (2003) have demonstrated that a nontarget DNA might enhance RNA-mediated resistance against TSWV by fusing a GFP gene with a viral sequence from the N gene of TSWV.

According to the threshold model of gene silencing (Doughtery *et al*., 1995), it is likely, that the GFP gene stabilizes the mRNA of 2bIR and prevents rapid degradation, or increases the efficiency of transport of mRNA from nucleus to cytoplasm to trigger defense response by dsRNA-mediated resistance. Notably, my gene construct confirmed that a direct fusion between

GFP and 2bIR is not necessary, since GFP was separated by a spacer of 17 nt. Consistent with this assumption Zeng *et al* (2005) reported that flanking non-structural sequences were required for the efficient processing of primary miRNA hairpins.

The 110 bp fragment from the TSWV N gene did not induce resistance at all, but it exhibited a low level of resistance after fusion with the GFP gene when inoculated with homologous TSWV (Pang *et al.*, 1997). It could be that the flanking GFP gene stabilizes and activates latent resistance ability of the otherwise not effective viral sequence. This raises the question whether GFP could be replaced by any other non-target sequence, without loosing the enhancement effect.

Unexpectedly, transgenic *N. benthamiana* plants derived from line11 of pBIN19-2bIR as well as line1 and line 3 of pBIN19-[GFP+2bIR] exhibited multiple-resistance against five different CMV isolates (Table 14), while Chen *et al* (2004) did not observe multiple-resistance when inoculating with CMV_{LAS} (subgroup II) on transgenic *N. benthamiana* plants containing an inverted repeat construct of CMV RNA2. Furthermore, nucleic acids alignments of the 2b ORFs of the five different CMV isolates which are used for challenging in transgenic plants, revealed a high degree of variation. This variation was somehow reflected by the different symptom development, which were induced from each CMV isolates on *N. benthamiana* and *C.quinoa*. It was surprising, that transgenic *N. benthamiana* plants from these 3 lines tolerated these sequence variability unexpectedly well, when inoculated with the different CMV isolates, because RNA-mediated resistance requires at least 90% sequence identity (Ritzenthaler, 2005).

Although nucleic acid alignment of all five different 2b genes revealed a lower level of identities, only a fragment of 23 nt in 2b gene among five isolates is very conserved. Thus, it can be assumed that siRNA/miRNA from these 23 nt is enough to destroy silencing activity of 2b gene as a VSR, but this needs to be verified experimentally. Qu *et al*. (2007) modified *N. benthamiana* miR171 precursor as a backbone to target the CMV 2b gene; Niu *et al*. (2006) modified *Arabidopsis thaliana* miR159 precursors as backbone to target the p69 gene encoded *Turnip yellow mosaic virus* (TYMV) and HC-Pro gene encoded *Turnip mosaic virus* (TuMV), respectively. Their studies were based on the theory, that the changes of several nucleotides within a miRNA

21-nt sequence do not affect its biogenesis and maturation (Vaucheret *et al*., 2004; Guo *et al*., 2005). Furthermore, in plants unlike in animals, most mRNAs only contain one single miRNA-complementary site (Carrington and Ambros, 2003; Kidner and Martienssen, 2005), because plant miRNA is extremely conserved (Zhang *et al*., 2006). Based on these reasons, it is assumed, that multiple-resistance against CMV would be mediated by modification of miRNA precursors with conserved 23nt of 2b gene as backbone to target CMV 2b gene from different isolates.

The specific resistance in transgenic plants was screened by a standard method, which revealed: (I) both tobacco species used for testing the constructs gave different results with the same construct, indicating that results can probably not transferred 1:1 to other plant species. (II) The screening method allowed a fast selection of the most appropriate constructs. (III) Resistance variation was independent of the plant species and/or binary vectors used for transformation. (IV) The most desired resistance phenotype was immunity, which was observed to the highest extent in *N. benthamiana* plants independent of the vector used. For *N. tabaccum*, much less tested plants remained virus free; (V) The most suitable construct was GFP+2bIR which is proposed for further experiments.

For practical applications of the described constructs, some modifications appear necessary. First, it should be tested, if the GFP part as asymmetric addition can be replaced by some other sequence. Second, the biosafety and stability of the construct must be evaluated. Probably, first evaluations should be done in the greenhouse, where biotic stresses could be controlled.

To obtain desirable and optimal results, a possible prediction of the function of gene constructs is necessary and important before transformation or transfection into the desired host. At present, a transient expression assay by agroinfiltration (Schöb *et al.*, 1997; Kopertekh *and* Schiemann, 2005; Tenllado *et al.*, 2003 a, b) and the spray application of crude extracts of bacterially expressed dsRNA in *E.coli* strain HT115 (DE3) on plant surfaces (Tenllado *et al.*, 2003 b) exist for a rapid testing of resistance efficiency, especially for constructs aiming for RNA-mediated resistance. The two methods have been applied to predict the resistance induction of different gene constructs. However, the transient expression by agroinfiltration can vary due to the

concentration and volumes of delivered bacteria. In fact, if transient expression by agroinfiltration of all gene constructs is suitable to evaluate their resistance variation in different host plants, it will facilitate further studies. Crude extracts of dsRNA from HT115(DE3) with deficit of RNase III can be sprayed or mechanically inoculated on surfaces of different host plants. This would be easier to control and quantify in comparison to agroinfiltration. It will be interesting, to compare the resistance obtained by transient expression with the results presented in this study from stable transformants. If both methods of transient expression show comparable results with stable transformants an efficient selection procedure for construct modification will be available.

5 SUMMARY

Cucumber mosaic virus (CMV) is an economically important pathogen on chili plants in Asia. So far, no durable resistant chili varieties were available to obtain virus resistant plants through a classical breeding program. Therefore, several biotechnological approaches to generate resistant plants via virus induced gene silencing (VIGS) were tested. However, due to different target plant species, different CMV isolates and different experimental testing systems, an evaluation of the most efficient construct is very difficult from the published data.

To evaluate a suitable construct for generating CMV resistance in chilli, several constructs using the regions of the coat protein from RNA 3 and the suppressors of gene silencing expressed from RNA 2 on CMV genome were introduced into two different binary vectors (pLH6000 and pBIN19) as single gene or as an inverted repeat. Additionally, a chimeric construct GFP+2bIR was generated in both binary vectors. These constructs were transformed in two different tobacco species (*N. benthamiana* and *N. tabaccum*). The resistance of these transformants was evaluated using different CMV isolates in a standardized testing system. Immunity, tolerance and recovery phenotypes were verified by symptom expression and virus detection by tissue print immunoblots.

Resistance screening on F1 generation revealed that resistance variation between gene constructs and tobacco plants: for single gene constructs (△CP, △2a+2b and △2a+△2b in which start codons of CP and 2b genes were deleted) in pLH6000, the given resistance efficiency rank was pLH6000-△2a+2b > pLH6000-△2a+△2b > pLH6000-△CP in *N. benthamiana*, while the given rank was not clear in *N. tabaccum*; however, the given resistance efficiency rank of single gene constructs in pBIN19 was not clear in both tobacco plants because of a lower resistant efficiency. For 2bIR construct, the resistant efficiency in *N. benthamiana* was higher than *in N. tabaccum*, and therefore the given rank was pLH6000-2bIR > pBIN19-2bIR in *N. benthamiana* but not in *N. tabaccum*. For CPIR, the resistance variation was not clear in both tobacco plants when challenged with heterologous isolate CMV_{AN}. For GFP+2bIR, resistance efficiency was obviously enhanced in both tobacco species with exception of *N. benthamiana* in pLH6000-GFP+2bIR, all resistant plants were further verified to be immune to CMV-AN by

symptom expression, tissue print immunoblot and back inoculation.

In addition, three transgenic *N. benthamiana* lines from pBIN19-2bIR (one line) and pBIN19-GFP+2bIR (two lines) were further challenged with five different CMV isolates. These three lines exhibited broad-resistance against five different CMV isolates.

Taken together, (I) the resistance efficiency in tobacco species was ranged from 0 to 100%, which is independent of vectors and/or plant species. (II) Resistance using RNA 3 fragments is lower than with RNA 2 fragments. (III) A chimeric construct with nontarget DNA as flanking sequence showed higher resistant efficiency even when these lines were challenged with heterologous CMV isolates when compared with 2bIR constructs. However the construct should be optimized by exchanging the GFP with a viral sequence before using it to obtain resistant vegetable against CMV in Asian agriculture.

Zusammenfassung

Cucumber mosaic virus (CMV) verursacht in Asien bedeutende ökonomische Schäden an Chilli. Bis heute sind keine Chilli-Varietäten bekannt, die eine dauerhafte Resistenz über klassische Züchtungsprogramme ermöglichen. Aus diesem Grund wurden verschiedene gentechnologische Ansätze für eine virusinduzierte Resistenz (virus induced gene silencing, VIGS) getestet. In verschiedenen Publikationen wurden unterschiedliche Wirtspflanzen, unterschiedliche CMV-solate und verschiedene Testsysteme verwendet, deswegen ist eine Evaluierung des besten Konstruktes auf der Basis von publizierten Daten sehr schwierig.

Zur Bestimmung eines geeigneten Konstruktes zur Generierung von CMV-resistentem Chilli wurden verschiedene Bereiche des Hüllproteins der RNA 3 und des gene silencing suppressors der RNA 2 des CMV-Genoms in zwei unterschiedliche binäre Vektoren (pBIN 19 und pLH 6000) als „single gene“ oder als „inverted repeat“ Konstrukt kloniert. Zusätzlich wurde ein chimäres Konstrukt (GFP-2b IR) in beide Vektoren kloniert. Beide Konstrukte wurden jeweils in zwei Tabakarten (*Nicotiana benthamiana* und *Nicotiana tabaccum*) stabil transformiert. Die Resistenz dieser Isolate wurde mit verschiedenen CMV-Isolaten in einem standardisierten Testsystem evaluiert. Die Phänotypen Immunität, Toleranz und Erholung wurde anhand von Symptomausprägung und Virusnachweis in Gewebeabdrücken mit Hilfe von serologischer

Detektion beobachtet.

Die Resistenztestung der F1 Generation zeigte eine Variation der Resistenz abhängig vom Genkonstrukt und der Wirtspflanze: Für die „single gene“ Konstrukte ΔCP, Δ2a+2b und Δ2a+ Δ2b (in denen das Startkodon von CP bzw. 2b entfernt wurde) im binären Vektor pLH6000 zeigte die Reihenfolge pLH6000- Δ2a+2b > pLH6000- Δ2a+ Δ2b > pLH6000- ΔCP in *N. benthamiana*, während in transformierten *N. tabaccum* die Reihenfolge unklar war. Die Rangfolge in beiden Pflanzenspezies war unklar, wenn mit dem binären Vektor pBIN19 transformiert wurde, da hier generell eine geringe Resistenz beobachtet wurde. Die Resistenz für das 2bIR Konstrukt war in *N. benthamiana* höher als in *N. tabaccum* und folglich war die Güte der Resistenz in der Reihenfolge pLH6000-2bIR > pBIN19-2bIR in *N. benthamiana* aber nicht in *N. tabaccum*.

Für das Konstrukt CPIR folgte die Resistenz keiner erkennbaren Regel in beiden Wirtspflanzen für den Fall, dass mit dem heterologen Isolat CMV_{AN} infiziert wurde. Bei Pflanzen, die mit dem Konstrukt GFP+2bIR transformiert waren, war eine signifikant bessere Resistenz in beiden Wirtspflanzen zu beobachten, allerdings mit der Ausnahme von N. benthamiana transformiert mit dem Konstrukt pLH6000-GFP2bIR. Die Abwesenheit von Virus wurde bei als immun bewerteten Pflanzen mit Gewebeabdrücken und serologischer Detektion sowie Biotests bestätigt.

Zusätzlich zur Testung mit dem homologen Isolat wurden drei Linien (1 x pBIN19-2bIR und 2 x pBIN19-GFP+2bIR) mit weiteren Isolaten auf Resistenz überprüft. Alle drei Linien zeigten eine breite Resistenz gegenüber fünf verschiedenen CMV-Isolaten.

Zusammengefasst ergab sich Folgendes: (I) die Reistenzgüte in den beiden transformierten Tabakarten variierte von 0 bis 100 %, unabhängig vom Vektor und Pflanzenart. (II) Die Resistenz, die mit Fragmenten der RNA 3 erhalten wurde, war niedriger als diejenige, die mit Fragmenten der RNA 2 erhalten wurde. (III) Ein chimäres Konstrukt mit einer virusunabhängigen DNA als flankierende Sequenz zeigte eine bessere Resistenz als 2bIR-Konstrukte, und zwar sogar dann, wenn mit nicht-homologen Isolaten getestet wurde. Trotzdem sollte dieses chimäre Konstrukt optimiert werden, indem das GFP gegen virale Sequenzen ausgetauscht wird, bevor es zum Einsatz zur Erzeugung von CMV-resistentem Gemüse in Asien kommt.

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7. Appendix

7.1 Sequences

7.1.1 Sequence of 2x35S/GFP/Nos

AAGCTTGCATGCCTGCAGGTCAACATGGTGGAGCACGACACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGG
GCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGA
AAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGG
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CGACACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGG
AAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGC
GATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGA
AGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAA
GACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGAGAATTCTCAACACAACATATACAAAACAAACGAATCTCAAGC
AATCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTCTGAAAATTTTCACCATTTACGAACGATA
GCCATGGGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCA
GTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCTTGGCCAACACT
TGTCACTACTTTCTGTTATGGTGTACAATGCTTTTCAAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCCATGCCTGAGG
GATACGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCA
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ATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCC
AACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAAGGATCCT
CTAGAGTCCGCAAAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGG
AATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAA
ATTTCTAATTCCTAAAACCAAAATCCAGTGACCTGCAGGCATGCAAGCTT

7.1.2 Sequence of △2a+2b

TAGAGCCATGGTGAATTCTTGTTTGCTCACTTCATGAGCTTTGTTGATCGATTGAAGTTTTTGGACAGAATGACTCAGTCTTGTATCGATCA
ACTTTCACTCTTTTTCGAGTTGAAATACAGGAAGTCAGGGGCCGAGGCTGCTTTAATGTTAGGCGCCTTTAAGAAATATACCGCTAATTTCC
AATCCTATAAAGAGCTCTACTATTCAGATCGTCGTCAGTGCGAATTGATCAATTCGTTTAGTTGTGTGGAGTTAAGGATTGAGCGTTCGATT
TCTACTAAGCAGCGAAAGAAGAAAGATGGAATTGAACGAAGGCGCAGTGACAAACGTCGAACTCCAGCTGGCTCGTATGATGGAGGTGA
AGAGACAGAGACGAAGGTCTCACAAGAAGAATCGACGGGAACGAGGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAATCTCAGACTG
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CCCCTTGTTTTCCATTATCAGCGGAAGAAGACCATGATTTTGACGATACGGATTGGTTCGCTGGTAATGAATGGGCGGAAGGTGTGTTTTG
AATCTCCCCTTCCTTTTCTCCCGCCATTTCTGAGGCGGGAGCTGAGTTGGCAGTATTGCTCTAAACTGTCTGAAGTCACTAAACGCTTTGCG
GTGAACGGGTTGTCCATCCGGATCCGACGTC

7.1.3 △CP sequence of AN

CTAGAGCCATGGTGGACAAATCTGGATCAACCAGTGCTGGTCGTAATCGCCGACGTCGTCCGCGTCGCGGTTCCCGCTCCGCTTCCTCCTCG
CGGATGCCACATTTAGAGTCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGTCCTACTATTAACCACCCAACCTTT
GTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTCACATCTATTACCCTGAAGCCACCAAAAATAGACAAAGGGTCTTATTATGGCAAAA
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GAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATGCGCTCGGGACGGATGAGTTAGTACTTCATGTCGACATTGAGCACCAACGCATTCC
CACATCTGGAGTGCTCCCAGTTTGAACTCGTGTTTTCCAGAACCCTCCCTCCATTTTCTGAGGCGGGAGCTGAGTTGGCAGTGTTATTATAA
ACTGCCTGAAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCATCCAGGGATCCGACGTC

7.1.4 2b sequence of RT52

ATGGAATTGAACGTAGGTGCAATGACAAGCGTCGAACTCCAACTGGCTCGTATGGTGGAGGCGAAGAAGCAGAGACGAAGGTCTCACAA
ACAGAATCGACGGGAACGAGGTCACAAAAGTCCCAGCGAGAGAGCGCGTTCAAATCTCAGACTATTCCGCTTCCTACCATTCCATCAAGT
GGATGGTTCGGAACTGACAGGGTCATGCCGCCATGTGAACGTGGCGGAGTTACCCGAGTCTGAGGCCTCTCGTTTAGAGTTATCGGCGGAA
GACCATGATTTTGACGATACAGATTGGTTCGCCGGTAACGAATGGGCGGAAGGTGCTTTCTGA

7.1.5 2b sequence of KS44

ATGGAATTGAACGAAGGCGCAATGACAAACGTCGAACTCCAGCTGGCTCGCATGATGGAGGTGAGGAGACAAAGACGAAAGTCTCACAA
GAAGAATCGACGGGAACGAGGTCACAAAAGTCCCAGCGAGAGAGCGCGTTCAAATCTCAGGCTATTCCGATTTTTACCGTTTTATCAGATA
GATGGTTCGGAACTGATAGAGATGTACCACCACGCGAGTGTGGTGGAATTGTCCGAGTCTGAGGCTCCTCGGTTTACGTTACCAGCGGAAG
AAGACCATGATTTTGACGACACAGATTGGTTCGCTGGTAATGAATGGGCGGAAGGTGCGTTTTGA

7.1.6 2b sequence of P3613

ATGGAATTGAACGCAGGCGCAATGACAAGCGTCGAACTCCAACTAGCCCGCATGGTGGAGGCGAAGAGACAGAGACGAAGATCTCACAA
GAAGAATCGACGGGAACGATGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAATCTCAGACTGTTCCGCTTCCTACCGTTCTTTCAAGTA
GATGGTTTGGAACTGATAGAGATGTACCGCCACGCGAGCGTGGCGGAATTGTCCGAGTCTGAGGCCCCTTGTTTTCCGTTGCCAGCGGAAG
ATGACCATGATTTCGACGATACAGATTGGTTCGCTGGTAACGAGTGGGCGGAAGGAGCATTCTGA

7.1.7 2b sequence of PV0420

ATGGATGTGTTGACAGTAGTGGTGTCGACCGCCGACCTCCACCTAGCCCATTTGCAGGAGGTGAAACGTCGAAGACGAAGGTCTCACGTCA
GAAACCGGCGAGCGAGGGGTTACAAAAGTCCCAGCGAGAGAGCGCGATCTATAGCGAGACTTTTCCAGATGTTACCATTCCACGGAGTAG
ATCCCGCGGATTGGTTTCCTGATGTCGTTCGCTCTCCGTCCGTTACCAGCCTTGTTTCTTATGAATCTTTTGATGATACTGATTGGTTTGCTG
GTAACGAATGGGCCGAAGGGTCGTTTTGA

7.1.8 2b sequence of AN

ATGGAATTGAACGAAGGCGCAGTGACAAACGTCGAACTCCAGCTGGCTCGTATGATGGAGGTGAAGAGACAGAGACGAAGGTCTCACAA
GAAGAATCGACGGGAACGAGGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAATCTCAGACTGTTCCGTTTTCTACCGTTTTATCAGATA
GACGGTTCGGAGCTGATAGAGATGCACCACCGTGCGCGCGCGGTGGAATTGTCCGAGTCTGAGGCCCCTTGTTTTCCATTATCAGCGGAAG
AAGACCATGATTTTGACGATACGGATTGGTTCGCTGGTAATGAATGGGCGGAAGGTGTGTTTTGA

7.1.9 Sequence of △2a+△2b

GGTGAATTCTTGTTTGCTCACTTCATGAGCTTTGTTGACCGATTGAAGTTTTTGGACAGAATGACTCAGTCTTGTATCGATCAACTTTCACTC
TTTTTCGAGTTGAAATACAGGAAGTCAGGGGCCGAGGCTGCTTTAATGTTAGGCGCCTTTAAGAAATATACCGCTAATTTCCAATCCTATA
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CCTTTTCTCCCTCCAGTTTTCTGAGGCGGGAGCTGAGTTGGCAGTATTGCTATAAACTGTCTGAAGTCACTAAACGCTTTGCGGTGAACGGG
TTGTCCATCC

7.1.10 Sequence of intron ST-LS1 IV2

TCTAGATAAGTTTCTGCTTCTMCCTTTGATATATATATAATAATTATCCATTAATTAGTAGTAATATAATATTTCAAATATTTTTTTTCAAAA
TAAAAAGAATGTAGTATATAGCAATTGCTTTTCTGTAGTTTATAAGTGTGTATATTTTAATTTATAACTTTTCTAATATATGACCAAAATTTG
TTGATGTGCAGGCGAGCGCCTGCAG

7.1.11 Sequence of 2bIR

ATACAGAGCTCCATGGGCCGAGGCTGCTTTAATGTTAGGCGCCTTTAAGAAATATACCGCTAATTTCCAATCCTATAAAGAGCTCTACTATT
CAGATCGTCGTCAGTGCGAATTGATCAATTCGTTTAGTTGTGTGGAGTTAAGGATTGAGCGTTCGATTTCTACTAAGCAGCGAAAGAAGAA
AGTGGAATTGAACGAAGGCGCAGTGACGAACGTCGAACTCCAGCTGGCTCGTATGATGGAGGTGAAGAGACAGAGACGAAGGTCTCACA
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GAAGACCATGATTTTGACGATACGGATTGGTTCGCTGGTAATGAATGGGCGGAAGGTGCGTTTTGAATCTCCCCTTCCTTTTCTCCCTCCAG
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CTTAGTAGAAATCGAACGCTCAATCCTTAACTCCACACAACTAAACGAATTGATCAATTCGCACTGACGACGATCTGAATAGTAGAGCTCT
TTATAGGATTGGAAATTAGCGGTATATTTCTTAAAGGCGCCTAACATTAAAGCAGCCTCGGATCCTGGGATCC

7.1.12 Sequence of CPIR

TCGACTAGATAAGGTTCCCGCTCCGCTCCCTCCTCCGCGGATGCTACTTTTAGAGTCTTGTCGCAGCAGCTTTCGCGACTCAATAAGACATT
AGCAGCTGGTCGTCCAACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAAACCTGGATACACGTTCACATCTATTACCCTGAAG
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AGAATCAAATTTCGGCAAAGGATTAACTCGAATTTGAATGCGCGAAACAAGCTTCTTATCGAACTCCGTGACTGAATCAGGTAGCAACAAC
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AAAGGTTGGGTGGTTAATAGTTGGACGACCAGCTGCTAATGTCTTATTGAGTCGCGAAAGCTGCTGCGACAAGACTCTAAAAGTAGCATCC
GCGGAGGAGGGAGCGGAGCGGGAACCTTATCTAGTCGA

7.1.13 Sequence of GFP+2bIR

CCATGGGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGT
GGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCTTGGCCAACACTTG
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ACCGTCTATCTGATAAAACGGTAGAAAACGGAACAGTCTGAGATTTGAACGCGCCCTCTCGCTGGGACTTTTGTGACCTCGTTCCCGTCGA
TTCTTCTTGTGAGACCTTCGTCTCTGTCTCTTCACCTCCATCATACGAGCCAGCTGGAGTTCGACGTTCGTCACTGCGCCTTCGTTCAATTCC
ACTTTCTTCTTTCGCTGCTTAGTAGAAATCGAACGCTCAATCCTTAACTCCACACAACTAAACGAATTGATCAATTCGCACTGACGACGATC
TGAATAGTAGAGCTCTTTATAGGATTGGAAATTAGCGGTATATTTCTTAAAGGCGCCTAACATTAAAGCAGCCTCGGATCCTGGGATCC

7.1.14 Sequence of PV0506-CP

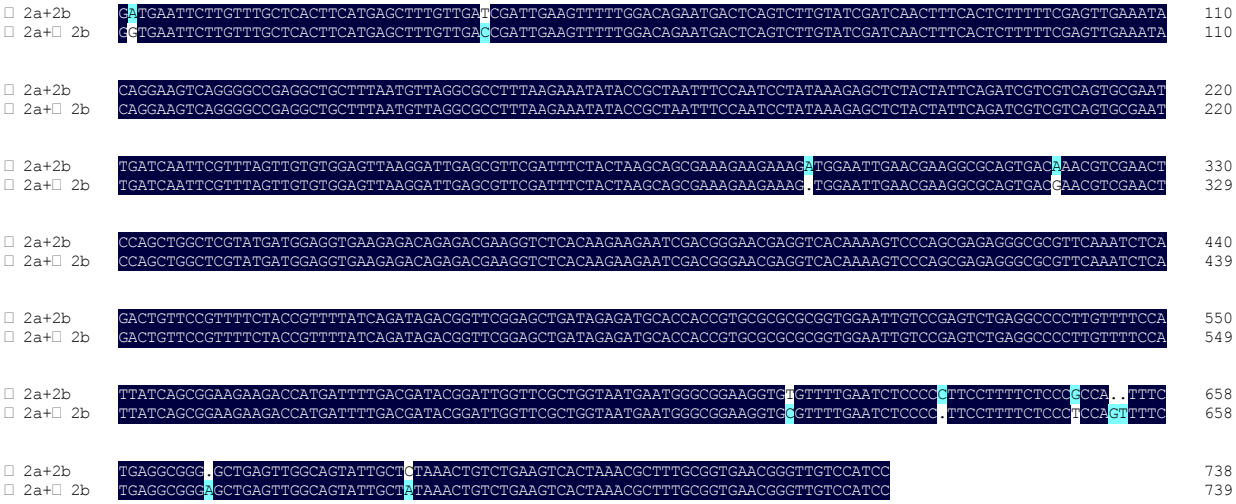
GTTCCCGCTCCGCTCCCTCCTCCGCGGATGCTACTTTTAGAGTCTTGTCGCAGCAGCTTTCGCGACTCAATAAGACATTAGCAGCTGGTCGT
CCAACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAAACCTGGATACACGTTCACATCTATTACCCTGAAGCCACCGAAAATAG
ACCGTGGGTCTTATTATGGTAAAAGGTTGTTGCTACCTGATTCAGTCACGGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTT
AATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTATCCGTTGCCGCTATCTCTGCTATG
TTTGCGGACGGAGCCTCACCGGTACTGGTTTATCAGTATGCTGCATCCGGCGTTCAAGCCAACAACAAATTGTTGTATGATCTTTCAGCGAT
GCGCGCTGATATTGGTGACATGAGAAAGTATAG

7.2 Alignments

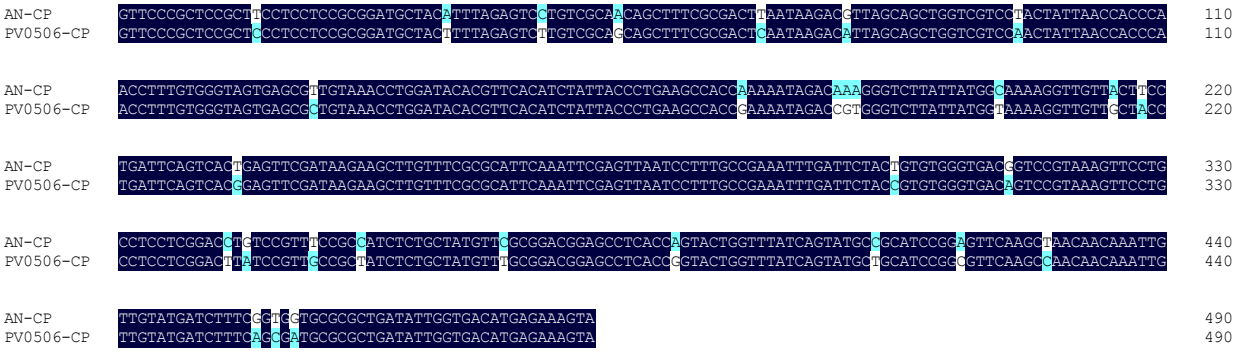
7.2.1 Alignment of AN-CP and AN-ΔCP

```
AN-CP     ATGGACAAATCTGGATCACCCAGTGCTGGTCGTAATCGCCGACGTCGTCCGCGTCGCGGTTCCCGCTCCGCTTCCTCCTCGCGGATGCTACATTTAGAGTCCTGTCGCA   110
AN-□ CP   GTGGACAAATCTGGATCAACCAGTGCTGGTCGTAATCGCCGACGTCGTCCGCGTCGCGGTTCCCGCTCCGCTTCCTCCTCGCGGATGCCACATTTAGAGTCCTGTCGCA   109

AN-CP     ACAGCTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTCACATCTATTA   220
AN-□ CP   ACAGCTTTCGCGACTTAATAAGACGTTAGCAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTCACATCTATTA   219

AN-CP     CCCTGAAGCCACCAAAAATAGACAAAGGGTCTTATTATGGCAAAAGGTTGTTACTTCCTGATTCAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGA   330
AN-□ CP   CCCTGAAGCCACCAAAAATAGACAAAGGGTCTTATTATGGCAAAAGGTTGTTACTTCCTGATTCAGCCACTGAGTTCGATAAGAAGCTTGTTTCGCGCACTCAAATTCGA   329

AN-CP     GTTAATCCTTTGCCGAAATTTGATTCTACTGTGTGGGTGACGGTCCGTAAAGTTCCTGCCTCCTCGGACCTGTCCGTTTCCGCCATCTCTGCTATGTTCGCGGACGGAGC   440
AN-□ CP   GTTAATCCTTTGCCGAAATTTGATTCTACTGTGTGGGTGACGGTCCGTAAAGTTCCTGCCTCCTCGGACCTGTCCGTTTCCGCCATCTCTGCTATGTTCGCGGCCGGAGC   439

AN-CP     CTCACCAGTACTGGTTTATCAGTATGCCGCATCCGGAGTTCAAGCTAACAACAAATTGTTGTATGATCTTTCGGTGGTGCGCGCTGATATTGGTGACATGAGAAAGTACG   550
AN-□ CP   CTCACCAGTACTGGTTTATCAGTATGCCGCATCCGGAGTTCTAGCTAACAACAAATTGTTGTATGATCTTTCGGTGGTGCGCGCTGATATTGGTGACATGAGAAAGTACG   549

AN-CP     CCGTGCTCGTGTATTCAAAAGACGATGCGCTCGAGACGGATGAGCTAGTACTTCATGTCGACATTGAGCACCAACGCATTCCCACATCTGGAGTGCTCCCAGTTTGAACT   660
AN-□ CP   CCGTGCTCGTGTATTCAAAAGACGATGCGCTCGGACGGATGAGTTAGTACTTCATGTCGACATTGAGCACCAACGCATTCCCACATCTGGAGTGCTCCCAGTTTGAACT   659

AN-CP     CGTGTTTTCCAGAACCCTCCCTCCATTTTCTGAGGCGGGAGCTGAGTTGGTAGTGTTACTATAAACTGCCTGAAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCATC   770
AN-□ CP   CGTGTTTTCCAGAACCCTCCCTCCATTTTCTGAGGCGGGAGCTGAGTTGGCAGTGTTATTATAAACTGCCTGAAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCATC   769

AN-CP     CAG                                                                                                            773
AN-□ CP   CAG                                                                                                            772
```

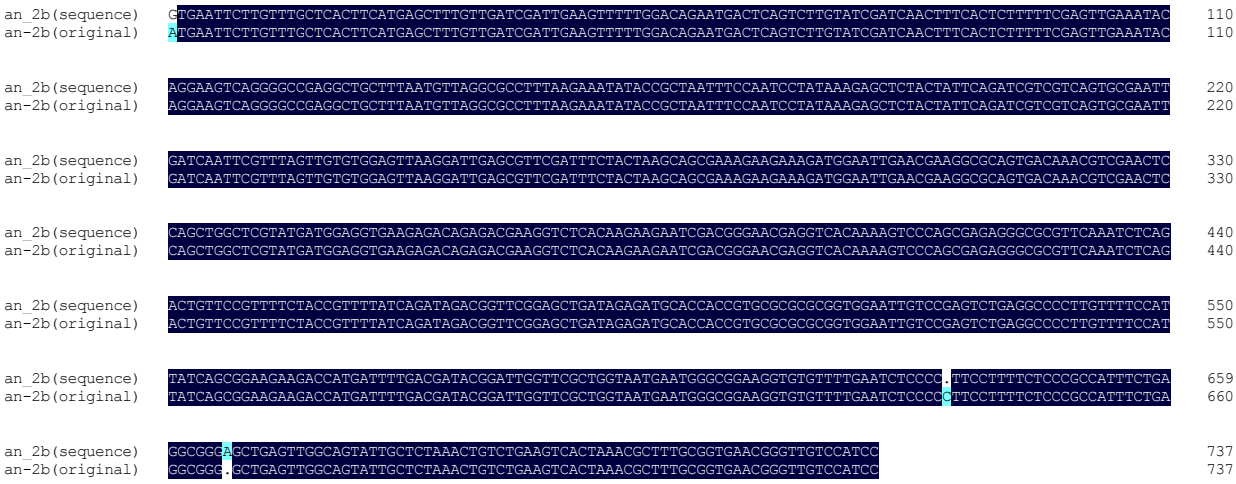
7.2.2 Alignments of AN-Δ2a+2b (original) and AN-Δ2a+Δ2b



7.2.3 Alignments of AN-CP and PV0506-CP (93.88% identities)

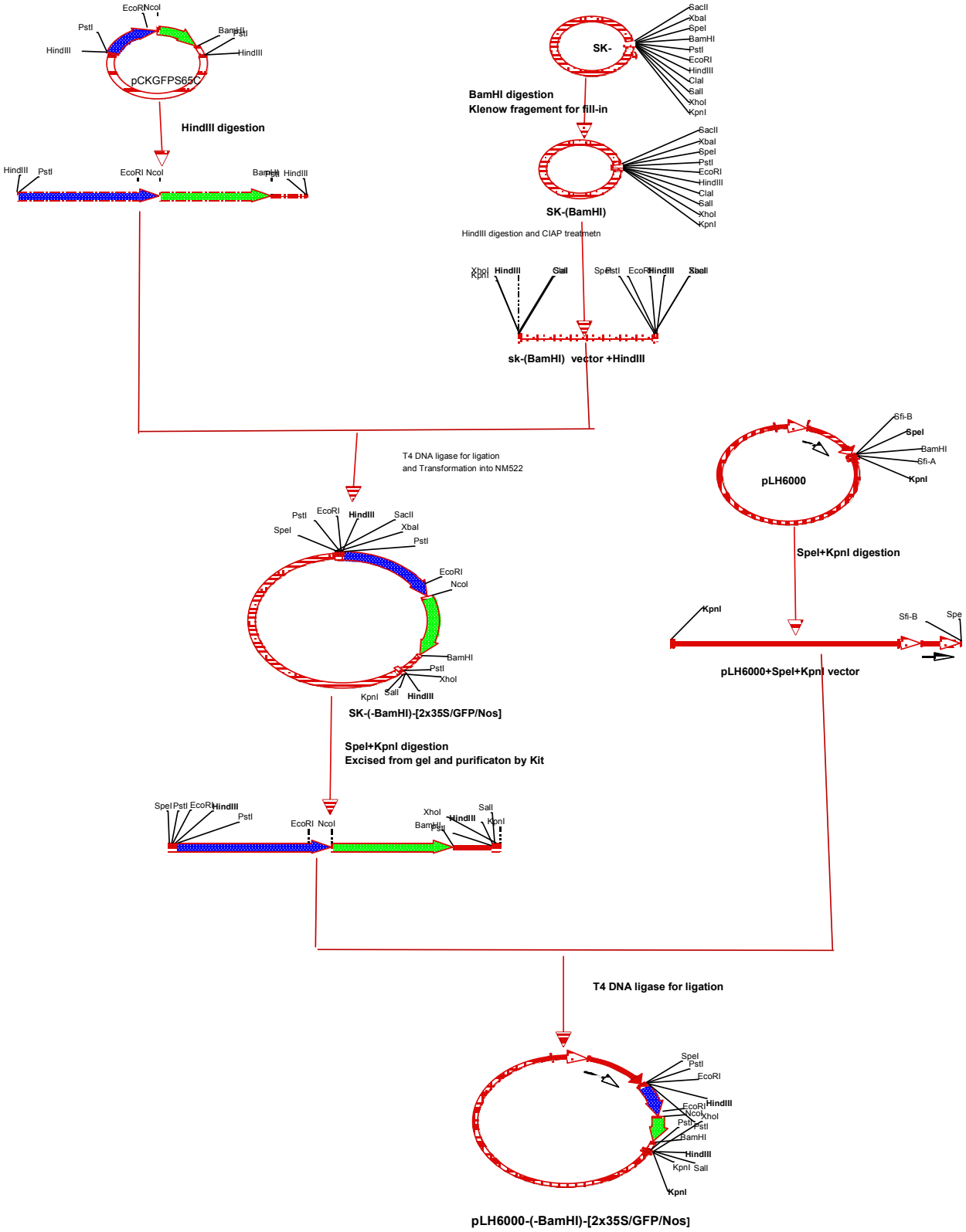


7.2.4 Alignment of AN-Δ2a+2b (original) and AN-Δ2a+2b (sequence)

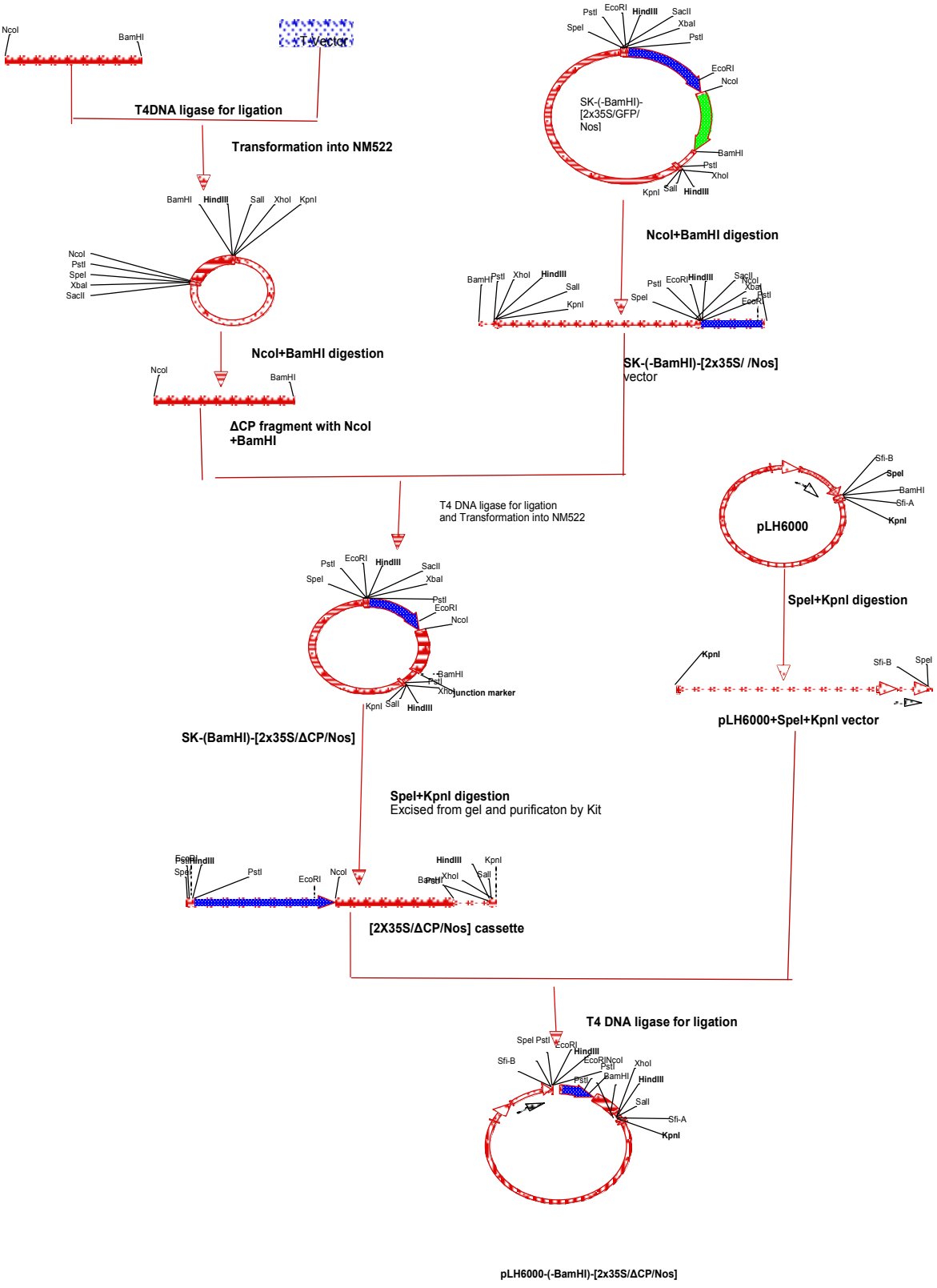


7.3 Maps of gene constructs

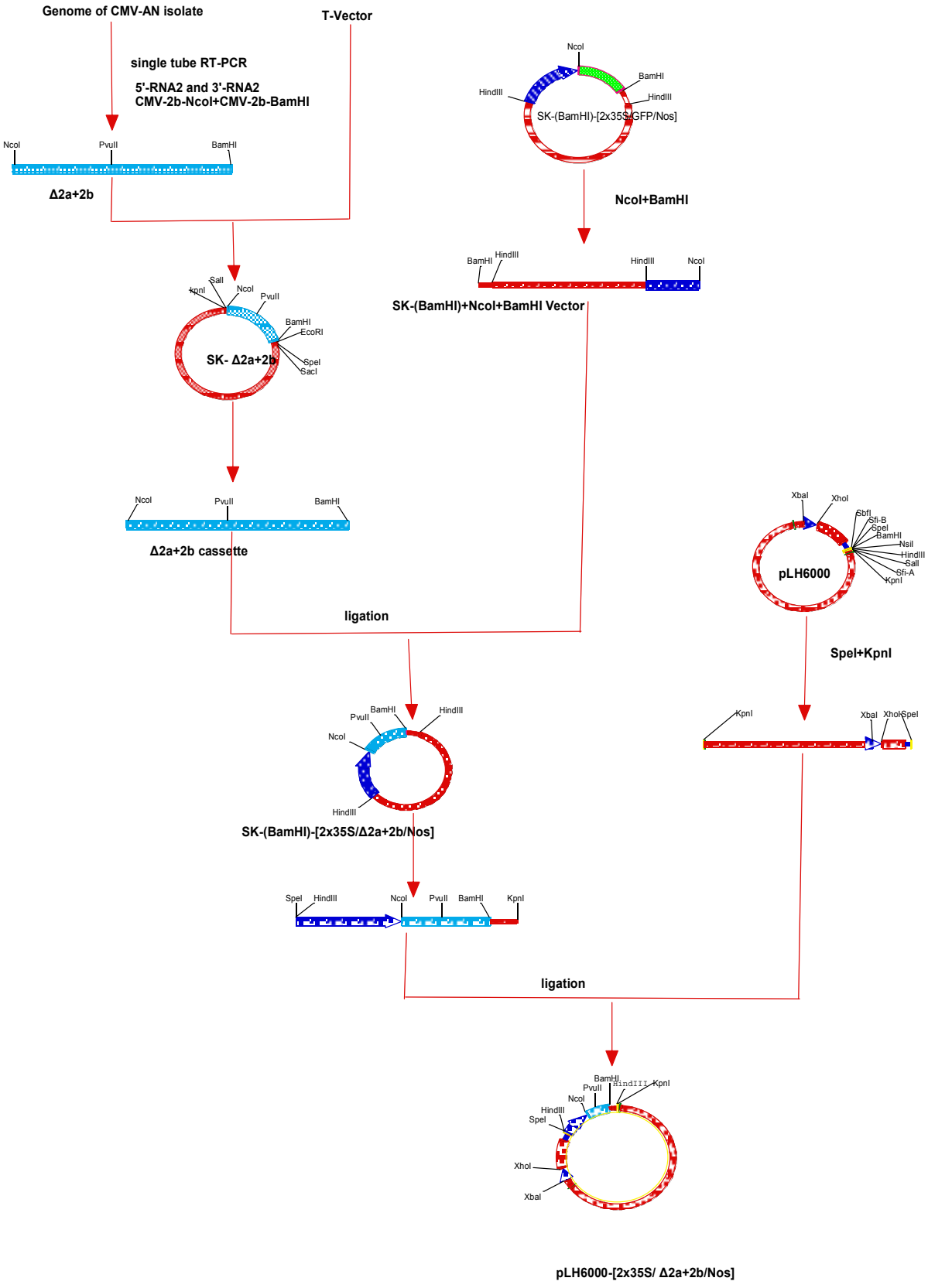
7.3.1 pLH6000-GFP



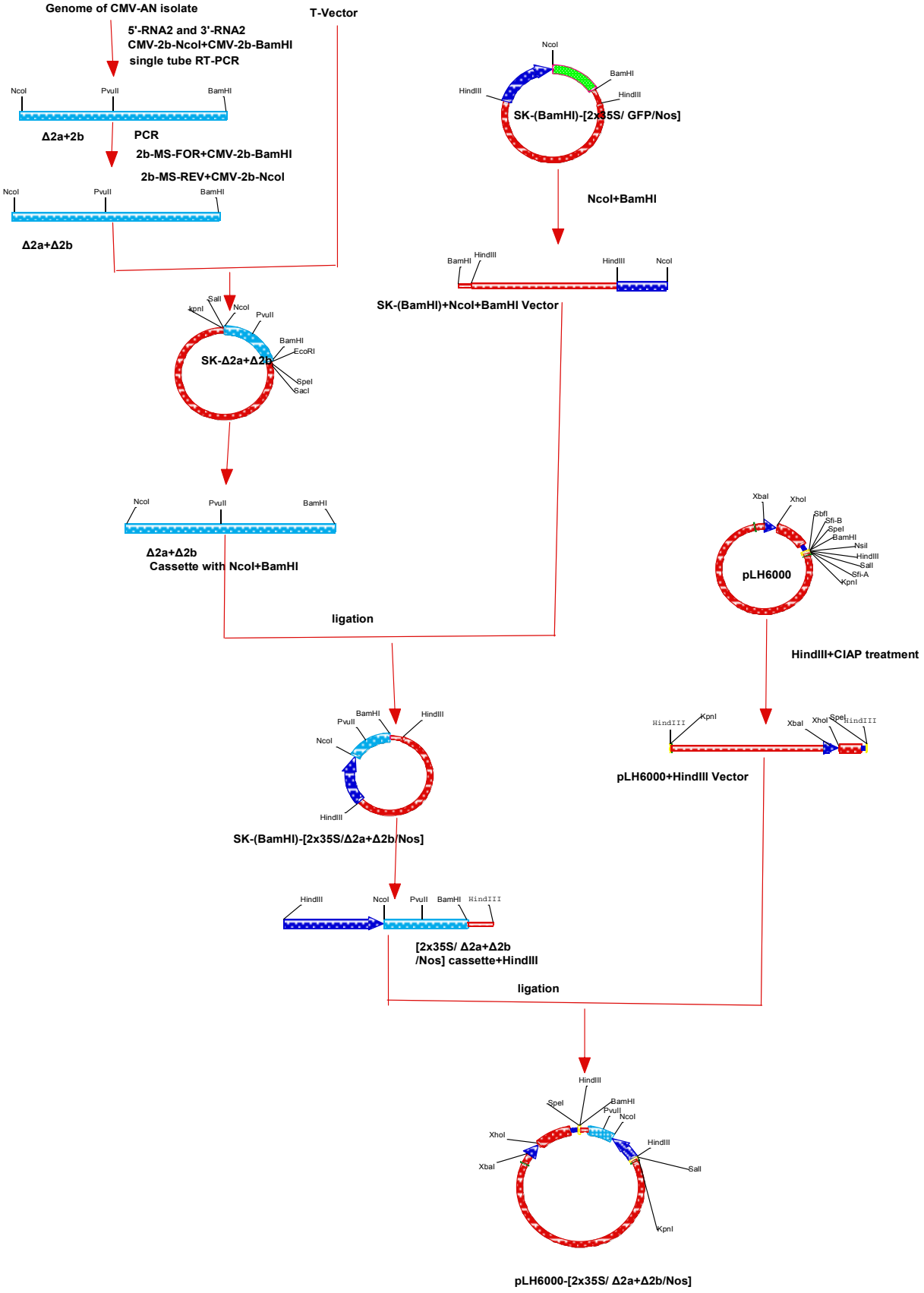
7.3.2 Map of pLH6000-ΔCP



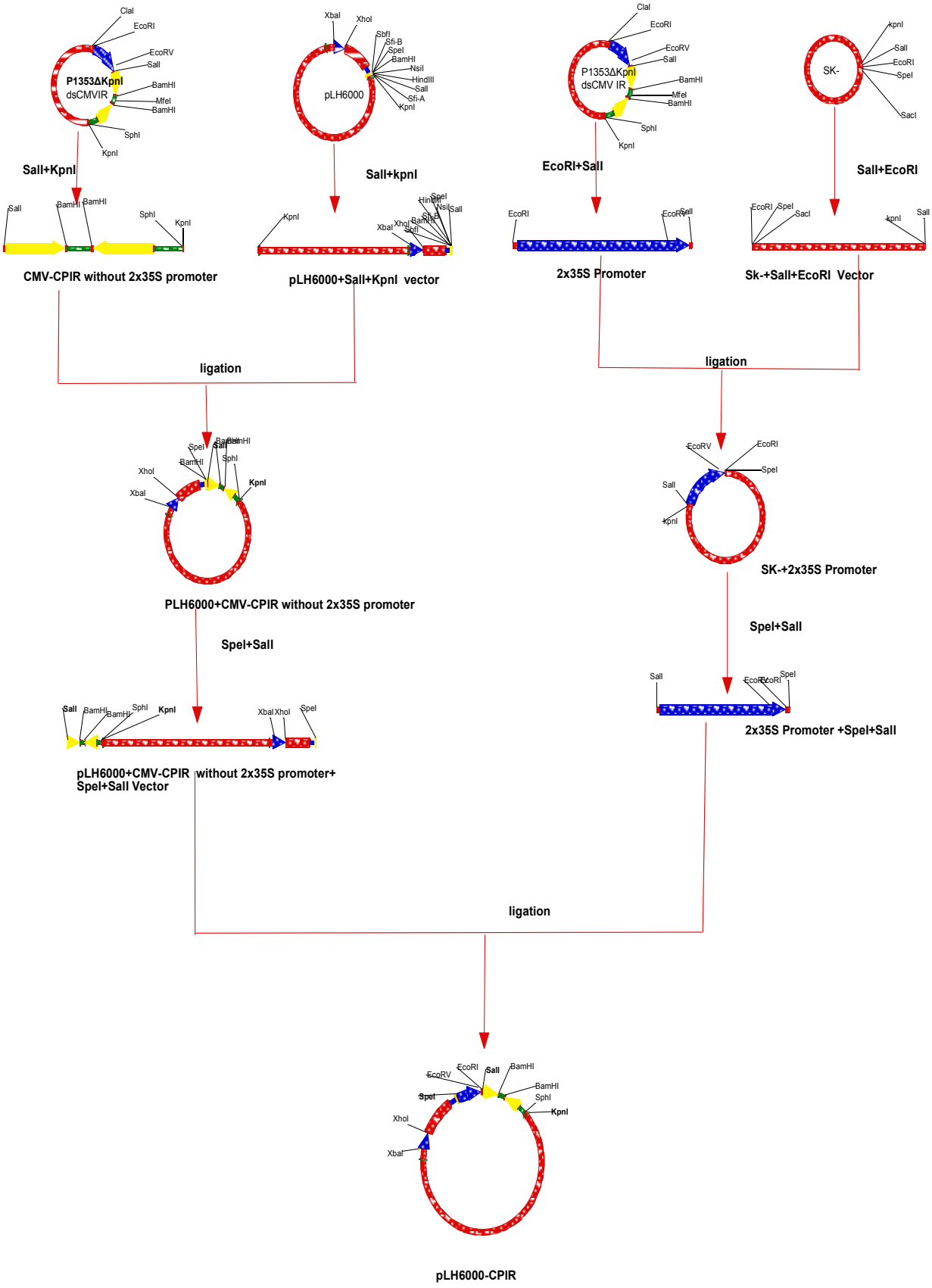
7.3.3 Map of pLH6000-Δ2a+2b



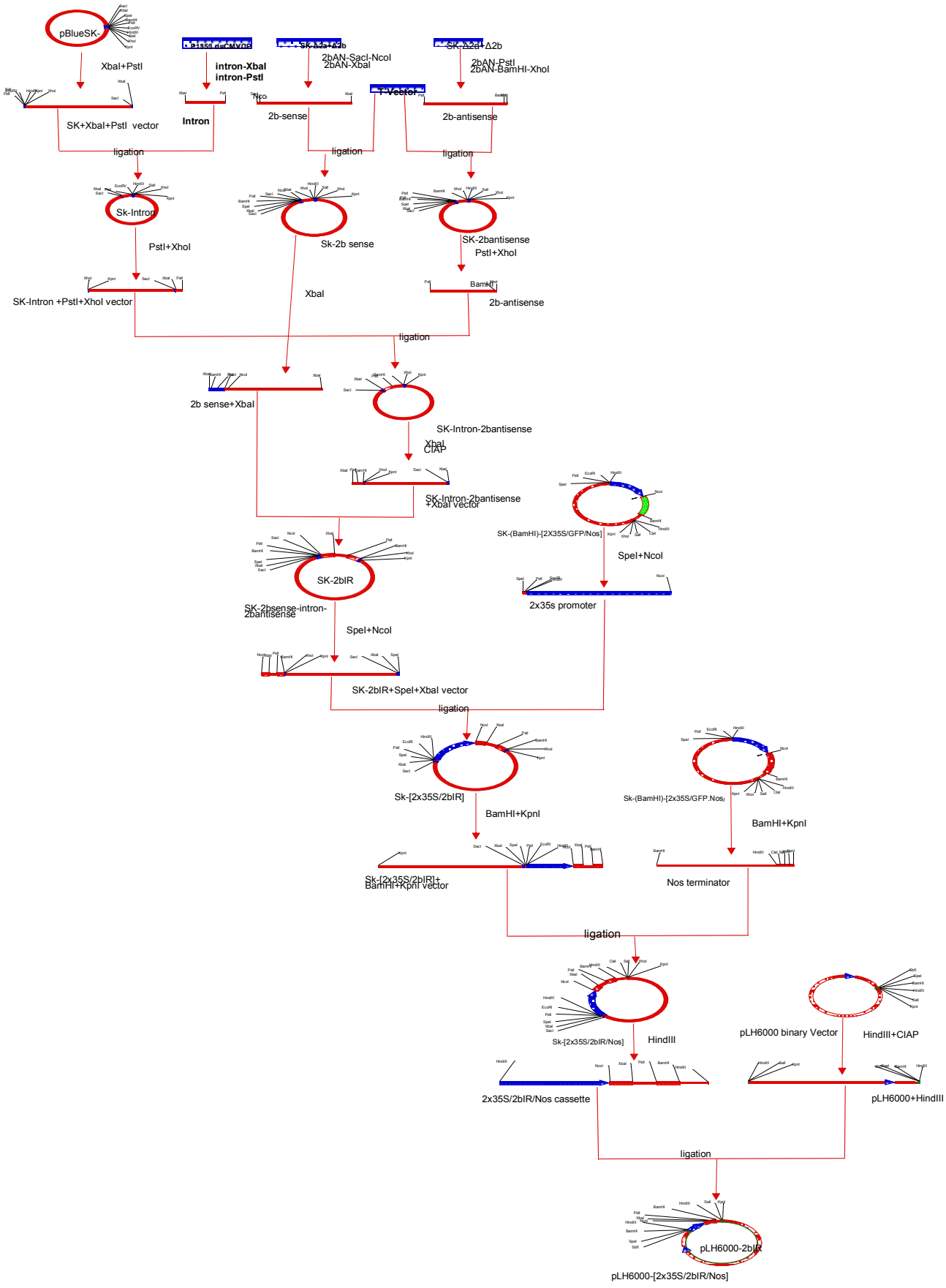
7.3.4 Map of pLH6000-Δ2a+Δ2b



7.3.5 Map of pLH6000-CPIR



7.3.6 Map of pLH6000-2bIR



Erklärung

Hiermit erkläre ich, dass ich die hier vorliegende wissenschaftliche Arbeit selbständig und ohne fremde Hilfe verfasst, nur die von mir angegebenen Quellen und Hilfsmittel benutzt habe und die in den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen, als solche kenntlich gemacht habe. Darüber hinaus erkläre ich, dass ich den Doktorgrad nicht besitze und mich auch früher nicht um den Doktorgrad beworben habe.

Hamburg, im Oktober 2008

Xinqiu Tan