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A Comparative Testing of *Cucumber mosaic virus* (CMV)-Based Constructs to Generate Virus Resistant Plants

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ABSTRACT

Among the viruses *Cucumber mosaic virus* (CMV) has been rated worldwide as one of the five most important viruses infecting vegetable species. CMV is a tripartite virus with high sequence variability, classified into three subgroups with 80% to 97% identical nucleotides in their coat protein. Due to the absence of natural resistance CMV is the plant virus with longest history in genetic engineering using pathogen induced approaches. However, the transformation and regeneration for some very important crops like chili is difficult. Therefore it will be an advantage to screen in model plants for gene constructs which might be independent of the target of final transformation and other parameters having an influence on the efficiency of a biotechnological approach. In our study we compared the resistance for all combinations of five different antiviral constructs, two different transformation vectors and two model host plants. From these approaches we identified the most effective construct which might also be applicable to transform eventually chili plants.

Keywords: *Cucumber mosaic virus*; Transgenic Plant; Resistance; Comparison

1. Introduction

The chili production has an economical impact in local as well as export markets in Asia and other parts of the world. More than one billion people consume chili in one or another form on a daily basis. The major diseases contributing to low yield and a reduced quality of fruits include bacterial wilt (*Ralstonia solanacearum*), phytophthora blight (*Phytophthora capsici* Leon.) powdery mildew (*Leceillula rurica*) and anthracnose (*Colletotrichum* sp.) [1]. In addition, several viruses are an important threat to chili production [2,3]. Due to its worldwide distribution and polyphagous vectors *Cucumber mosaic virus* (CMV) is one of the five most important viruses infecting vegetable species worldwide [4-6].

CMV is the type species of the genus *Cucumovirus*, family *Bromoviridae*. It has a tripartite ssRNA genome coding for one structural and four functional proteins. The RNA dependent RNA-polymerase (RdRP) is encoded on RNA 1 (ORF 1a) and RNA 2 (ORF 2a). The gene silencing suppressor (ORF 2b) overlaps with ORF 2a. RNA 3 encodes the cell to cell movement protein (ORF 3a) and the CP, which forms together with the RNA icosahedral particles. CMV is classified into subgroups Ia, Ib and II with 97% and 80% identical amino acids (aa) in the coat protein (CP), respectively [5,7,8].

Almost all known resistance genes found in several natural sources of *Capsicum* sp. are partial or polygenic [9-11] only few of them confer sustainable resistance [12,13]. Kang *et al*. [14] described a single dominant gene controlling CMV resistance in peppers which was effective against two virus isolates, however, a third one caused infection due to the extreme high variability of CMV. Lee *et al*. [15] identified the CMV strain CMVP1 that infected commercially available CMVP0 resistant pepper plants in the mid 1990th. Another promising resistant chili variety, breeding line VC246 from the World Vegetable Research and Development Center (WVRDC, Taiwan), revealed upon screening with several isolates from different serogroups five out of 28 isolates that overcame this resistance [16].

Several transgenic CMV resistant or tolerant plants were reported using the coat protein and the RNA replicase gene [17] and references therein [18]. In addition, extensive studies to induce resistance against CMV with

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truncated CP or 2a protein expressed in transgenic plants have been reported [19].

For chili, reports of tolerant plants using the coat protein gene are available [20-23]. However, the biotechnological production of CMV resistant chili plants is difficult, because the efficient transformation on chili pepper is inhibited since the shot regeneration rate is genotype specific [24] and the gene transfer via *Agrobacterium* infection into cotyledon and hypocotyls tissue is partly blocked for unknown reasons [25]. Therefore testing the efficiency of constructs directly in the chili lines is useless since the transformation efficiency is very low and/or not reproducible [26-28].

To circumvent the problem of the low transformation and regeneration efficiency we used as model plants *N. benthamiana* and *N. tabacum* cv. Samsun for transformation with five constructs each in two different vectors and screened all combinations with up to five CMV isolates from all subgroups Ia, Ib and II to identify constructs that conferred the immunity type of resistance independent of model plant, virus isolate and transformation vector.

2. Materials and Methods

2.1. CMV Isolates, Maintenance and Purification

In this study five CMV isolates representing all subgroups were included. CMV_{AN} (subgroup Ib) was used for generating the constructs for plant transformation. Lines were challenged with the homologous isolate CMV_{AN} as well as with the heterologous isolates CMV_{P3613} and CMV_{KS44} (subgroup Ib), CMV_{RT52} (subgroup Ia) and the subgroup II isolate CMV_{PV0420}.

Virus isolates were propagated on *N. glutinosa*. For inoculum preparation virus particles were purified as described [29]. Particles were checked for specific infectivity on the local lesion hosts *Vigna unguiculata* and *Chenopodium quinoa* by mechanical inoculation [16]. All plants were incubated in the greenhouse at 25˚C +/– 1˚C with a photoperiod of 16 hours light and 8 hours dark.

2.2. Plant Inoculation and Resistance Screening

Two leaves of plants at the 4 to 6 leaf stage were rubbed with virus particles diluted in 20 mM phosphate buffer (pH 7) and 5% (w/v) carborundum (600 mesh). The infectivity of the diluted particles was adjusted to induce 30 to 60 local lesions when using a total volume of 10 µl on *Vigna unguiculata*. This dose of inoculum infected 100% of the *N. benthamiana* and *N. tabacum* plants. For each plant-vector-insert combination 8 selection marker resistant plants of four to six independent lines were tested in the F1 generation with one repetition. Symptom expression was checked visually 20 and 35 days post infection (dpi) and virus presence/absence was verified by tissue print immunoblot of transverse sections of non-inoculated leaves using coat protein-specific polyclonal antiserum AS-0475 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) as described [30].

The visual screening of symptoms identified four phenotype classes: immune (no symptoms, negative tissue print), tolerant (no, mild or delayed mild symptoms, positive tissue print), recovery (early symptoms but in late infection no symptoms, positive/negative tissue print) and susceptible (symptoms, positive tissue print). Resistance was defined as the sum of immune, tolerant and recovered plants. Absence of virus was confirmed by RT-PCR as described in [8] using primers CMV-CPfor (5'-atg gac aaa tct gra tcw mcc-3') and CMVrev (5'-ctg gat gga caa ccc gtt c-3'). As an internal control using primers Nad_{sense} (5'-gatgcttcttggggcttcttgtt-3') and $Nad_{antisense}$ (5'-ctccagtcaccaacattggcataa-3') a plant specific fragment was amplified from total RNA simultaneously to the virus specific fragment as described in [31].

2.3. Gene Constructs in pLH6000 and pBIN19 Binary Vectors and Plant Transformation

The constructs are based on the coat protein gene (CP) or the 2b gene silencing suppressor gene including the overlapping region with the 2a of the isolate CMV_{AN}. Genes were used either in a translatable and/or a non-translatable form as well as in an inverted repeat form. In addition a chimeric construct containing a translatable GFP upstream of the inverted 2b repeat was prepared. The constructs are listed in **Table 1** and the details of their generation are given in the supporting information. The constructs were transformed using *Agrobacterium tumefaciens* LBA4404 (pBIN19) or GV3101 (pLH6000) by electroporation.

Leaf discs of *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Samsun nn were transformed with *Agrobacterium tumefaciens* LBA4404 according to [32] using for pLH6000 constructs hygromycin (20 mg/l) and for pBIN19 constructs kanamycin (50 mg/l) as a selection marker, respectively. Integration of the transgene and absence of *Agrobacterium* was verified by PCR. Transgene F1 were identified by germinating seeds on kanamycin (150 mg/l) and hygromycin (100 mg/l) containing MS-agar [33] before transferring seedlings to soil.

3. Results

3.1. Description of the Constructs

Virus specific inserts were derived from subgroup Ib isolate CMV_{AN}. The inserts of the single gene constructs ΔCP, Δ2a2b and Δ2aΔ2b had a length of 773 bp (ΔCP),

Table 1. Names, origin and length of viral sequences used in pLH6000 and pBIN19 binary vectors.

construct name	Origin/length (in bp) of CMV derived sequence	Specification
ΔCP	CP/773	not translatable
Δ2a2b	2a/2b/735 (641 2a/336 2b)[1]	2a not translatable, 2b translatable
Δ2aΔ2b	2a/2b/735 (641 2a/336 2b)[1]	2a not translatable, 2b not translatable
2bIR	2a/2b/549 (399 2a/336 2b)[1]	(Δ2aΔ2b) as inverted repeat, separated by an intron sequence
GFP_2bIR	2a/2b/549 (399 2a/336 2b)[1]	(2bIR) is fused at the 3´ end of a translatable GFP gene

[1]with a 242 bp overlap of 2a and 2b gene.

739 bp (Δ2a2b) and 738 bp (Δ2aΔ2b). In construct ΔCP the start codon ATG was changed to GGT and for Δ2aΔ2b construct the adenine from the start codon of the 2b silencing suppressor was removed resulting in non-translatable CP- and Δ2aΔ2b-genes, respectively. The inverted repeat constructs 2bIR and GFP_2bIR were based on the Δ2aΔ2b construct containing no translatable ORF. The only translatable gene of all constructs was the 2b silencing suppressor in the construct Δ2a2b. The ΔCP is based on RNA 3 while all 2b and Δ2b containing constructs are based on sequences of RNA 2. A more detailed description of the cloning procedure is given in the supporting information. Due to antibiotic resistance conflicts constructs based on pLH6000 were transformed with *Agrobacterium* strain GV3101, while the pBIN19 constructs were introduced into plants with *Agrobacterium* strain LBA4404.

3.2. Sequence Comparison of Virus Specific Inserts with the Respective Regions of Heterologous Isolates Used for Resistance Screening

All subgroup Ib isolates (KS_{44}, P_{3613}) used for heterologous resistance screening showed a nucleic acid identity of 90% - 93% for the Δ2a2b region when compared with the respective region of CMV_{AN}, while for subgroup Ia isolate CMV_{RT52} the identity was 86% and for subgroup II isolate CMV_{PV0420} 69% when compared with CMV_{AN}. For the 2b region the identity of the Ib isolates ranged between 88% and 92%, subgroup Ia isolate CMV_{RT52} showed 84% and subgroup II isolate CMV_{PV0420} 65% identity. Roughly the same gradation in the identity is present on the CP genes, 93% - 96% for the Ib isolates, 91% for IA and 76% identical nucleotides for the subgroups II isolate, respectively. The identities are summarized in **Table 2**.

3.3. Analysis of Transgenic Lines

In total, 249 lines were selected from independent calli transformed with five constructs harboring viral or chimeric sequences either in the vector pLH6000 or pBIN19. As controls served plants transformed with either the empty vector or the vector with GFP as insert. All of the control lines showed signs of infection and became infected comparable to the non-transformed plants.

Table 2. Nucleic acid identity (%) of genes Δ2aΔ2b (641 bp 2a and 336 bp 2b with a 241 bp overlap), 2b and CP from subgroups Ia, Ib and II.

Isolate	subgroup	Δ2aΔ2b	2b	CP
CMV_{AN}	Ib	100	100	100
CMV_{KS44}	Ib	93	92	96
CMV_{P3613}	Ib	90	88	93
CMV_{RT52}	Ia	86	84	91
CMV_{0420}	II	69	65	76

Stable integration was verified by PCR and by segregation patterns after self pollination of the parental generation and subsequent seed germination on selective medium. Seedlings from lines following a segregation pattern of 3:1 (X^2 confidence value of $P_{0.05} \leq 3.84$) were transferred into soil in the greenhouse for resistance testing. Each line was tested with 4 individual plants and the testing was repeated.

3.4. Influence of Plant Species and Vector on the Resistance When Inoculated with the Homologous Isolate CMV_{AN}

The overall resistance from *N. benthamiana* (31% immune plants, **Table 3(a)** line 11) and *N. tabacum* cv. Samsun (20% immune plants, **Table 3(b)**, line 11) differed. In *N. benthamiana* all single gene constructs ΔCP, Δ2a2b and Δ2aΔ2b transformed with the pLH6000 vector resulted in 34% to 50% immune plants derived from 14 lines (**Table 3(a)**, lines 1 - 3) whereas no immune plants were obtained from the 14 lines harboring the same inserts but transformed with the pBIN19 vector. Also no immune plants were obtained when transformed with pLH6000 and pBIN19 in *N. tabacum* (**Table 3(b)**,

Table 3. (a) Resistance of *N. benthamiana* transformed with different constructs; (b) Resistance of *N. tabacum* cv. Samsun transformed with different constructs.

(a)

line	number of lines	construct	% immune	number of plants				% resistant i + t + r[2]
				immune	susceptible	recovery	tolerant	
1	4	pLH ΔCP	34	11	21	0	0	34
2	5	pLH Δ2a2b	32.5	13	12	15	0	70
3	5	pLH Δ2a Δ2b	50	20	19	1	0	52.5
4	5	pLH 2bIR	42.5	17	23	0	0	42.5
5	3	pLH GFP_2bIR	4	1	23	0	0	4
6	4	pBINΔCP	0	0	32	0	0	0
7	3	pBIN Δ2a2b	0	0	24	0	0	0
8	4	pBIN Δ2a Δ2b	0	0	32	0	0	0
9	4/1[1]	pBIN 2bIR	28	9	23	0	0	28
10	9/4	pBIN GFP_2bIR	60	43	29	0	0	60
total			31	114	238	16	0	35

[1]4/1 = out of 4 tested plant lines 1 was completely immune to infection; [2]i + t + r = immune + recovered + tolerant (= resistant) plants.

(b)

line	number of lines	construct	% immune	number of plants				% resistant i + t + r[2]
				immune	susceptible	recovery	tolerant	
1	5	pLH ΔCP	0	0	36	4	0	10
2	4	pLH Δ2a2b	0	0	29	3	0	9
3	5	pLH Δ2a Δ2b	0	0	37	3	0	7.5
4	7	pLH 2bIR	0	0	49	7	0	12.5
5	7/5[1]	pLH GFP_2bIR	87.5	63	0	0	9	100
6	4	pBINΔCP	0	0	28	4	0	12.5
7	6	pBIN Δ2a2b	0	0	39	7	2	19
8	4	pBIN Δ2a Δ2b	0	0	29	0	3	9
9	6	pBIN 2bIR	10	1	36	2	1	10
10	10/2	pBIN GFP_2bIR	34	27	53	0	0	34
total			20	91	336	16	15	26

[1]7/5 = 5 out of 7 tested plant lines were completely immune to infection. [2]i + t + r = immune + recovered + tolerant (= resistant) plants.

lines 1 - 3 and 6 - 8).

The 2bIR construct cloned in pLH6000 resulted in 42.5% immune *N. benthamiana* plants (**Table 3(a)**, line 4), while all seven *N. tabacum* lines were susceptible to CMV infections when transformed with the pLH6000 vector (**Table 3(b)**, line 4). The only observed effect was recovery of seven plants. Similar results were obtained using the pBIN19 vector (**Table 3(a)**, line 9 and **Table 3(b)**, line 9). From *N. benthamiana* plants transformed with pBIN19 28% immune plants were obtained. From these lines one line was 100% immune to infection.

In summary, *N. benthamiana* lines showed more immune plants when transformed with single gene- or 2bIR-constructs than comparable *N. tabacum* lines. Transformation of *N. benthamiana* plants with single gene constructs led to resistance when using the pLH6000 vector but not with the pBIN19 vector. When comparing plants transformed with the inverted repeat construct (2bIR) both plant species, *N. benthamiana* and *N. tabacum*, showed immune plants, however in the combina-

tion of *N. benthamiana* plants and pLH6000 vector we obtained with 42.5% the highest score of immune plants.

In contrast to the high number of immune *N. benthamiana* plants, when transformed with single gene or inverted repeat constructs (2bIR) using the pLH6000 vector, only one out of 24 plants from three lines was not infected harboring the 2bIR construct flanked upstream by the GFP gene (GFP_2bIR, **Table 3(a)**, line 5). All other host species/vector combinations resulted in a high number of resistant plants ranging between 34% and 87% (**Table 3(a)**, line 10 and **Table 3(b)**, lines 5 and 10). With the exception of the *N. benthamiana*/pLH6000 combination with all other host/vector combinations between one and four lines resulted in 100 % immune plants.

3.5. Testing Immune Lines with Heterologous Isolates from Subgroups Ib, Ia and II

Only plants of lines which were observed to be 100% immune to CMV_{AN} were challenged with heterologous isolates from the subgroups 1b (CMV_{P3613} and CMV_{KS44}), 1a (CMV_{RT52}) and II (CMV_{PV0420}).

With the exception of one plant which became infected (**Table 4B**, line 3) the three other *N. benthamiana* and *N. tabacum* plant lines transformed with the GFP_2bIR in pBIN19 and pLH6000, respectively, were immune to infection when challenged with the 1b isolates CMV_{P3613} and CMV_{KS44} (**Table 4A-D**, lines 2 and 3). A lower percentage of immune *N. tabacum* plants, ranging from 12.5% to 100%, was obtained from lines transformed with the pBIN19 vector (**Table 4E**, **F**, lines 2 and 3).

When plants of lines transformed with GFP_2bIR in pBIN19 were challenged with the subgroup 1A (CMV_{RT52}) and subgroup II (CMV_{PV0420}) isolates *N. benthamiana* plants showed 87.5% to 100% immune plants (**Table 4A** and **B**, lines 4 and 5) while *N. tabacum* plants showed not more than 37.5% immune plants (**Table 4C-F**, lines 4 and 5). *N. tabacum* plants transformed with GFP_ 2bIR in pBIN19 became completely infected with the serogroup II isolate PV_{0420} (**Table 4E** and **F**, line 5).

In summary, *N. benthamiana* plants transformed with GFP_2bIR in pBIN19 showed the highest number of immune plants when challenged with isolates from all subgroups.

4. Discussions

Defence against virus infection based on gene silencing may be the result of different strategies. As reviewed [34] the cytoplasmatic pathway is important in virus infection. The endogenous messenger RNA pathway negatively regulates viral gene expression and the suppression of viral transcription may be a result of siRNA guided DNA methylation, which might be activated via the introducetion of a transgene. Since different plant species and even different cultivars have a different genetic background, they might respond to the same virus in different ways although being transformed with the same antiviral construct.

This host dependence became clearly evident in our study when using the single gene constructs ΔCP, Δ2a2b and Δ2aΔ2b and inverted repeat construct (2bIR). Immune plants were only obtained when *N. benthamiana* plants were transformed, regardless of the translatability of the transgene and origin of the genome segment. *N. benthamiana* plants express a non functional salicylic acid (SA) inducible RdRP. Yang *et al*. [35] discussed that due to the missing RdRP the SDE 1-like RdRP produces increasing amounts of siRNAs from the pool of aberrant RNAs in *N. benthamiana*. This might lead to hyperactive gene silencing and might explain the superiority of *N. benthamiana* transgenic lines compared to the *N. tabacum* ones.

Because of antibiotic resistance conflicts the constructs based on pLH6000 were transformed with *Agrobacterium* strain GV3101, while the pBIN19 constructs were transformed with strain LBA4404. This implies that the influence on resistance of the vector and the Agrobacterium strain have to be discussed together.

Waterhouse *et al*. [36] summarized that only in plants with multiple, methylated copies of the transgene a co-suppression and virus resistance can be observed. From unpublished data [37] was stated that the copy number in single loci is correlated with *Agrobacterium* strains, which have different efficiencies to make single or multicopy T-DNA insertions. This is supported by our observation that only for pLH6000 constructs segregation patterns of 15:1 were observed, suggesting multicopy inserts at two different loci (data not shown).

According to [37] it might be possible, that the strain GV3101 did produce plant cells with more than one copy in a single locus arranged in a tandem array. As a consequence gene silencing and resistance is enhanced when comparing the single gene constructs ΔCP, Δ2a2b and Δ2aΔ2b. T-DNA delivery might also be a consequence of stability and replication efficiency in the *Agrobacterium* due to the origin of replication of the vector, which is pVS1 from Pseudomonas for pLH6000 [38] and RK2 for pBIN 19 [39]. The pVS1 origin of replication ensures good plasmid persistency in *Agrobacterium* sp. [38]. However, since we did not check for multicopy insertion, this explanation for significantly higher resistance remains highly speculative.

It is, however, reassuring that neither the translatability nor the genome segment origin (ΔCP from RNA 3 and all 2b containing constructs from RNA 2) of the transformed constructs seem to influence virus infection.

Table 4. Resistance against heterologous isolates of plant lines transformed with GFP_2bIR (A-F) and 2bIR (G), which were 100 % immune against the homologous isolate.

	line	Isolate	subgroup	host/vector/	immune	susceptible	% i
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		8	0	100
A	3	CMV_{KS44}	Ib	*N.b.*[1]/pBIN19	8	0	100
	4	CMV_{RT52}	Ia		7	1	87.5
	5	CMV_{PV0420}	II		8	0	100
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		8	0	100
B	3	CMV_{KS44}	Ib	*N.b.*/pBIN19	7	1	87.5
	4	CMV_{RT52}	Ia		8	0	100
	5	CMV_{PV0420}	II		8	0	100
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		8	0	100
C	3	CMV_{KS44}	Ib	*N. t.*[2]/pLH6000	8	0	100
	4	CMV_{RT52}	Ia		3	5	37.5
	5	CMV_{PV0420}	II		0	8	0
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		8	0	100
D	3	CMV_{KS44}	Ib	*N. t.*/pLH6000	8	0	100
	4	CMV_{RT52}	Ia		0	8	0
	5	CMV_{PV0420}	II		0	8	0
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		6	2	75
E	3	CMV_{KS44}	Ib	*N. t.*/pBIN19	8	0	100
	4	CMV_{RT52}	Ia		n.t.	n.t.	n.t.
	5	CMV_{PV0420}	II		0	8	0
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		2	6	25
F	3	CMV_{KS44}	Ib	*N. t.*/pBIN19	1	7	12.5
	4	CMV_{RT52}	Ia		n.t.	n.t.	n.t.
	5	CMV_{PV0420}	II		0	8	0
	1	CMV_{AN}	Ib		8	0	100
	2	CMV_{P3613}	Ib		8	0	100
G	3	CMV_{KS44}	Ib	*N.b.*/pBIN19	8	0	100
	4	CMV_{RT52}	Ia		8	0	100
	5	CMV_{PV0420}	II		5	3	62.5

[1]= *Nicotiana benthamiana*; [2]= *Nicotiana tabacum* cv. Samsun.

On the other hand the influence of the *Agrobacterium* strain and/or the vector is not evident when comparing plants transformed with the 2bIR inverted repeat construct. Obviously, the impact of multicopy inserts might be reduced, since the construct itself provides dsRNA. This is in good accordance with findings from [40] who proved the superiority of dsRNA constructs to obtain resistance against CMV. Their construct, based on the CP sequence and transformed into *N. tabacum*, resulted in 25% to 35% resistant plants, depending on the promoter for transgene expression. Chen *et al*. [41] extended their studies to several constructs based on the CP as well as the 2a2b sequence in long or short variants and compared the resistance achieved in their transgenic plants transformed with the respective viral regions as single gene constructs. The differences between their and our transgenic plants transformed with the 2a2b single gene constructs in the combination with pBIN19 as the vector and *N. benthamiana* are the length of the viral part of their constructs and the CMV isolate used for transformation and challenging. The difference in the viral sequence length might explain that we did not get any resistant plants while [41] obtained between 11% and 21% resistant plants in their experiments. Using the CP sequence for transformation, neither [41] nor we got any resistant *N. benthamiana* plant when using the pBIN19 vector system.

Chen *et al*. [41] used a CMV RNA2-based inverted repeat construct containing the 3' part of 2a gene and the 2b gene. The resistance variation of their transgenic *N. benthamiana* plants against CMV revealed that the differences might be due to the length of the two sequences they used. Their long inverted repeat (LIR) covers 1534 bp and induced resistance in 75% of the plants, while their small inverted repeat (SIR) covered 490 bp and induced only 30% resistant plants. The resistance of the SIR is in good accordance with the data of our 2bIR construct with a viral sequence length of 549 bp. In our study, the resistance frequency ranged between 30% to 40% in *N. benthamiana* plants derived from pLH6000-2bIR and pBIN19-2bIR.

When comparing the resistance induced by 2bIR with that of GFP_2bIR, an enhancement for the latter was clearly observed in both tobacco species. 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 [42,43]. While all other constructs transformed with the vector pLH6000 in *N. benthamiana* resulted in highest numbers of immune plants, the performance of this plant/vector combination using GFP_2bIR as the antiviral insert resulted in a low number of immune plants. This observation cannot be explained by us.

According to the threshold model of gene silencing [44] it appears possible, that the GFP gene stabilizes the mRNA of 2bIR and prevents rapid degradation, or increases the transport of mRNA from nucleus to cytoplasm to trigger defense response by dsRNA-mediated resistance more efficiently. Probably the effect is not due to the GFP sequence itself, since [45] demonstrated a similar effect caused by a fusion of the NIb gene of Potato virus Y (PVY) with a blue fluorescence protein gene and [30] fusing a nonsense sequence to the N protein gene of *Tomato spotted wilt virus* (TSWV). However, the nonsense sequence used by [30] was translatable and it cannot be excluded that the effect was protein rather than RNAi mediated. The inoculum dose-dependence for breaking the resistance is supported by our observation of 100% infection when inoculating the GFP_2bIR lines 1 and 6 with two different methods. An immunity of 100% challenged plants was obtained by mechanical inoculation, whereas inoculation by grafting broke resistance (data not shown). A similar effect has been shown by [46] in *Solanum lycopersicum* L.

In respect to the statement of [47] that for RNA mediated resistance a minimum of 90% identical nucleotides is necessary, our results are unusual. None of the heterologous isolates of subgroup Ia and II did fullfill this condition. However, a common stretch of 23 conserved nucleotides (data not shown) might have led to variable degrees of RNAi mediated immunity. None of the lines with 100% immunity to a challenge with the homologous virus isolate CMV_{AN} used for transformation, revealed a similar level of resistance when challenged with non-homologous isolates. Only *N. benthamiana* plants transformed with the pBIN19-GFP_2bIR construct showed 87.5% to 100% immune plants when challenged with isolates from subgroups Ib, Ia and II. All other lines that were immune to the homologous isolate showed a lower degree of resistance when challenged with non-homologous isolates. Possibly, the degree of sequence identity has a similar effect as the dose of inoculum for the resistance effect. A possible explanation might be the different efficiency of the gene silencing due to lower sequence identities between the transgene and the challenging viruses, all applied as inoculum with comparable specific infectivity.

From our comparisons we can conclude that the kind of insert has highest influence for the generation of immune plants. However, also the vector/Agrobacterium system seemed to be highly important—at least when the challenging virus was not the homologous one.

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Supporting Information

Construction of pLH6000-GFP

The BamHI-restriction recognition site of the plasmid pBlueScript II SK-(Stratagene) was removed by digestion and filling in SK-/ΔBamHI. A fragment of 1867 bp, containing the GFP-gene driven by a double 35S promoter and with a Nos terminator 2x35S/GFP/Nos, was released by HindIII digestion from the plasmid pCKGFPS65C [48], ligated into a HindIII linearized SK-/ΔBamHI vector and transformed in *E.coli* . In a correct orientation the SpeI site from SK-/ΔBamHI is located upstream of the 35S promoter. The resulting clone was named SK-ΔBamHI-(2x35S/GFP/Nos). The cassette of ΔBamHI-(2x35S/GFP/Nos) from this clone was isolated by SpeI and KpnI digestion and cloned into the SpeI/KpnI site of pLH6000. The resulting plasmid was named pLH6000-GFP.

Construction of pLH6000-ΔCP

The CP gene of CMV_{AN} was amplified by RT-PCT using primers CMV-CP ΔNcoI (5'-ctagagccatggtggacaaatctgg at-3') and CMV-CP BamHI (5'-gacgtcggatccctggatggac aaccc-3'). To join the ΔCP fragment with the 2x35S promoter and NOS terminator, the GFP gene from the clone SK-ΔBamHI-(2x35S/GFP/Nos) was removed by NcoI/BamHI digestion and the remaining vector was used SK-ΔBamHI-(2x35S/ΔCP/Nos). From this clone the 2x35S/ΔCP/Nos cassette was isolated and ligated into the SpeI/KpnI site of pLH6000 to obtain pLH6000-ΔCP.

Construction of pLH6000-Δ2a2b 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} with a 242 bp overlap of 2a and 2b, located between nucleotide position 2130 and 2864 on the CMV_{AN} RNA 2 was amplified by RT-PCR with primers 5'-RNA2 (5'-gat gaattcytgtttgctcac-3') and 3'-RNA2 (5'-ggatggacaacccgt tc-3') and subcloned (SK-Δ2a + 2b). Two new restriction sites for further subcloning, NcoI and BamHI, were introduced by PCR with the primers CMV-2b-NcoI (5'-cta gagccatggtgaattcttgtttgc-3') and CMV-2b-BamHI (gacgtc ggatccggatggacaacccgt-3') using plasmid SK-Δ2a + 2b) as template. The plasmid SK-Δ2a + 2b was digested with NcoI/BamHI and the Δ2a + 2b was isolated and ligated into the NcoI/BamHI linearized plasmid (SK-ΔBamHI-(2x35S/ΔCP/Nos) and transformed into *E. coli* (SK-ΔBamHI-(2x35S/Δ2a + 2b /Nos). The cassette ΔBamHI-(2x35S/Δ2a + 2b/Nos was isolated by SpeI/KpnI digestion and ligated into the SpeI/KpnI site of pLH6000 (pLH6000-Δ2a2b).

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 adenine of the start codon. Using the plasmid SK-Δ2a + 2b as template, the start codon of 2b gene was removed by site-directed mutagenesis according to [49] with the two primer pairs 2b-MS-FOR (5'-gaagaaagtggaattgaacgaaggcgc-3')/CMV-2b-BamHI (5'-gacgtcggatccggatggacaacccgt-3') and CMV-2b-NcoI (5'-ctagagccatggtgaattcttgtttgc-3')/2b-MS-REV (5'-cgttcaattccactttcttctttcgctgc-3') to generate the Δ2a + Δ2b fragment. The Δ2a + Δ2b fragment was reamplified by primers CMV-2b-NcoI and CMV-2b-BamHI with the Δ2a + Δ2b fragment as template and subcloned (SK-Δ2a + Δ2b). The fragment Δ2a + Δ2b from plasmid SK-Δ2a + Δ2b was digested by NcoI/BamHI, isolated and subcloned into the NcoI/BamHI linearized vector (SK-ΔBamHI-(2x35S/GFP/Nos) to obtain SK-ΔBamHI-(2x35S/Δ2a + Δ2b/Nos). The insert of SK-ΔBamHI-(2x35S/Δ2a + Δ2b/Nos) was digested by HindIII and cloned into pLH6000. The resulting clone was named pLH6000-Δ2aΔ2b.

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.

The 198 bp intron from plasmid p1353dsCMVIR was amplified by primers Intron PstI (5'-tatacgatctgcaggcg ctcgcc-3') and Intron_XbaI (5'-ccctctagataagtttctgc-3') and ligated into the PstI/XbaI site of SK- (SK-Intron). Antisense and sense strand of the 2b gene, a fragment of 549 bp (containing 335 bp upstream from 2b gene and 455bp downstream 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, were amplified with primers 2bAN_ PstI (5'-aatactgcagactcagccc-3') and 2bAN_BamHI_ XhoI (5'-tacaggatcccaggactcgaggctg-3') for antisense, 2b_AN_SacI_NcoI (5'-atacagagctccatgggccgaggctgc-3') and 2b_AN_XbaI (5'-gacagtctagagcaatactgcc-3') for sense using plasmid SK-Δ2a + Δ2b as template. The two fragments were subcloned to obtain SK-anti2b) and SK-sense2b. The anti2b fragment from plasmid SK-anti2b was isolated by PstI/XhoI digestion and ligated with in a PstI and XhoI linearized (SK-Intron) to obtain SK-Intron-anti2b. The sense2b fragment was isolated from plasmid SK-sense2b by XbaI and ligated in a XbaI linearized SK-Intron-anti2b vector (SK-ds2bIR). The orientation of the recombinant was identified by BamHI digestion.

The DNA fragments of 2x35S promoter and Nos terminator from plasmid SK-ΔBamHI-(2x35S/Δ2a + Δ2b/ Nos) were isolated by SpeI/NcoI (35S promoter) and

BamHI/KpnI (Nos terminator) digestion, respectively. The 2x35S promoter fragment was ligated with the SpeI/NcoI linearized plasmid SK-ds2bIR to obtain SK-2x35S/2bIR. The Nos terminator was ligated in the BamHI/KpnI linearized plasmid SK-2x35S/ds2bIR to obtain SK-2x35S/2bIR/Nos. Then the cassette 2x35S/2bIR/Nos from plasmid SK-2x35S/2bIR/Nos was isolated by HindIII digestion and ligated with HindIII linearized pLH6000 (pLH6000-2bIR).

Construction of the Chimeric Construct pLH6000-GFP_2bIR

To join 2bIR with GFP from pCKGFPS65C, a fragment of 2bIR from plasmids of SK-2bIR was isolated with BamHI and ligated in a BamHI linearized SK-ΔBamHI-(2x35S/GFP/Nos). The correct orientation of the recombinant plasmid, GFP: sense2b: intron: antisense2b, was determined by NcoI digestion (SK-ΔBamHI-(2x35S/GFP_2bIR/Nos). The fragment GFP_2bIR from plasmid SK-ΔBamHI-(2x35S/GFP + 2bIR/Nos) was isolated by SpeI/KpnI digestion and cloned in a SpeI/KpnI linearized pLH6000 (pLH6000-GFP_2bIR). In this construct GFP is translatable.

Transfer of Cassettes into pBIN19

The ΔCP and GFP_2bIR cassettes were cloned in the SpeI/KpnI and the Δ2a2b, Δ2aΔ2b and 2bIR cassettes in the HindIII site of pBIN19.

