UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Dept. of Agroforest Ecosystems

Development of an Artificial Recombination Test System Under High Selection Pressure for Beet- Infecting Poleroviruses

Master's Thesis

European Master Degree in Plant Health in Sustainable Cropping Systems

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Abstract

Beet chlorosis virus (BChV) and beet mild yellowing virus (BMYV) are closely related species of the genus *Polerovirus*, transmitted by aphids and are a part of the virus yellows disease complex in *Beta* species. Although frequently detected together in mixed infections, these two viruses have distinct host ranges. BMYV and BChV natural recombinants were already documented, and in the context of the highly complex genome expression strategy of poleroviruses, the potential recombination system of these species could become a valuable research tool.

Recombination systems have been developed for other viruses, such as Potato virus X (Draghici & Varrelmann, 2010), but not for poleroviruses. In this work, an artificial recombination system under high selection pressure for beet-infecting poleroviruses was developed by cloning a partial virus genome into the binary vector using Gibson assembly. *In planta* assay by means of *Agrobacterium*-mediated inoculation of *Nicotiana benthamiana* was done to test the system's viability.

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Abbreviations

A. tumefaciens	*Agrobacterium tumefaciens*
AP	Alkaline phosphatase
B. macrocarpa	*Beta macrocarpa*
B. vulgaris	*Beta vulgaris*
BChV	Beet chlorosis virus
BMYV	Beet mild yellowing virus
BtMV	Beet mosaic virus
BWYV	Beet western yellows virus
BYV	Beet yellows virus
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CP	Capsid protein
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSMZ	Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
E. coli	*Escherichia coli*
HDVrbz	Hepatitis delta virus ribozyme
IfZ	Institute of Sugar Beet Research
IgG	Immunoglobulin G
Kan	Kanamycin
LB	Lysogeny broth
Mab	Mouse antibody
N. benthamiana	*Nicotiana benthamiana*
OD	Optical density
ORF	Open reading frame
ORI	Replication origin
PBS-Tween	Phosphate-buffered saline-Tween
PCR	Polymerase chain reaction
pNPP	para-Nitrophenylphosphate
RAM-AP	Rabbit anti-mouse alkaline phosphatase linked
RdRp	RNA-dependent RNA polymerase
Rif	Rifampicin
RNA	Ribonucleic acid
RTD	Read-through domain
sgRNA	Subgenomic RNA
SOB-medium	Super optimal broth
SOC	Super Optimal broth with Catabolite repression
T. expansa	Tetragonia expansa
Ta	Annealing temperatures
TAS-ELISA	Triple antibody sandwich-enzyme linked immuno sorbent assay
TBSV	Tomato bushy stunt virus
Tc	Tetracycline
T-DNA	Transfer DNA
Tm	Melting temperatures
UTR	Untranslated region
VPg	Viral protein genome-linked
VY	Virus yellows

1 Introduction

Beet chlorosis virus (BChV) and beet mild yellowing virus (BMYV) are a part of the virus yellows (VY) disease complex in *Beta* species. These are positive-sense single-stranded RNA viruses that belong to the genus *Polerovirus*, family *Solemoviridae* (Current ICTV Taxonomy Release). They are transmitted by aphids in a persistent, non-propagative manner and are restricted to the vascular tissue of the host plant.

Green peach aphid (*Myzus persicae*), the primary vector of the VY disease, was successfully controlled until the 2019 EU ban on neonicotinoid seed coating in sugar beet cultivation (Hossain et al., 2021). Without pesticide presence, aphid colonisation can occur earlier in the season. Furthermore, as it was shown, early infection with BChV or BMYV significantly decreases (24-27%) sugar yield (Stevens et al., 2004). Growing insect vector resistance to the number of remaining pesticides (Bass et al., 2014) leaves breeding for the host plant resistance the most viable VY disease control option. Unfortunately, out of the few partially resistant to BMYV sources identified, even less showed additional resistance to BChV (Asher et al., 2001).

The host range of BMYV is reported to be quite broad. It includes, besides *Beta* species, some common weeds like *Viola arvenisis*, *Stellaria media*, *Capsella bursa-pastoris*, *Spergula arvensis,* as well as minor crops like oilseed *Crambe abyssinica* (Stephan & Maiss, 2006). On the other hand, host range of BCHV seems to be much narrower and includes *Beta vulgaris*, *Spergula arvensis*, *Spinacia oleracea* and *Chenopodium capitatum* (Hauser et al. 2002). This host specificity indicates the presence of certain genomic factors that can be investigated by reverse genetic methods, that employs artificial recombination.

The occurrence of interspecific recombination between BChV and BMYV has already been documented (Kozlowska-Makulska et al., 2015) and can be a source of virus variability and host plant resistance breaking. Considering the broad host range of the two viruses and frequent occurrence together in mixed infections (Hauser et al., 2000) as well as the potential to be present together with other beet-infecting viruses like Beet yellows virus (BYV), and beet mosaic virus (BtMV), recombination study of poleroviruses is of great importance.

1.1 Polerovirus genome expression

To design a recombination system, one should understand the properties of the genome of the organisms, they are working with, as gene expression strategies utilized by poleroviruses are extremely complex and require additional explanation.

BMYV and BChV share a similar monopartite single-stranded positive-sense RNA genome of around 5,7 kbp, that includes 6 (Stephan & Maiss, 2006) and 7 (Wetzel et al., 2018) open reading frames

(ORFs), respectively. 5’ end of the virus genome, containing ORF0, 1, and 2 can be transcribed directly and expressed by leaky scanning and ribosomal frameshift strategies. Additionally, a viral genome-linked protein (VPg) is connected to the RNA 5’end and is involved in translation initiation. On the other hand, 3’ end of the genome, with ORF3, 4, 5 and 3a for BChV, is transcribed via subgenomic RNA (sgRNA) and lacks a poly(A)-tail. The proteins expressed by poleroviruses include P0, a suppressor of post-transcriptional gene silencing, P1 and P2 that are required for viral replication and are expressed by ribosomal shift as a fusion protein, containing VPg and RNA-dependent RNA polymerase (RdRp). Recently described P3a protein that is believed to be involved in a long-distance movement (Smirnova et al., 2015) and translated from a non-AUG start codon. Major capsid protein (CP), encoded by ORF3 and required to assemble the virus capsid. P3-P5 fusion protein, also called read-through domain (RTD), is believed to be essential for aphid acquisition. And P4 protein, expressed by leaky scanning from the ORF4, is a putative movement protein (Mayo & Ziegler-Graff, 1996).

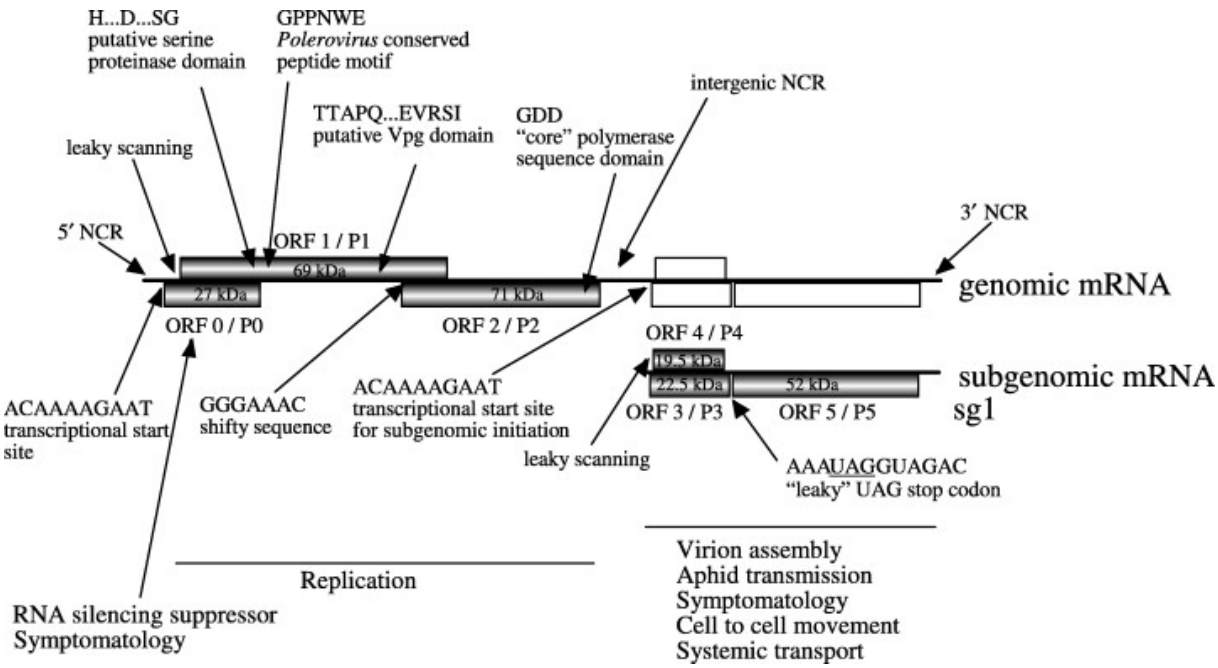

Figure 1. Organization and expression of a polerovirus genome

Taken from Stevens et al., 2005.

It is clear, that such a fascinating genome should be studied more deeply, both for practical use, e.g. in agriculture for resistant cultivar development, and for fundamental virus genomes research. Thus, developing an instrument that can potentially be used in reverse genetic research or modelling of recombination events in nature is important.

1.2 Aim of the thesis

Even though BMYV and BChV recombination was already documented by Kozlowska-Makulska et al. (2015), there is a reservation about the recombinant detection methods used and how they could create recombinants themselves. PCR methods, employed by Kozlowska-Makulska et al. (2015), can produce artefacts, due to polymerase ability of template switching, resulting in false positive artificial in vitro recombinants (Kanagawa, 2003; Odelberg et al., 1995).

Besides, natural recombination occurs under low selection pressure due to multiple variations of functional full-length viral genome present. Therefore, the development of the system, where partial viral genomes cannot replicate without creating a viable recombinant, under high selection pressure, allows to prove the recombination possibility for chosen viruses unequivocally and opens the prospect for studying desired virus properties, like host specificity, resistance breaking, etc.

Thus, with the growing economic importance of the viruses, to overcome PCR bias occurring due to polymerase template switching, and to facilitate the study of the virus genomes, the present work was developed with the following objectives:

1. Compare BChV and BMYV genomes and design virus fragments that potentially can recombine.

2. Develop a cloning procedure to create virus constructs in an appropriate vector.

3. Transform *A. tumefaciens* with developed virus constructs and asses their viability *in planta*.

2 Materials

2.1 Virus source

The viral constructs were produced using BMYV and BChV infectious full-length cDNA clones in the binary vector pDIVA from the "Institute of Sugar Beet Research" (IfZ) collection. BMYV infectious clone is based on the isolate P9-9E-W2_BMYV (Acc. No. ON924242.1), produced and kindly provided by Dr. Roxana Hossain. BChV infectious clone is based on the isolate BChV-2a (Acc. No. MH271171.1), produced by Wetzel et al. (2018).

Additionally, sugar beet (*Beta vulgaris* subsp*. vulgaris*) plants systemically infected with BChV were used as a virus source for the production of BChV P02 construct. The plants are a part of the IfZ collection.

2.2 Cloning vector

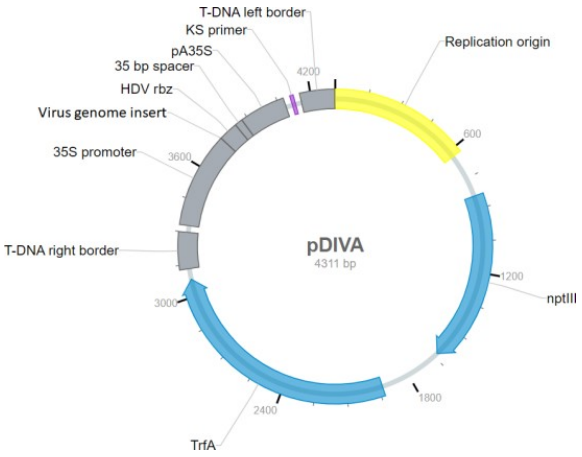

Figure 2. Vector map of pDIVA (Acc. No. KX665539.1)
Modified from https://novoprolabs.com/vector/Vgq2tqoa

The binary vector pDIVA (Acc. No. KX665539.1) was constructed by Prof. Dr. Edgar Maiss and acquired for this work from IfZ vector collection.

As shown in Figure 2, the cauliflower mosaic virus (CaMV) 35S promoter is situated directly before the inserted viral sequence and increases its expression in plant cells. The hepatitis delta virus ribozyme (HDVrbz) is a non-coding RNA that can catalyse cleavage and separate the insert sequence from the vector sequence. Mentioned features are all part of T-DNA, which is integrated into the plant nucleus after infection due to *vir*-genes of helper plasmid of *Agrobacterium tumefaciens* (Hellens, 2000). The replication origin (ORI) and TrfA plasmid replication initiation protein ensure a high rate of plasmid replication within both *Escherichia coli* and *A. tumefaciens*. Kanamycin resistance is an important selection tool for discriminating successful bacterial transformants. pDIVA

Table 1. Description of the selected features of pDIVA binary vector

Feature	Description	Position
Replication origin	sequence with high content of A and T nucleotides	1-630
nptIII	kanamycin resistance	839-1633
TrfA	plasmid replication initiation protein	1932-3080
35S promoter	CaMV 35S RNA promoter	3330-3754
Virus genome insert	a place of viral genome fragment insertion	3754 ↓ 3755
HDV rbz	hepatitis delta virus ribozyme	3755-3839
pA35s	cauliflower mosaic virus 35S RNA terminator	3875-4079

2.3 Plant material

Plants used in the experiments are listed below (Table 2).

Table 2. Test plants summary

Latin Name	Common Name	Cultivar	Source	Experiment
Beta vulgaris subsp. *vulgaris*	White beet	Albina Vereduna	Pieterpikzonen B.V. Holland	experimental host screening
Beta macrocarpa	Wild beet	-	IfZ collection	experimental host screening
Tetragonia tetragonioides (syn. *T. expansa*)	New Zealand spinach	G 865	NBB egesa GmbH	experimental host screening
Nicotiana benthamiana	Bentham tobacco	-	IfZ collection	constructs viability evaluation

3 Methods

3.1 *In silico* primer design

The sets of PCR primers (Table 3) were designed in the program SnapGene (GSL Biotech LLC) using sequence data of BMYV and BChV infectious clones (from 2.1). Two types of virus constructs were intended to be synthesised. One that contains functional RdRp, but without CP sequence, denoted throughout the work as P02. The second kind of construct, denoted as RO, corresponded to the genomic 3' end of each polerovirus, which was not able to produce a fully functional RdRp. However, an overlapping sequence of ~820 nucleotides in the highly conserved RdRp sequence was included in these constructs to allow a potential intra- and interspecies recombination.

Table 3. Primer list

Name[1]	**Sequence (5'->3')**[2]	**Fragment**[3]	**Tm**[5]**,°C**	**Ta**[5]**,°C**
ARP1_35S_as (forward)	CCTCTCCAAATGAAATGAACTTCCTTATATAG	Linearised pDIVA	64,9	64,9
ARP2_HDV_s (reverse)	GGGTCGGCATGGCATCTC		65,8	
ARP15_BChV_P02_fw	GTTCATTTCATTTGGAGAGGacaaaagaatactaggaggacagtgaatgaactttg	BChV P02	69,2	68,4
ARP16_BChV_P02_rv	GAGATGCCATGCCGACCCgtatgcttgatcctcctgaattagttttgtgg		68,4	
ARP5_BChV_RO_fw	GTTCATTTCATTTGGAGAGGtccctagtggatcagttggtagc	BChV RO	66,3	66,3
ARP6_BChV_RO_rv	GAGATGCCATGCCGACCCacaccgaaatgccagaaggaatc		66,3	
ARP7_BMYV_P02_fw	GTTCATTTCATTTGGAGAGGacaaaagaaaccagcgaggatctag	BMYV P02	65,8	65,8
ARP8_BMYV_P02_rv	TGGAGATGCCATGCCGACCCgctgtttatgttatccctcgttatatcttttgt		66,6	
ARP9_BMYV_RO_fw	GTTCATTTCATTTGGAGAGGtcattggttgatcaactggtagccc	BMYV RO	67,8	67,1
ARP10_BMYV_RO_rv	TGGAGATGCCATGCCGACCCacaccgaagtgccgtaggg		67,1	
ARP11_pDIVA_seq_fw	GCCTCTGCCGACAGTGGTCC	Construct sequencing[4]	70,1	70,0
ARP12_pDIVA_seq_rv	GATCGAGCTCGGGCCAGCTTC		70,0	

1. Primer name contains information about target region or fragment to be amplified (e.g. BChV_P0_P2 - BChV P02), "fw" indicates forward primer, "rv", reverse.

2. Uppercase letters represent pDIVA "overlap" sequence, and lowercase, viral genome.

3. Fragment column refers to the intended product of the PCR reaction, where the primers are used.

4. For more details, see chapter 3.3.4.

5. Tm - melting temperature; Ta - annealing temperature.

In such a way, four virus fragments need to be amplified: BChV P02, BChV RO, BMYV P02, BMYV RO. As these fragments were meant to be cloned into pDIVA binary vector via Gibson assembly, an "overlap" ends with pDIVA sequence needed to be added to the primers and subsequently to the

fragment amplicons to facilitate the cloning. Additionally, primers to "open" or linearise pDIVA vector were made, as well as sequencing primers, to control the quality of the cloning procedure.

Thermo Fisher Tm Calculator was used to compute melting temperatures (Tm) and estimate proper annealing temperatures (Ta) of the primer pairs for "Phusion or Phire DNA polymerase" using the default setting. In primers that contain additional vector overlap sequence for Gibson assembly, only the viral genome part of the primer (lowercase in Table 3), which binds to the template, was taken to calculate Tm and Ta.

The primers were ordered online from Eurofins Genomics. Upon arrival, following indications in the manufacturer-provided "Oligonucleotide Synthesis Report", primers were redissolved in DEPC-treated water (Carl Roth) to achieve 100 pmol/µL concentration. Further, a 1:10 dilution was made for every primer to achieve a working concentration of 10 pmol/µL.

3.2 Synthesis of the virus constructs

3.2.1 RNA extraction

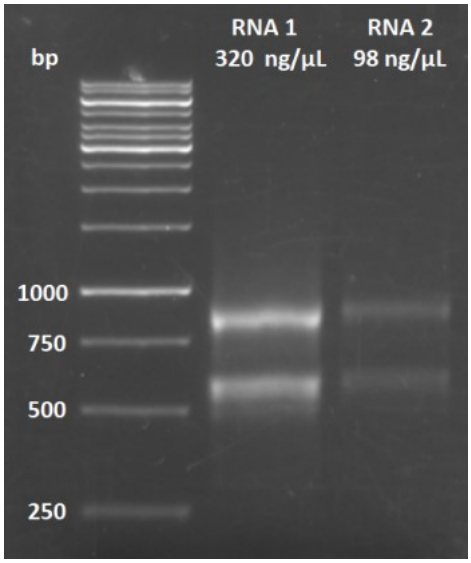

Figure 3. Gel electrophoresis of the total extracted RNA

Conditions:
1% agarose gel;
140 V, 45 min;
8 µL volume per well
with 1 µL of RNA.

To extract total plant RNA, 100 µg of leaf tissue was taken from BChV-infected sugar beets (*B. vulgaris* plants). Samples were put into 1,5 ml tubes and frozen in liquid nitrogen, then homogenized with cooled metal pestles. Total plant RNA extraction was performed using NucleoSpin® RNA Plant Kit and following the protocol by MACHEREY-NAGEL (2022).

Absorbance values at 260 nm were measured, and the concentration of the obtained total RNA was quantified using a DeNovix spectrophotometer:

1) Measuring surfaces were cleaned before usage, between samples and after the last measurement.

2) 1 µL of DEPC-treated water (Carl Roth) was applied to the lower measuring surface of the machine and measured as "blank".

3) 1 µL aliquot of total extracted RNA was similarly applied and measured as a "sample".

4) Concentrations were recorded.

The quality of the extracted RNA was assessed via agarose gel electrophoresis (Figure 3). The procedure will be detailed in chapter 3.2.5. The sample with the highest concentration, RNA 1 (320 ng/µL), was selected for further cDNA synthesis. For storage, samples were put into liquid nitrogen and later into a -80°C freezer.

3.2.2 cDNA synthesis

cDNA synthesis was performed using RevertAid H Minus reverse transcriptase (Thermo Fisher Scientific™) and random hexamer primers (Thermo Fisher Scientific™), following the protocol for the first strand cDNA synthesis by Thermo Scientific™ (2016):

1) Indicated components (Table 4) were added into a sterile, nuclease-free 200 µL tube on ice.

Table 4. cDNA synthesis. First step recipe

Reagent	Volume, µL
Total RNA (RNA 1, 320 ng/µL)	1
Random hexamer primer (10 pmol/µL)	1
DEPC-treated water (Carl Roth)	10,5

2) The solution was briefly mixed, centrifuged, then incubated at 65°C for 5 min in the thermocycler and placed back on ice.

3) Further, the following components (Table 5) were added to the mix.

Table 5. cDNA synthesis. Second step recipe

Reagent	Volume, µL
5X Reaction Buffer (Appendix 1)	4
Thermo Scientific™ RiboLock™ RNase inhibitor	0,5
dNTP Mix 10mM (Thermo Fisher Scientific™)	2
RevertAid H Minus Reverse transcriptase	1

4)The solution was again briefly mixed, centrifuged, and then incubated in a thermocycler according to the following program (Table 6).

Table 6. cDNA synthesis. Incubation program

T,°C	Time
25	00:10:00
42	00:60:00
70	00:10:00

5)Obtained cDNA was stored in a -20°C freezer.

3.2.3 PCR amplification

Virus and vector fragments amplification was done using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific™) and the sets of primers described in chapter 3.1 (see Table 3). Information on the amplified fragments, along with corresponding primers and template DNA is provided in Table 7.

Table 7. Fragment amplification PCR. Products and components

Amplified Fragment	Length, bp	Primers	Template DNA
pDIVA (linearised)	4311	ARP1_35S_as (forward) ARP2_HDV_s (reverse)	pDIVA binary vector[1], 1:20 dilution
BChV P02	3462	ARP15_BChV_P02_fw ARP16_BChV_P02_rv	BChV cDNA synthesised from total extracted RNA[2], not diluted
BChV RO	3212	ARP5_BChV_RO_fw ARP6_BChV_RO_rv	BChV infectious clone[1], 1:20 dilution
BMYV P02	3460	ARP7_BMYV_P02_fw ARP8_BMYV_P02_rv	BMYV infectious clone[1], 1:20 dilution
BMYV RO	3170	ARP9_BMYV_RO_fw ARP10_BMYV_RO_rv	BMYV infectious clone[1], 1:20 dilution

1. For information on virus and vector source, see chapter 2.1.

2. See chapters 3.2.1 and 3.2.2.

All primers were used in a working concentration of 10 pmol/µL. Template DNA was diluted with DEPC-treated water (Carl Roth) to 1:20 in all cases, except for BChV cDNA, which was used undiluted after synthesis. Volume per reaction of the template cDNA for BChV P02 fragment differs as well (see Table 8, where appropriate recipes are given in separate columns).

Table 8. Fragment amplification PCR. Reaction recipe

Template DNA Type Reagent	Plasmid DNA[1] µL/reaction	cDNA µL/reaction
Phusion™ High-Fidelity PCR Master Mix (Thermo Fisher Scientific™)	10	10
Forward primer	1	1
Reverse primer	1	1
DEPC-treated water (Carl Roth)	7	6
Template DNA	1	2
TOTAL	20	20

1. Plasmid DNA here refers to BChV and BMYV infectious clones and pDIVA plasmid

In the thermocycler program, the temperature for the annealing step was taken from the Thermo Fisher Tm Calculator recommendation (see Table 3, "**Ta**" column), time for the elongation step was calculated using Phusion High-Fidelity DNA polymerase characteristics (namely, elongation speed of 1000 bp per 15 s, Thermo Scientific™ Product Information) and expected fragment length (see Table 7), according to the following equation (1).

$$\text{elongation time, s} = \frac{\text{fragment lengt, bp}}{\text{1000, bp}} * 15, \text{s} \quad (1)$$

Thus, summarised thermocycler program is given in Table 9.

Table 9. Fragment amplification PCR. Thermocycler program

Step	Temperature,°C	Time	
Initial denaturation	98	00:00:30	
Denaturation	98	00:00:10	30x cycles
Annealing	**Ta**, see Table 2	00:00:10	
Elongation	72	**calculated**, see (1)	
Final elongation	72	00:10:00	
Temporary storage	12	∞	

3.2.4 Template DNA digestion

The PCR product was digested with DpnI restriction enzyme (Thermo Fisher Scientific™) to avoid contamination with full-length infectious clones, as their plasmids were used as template DNA in the PCR reaction. For this, 1 µL of the enzyme (10 U/µL) was added into the tubes after PCR, mixed by pipetting, and incubated at 37°C for 1 hour.

DpnI digestion was used to generate BChV RO, BMYV P02 and BMYV RO fragments. The enzyme recognizes adenine methylation at GATC sites of the plasmid DNA (Thermo Scientific™ Product Information) and cuts as shown below:

5'...G A^{m6} ↓ T C...3'

3'...C T ↑ A^{m6} G...5'

In the BChV P02 fragment amplification case, cDNA was used as a template. A DpnI digest is not necessary in that case, cDNA cannot be transformed directly into bacteria, so it does not pose danger of contamination and won't result in false negative bacterial colonies after transformation.

3.2.5 Agarose gel electrophoresis

Through the course of this work, agarose gel electrophoresis was used for:

1) quality check of total extracted RNA (see chapter 3.2.1);

2) separation and length assessment of DNA amplicons after PCR (see chapter 3.2.3);

3) quality and fragment length check of DNA after gel extraction (see chapter 3.2.6);

4) visualisation of the results of the analytic restriction endonuclease digestion (see chapter 3.3.3).

Agarose gel was prepared following the protocol:

1) 0,6 g of agarose (Carl Roth) and 60 ml of 1xTAE buffer (Appendix 1) were added into a flask.

2) The flask was heated in a microwave until the agarose was completely dissolved.

3) The agarose solution was cooled down to approximately 60°C.

4) 4 µL of an intercalating nucleic acid stain GelRed™ (VWR) was added, and the solution was gently stirred.

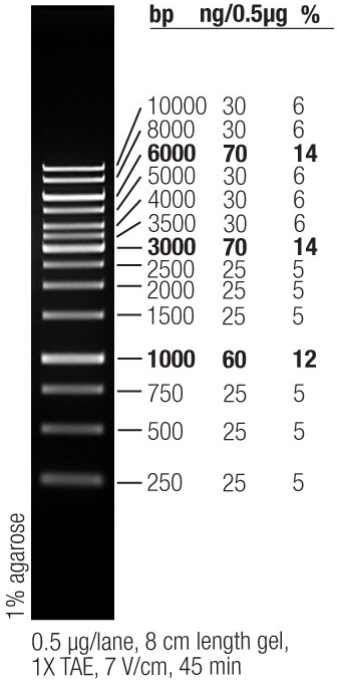

Figure 4. Thermo Scientific™ Gene-Ruler™ 1 kb DNA Ladder

5) The solution was poured into a casting tray, and a comb was inserted to create wells for sample loading.

6) The gel slab was allowed to solidify at room temperature.

7) Electrophoresis chamber was filled with 1xTAE buffer.

8) The comb was carefully removed, and the agarose slab was placed in the buffer-filled chamber to completely cover the gel surface .

9) For RNA electrophoresis, a separate set of tools (casting tray, comb, electrophoresis chamber) was used to avoid RNA degradation by external RNases.

10) 1 µL of DNA gel loading buffer was added to every sample, then the samples were loaded into the wells. Only in the case of analytic restriction endonuclease digestion this step was avoided due to using FastDigest Green Buffer (Thermo Fisher Scientific™), which already contains the loading buffer.

11) 7 µL of GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific™) ready-to-use solution was loaded for the size reference (Figure 4).

12) The electrophoresis was launched with the appropriate voltage and time. In this work, they are specified under every gel electrophoresis picture.

13)The bands were visualized with a UV transilluminator at 320 nm and documented using Intas GelDoc.

3.2.6 DNA extraction from agarose gel

PCR amplicons were separated according to their size via agarose gel electrophoresis to ensure the success of the procedure (expected signal is present) and to separate impurities. However, in order to use amplified fragments, DNA needed to be extracted from the gel.

Directly after documenting PCR gel electrophoresis, the band of interest was cut out of the gel under FastGene Blue/Green LED flashlight (Nippon Genetics). The cut-out gel was then placed in 1,5 ml tube and weighed. Gel extraction was performed using Monarch® DNA Gel Extraction Kit and following protocol by New England BioLabs (2021). The incubation of a gel piece was done at 50°C.

Centrifugation was carried out as instructed for 1 minute at 16,000 x g. DNA was eluted in 20 µL of DNA dilution buffer (supplied with kit), the buffer was incubated for 1 minute at 50°C prior tu use.

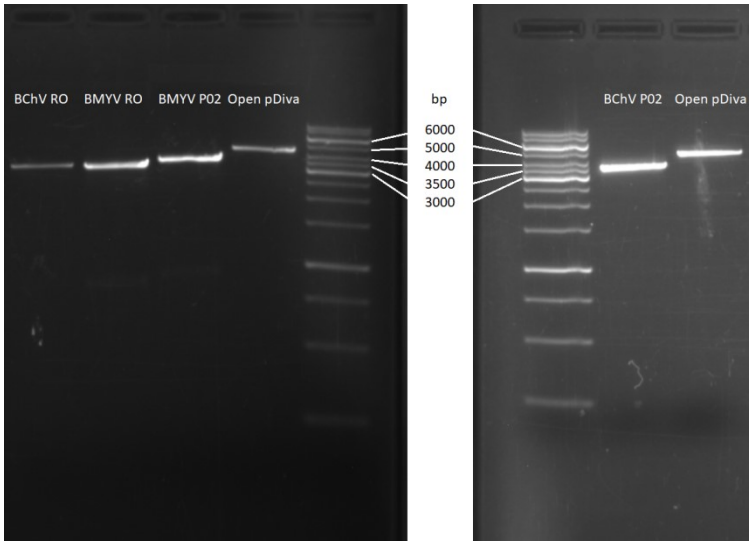

Figure 5. Amplified PCR products separated by gel electrophoresis after DNA gel extraction

Conditions:
1% agarose gel;
140 V, 45 min;
8 µL volume per well
with 1 µL of DNA.

Conditions:
1% agarose gel;
120 V[1], 45 min;
8 µL volume per well
with 1 µL of DNA.

1. The voltage was changed due to the requirements of the new electrophoresis chamber, which allowed a maximum of 120 V.

Gel electrophoresis of the extracted DNA was performed (Figure 5). The expected fragment size (Table 7) corresponded to the size of the obtained fragments.

3.2.7 Gibson assembly

Gibson assembly is a powerful and widely used technique for the rapid and seamless assembly of DNA fragments. Developed by Gibson et al. (2009), it is an isothermal, single-reaction method for synthesizing complex genetic constructs, including plasmids, synthetic genes, etc. Using overlapping homologous ends produced by the T'5 exo-nuclease, Gibson assembly allows for the efficient and precise joining of DNA fragments without the need for restriction enzymes.

Table 10. Gibson assembly. Reaction recipe

Reagent	Volume, µL		
	1:3 ratio	1:5 ratio	pDIVA control
Gibson Master Mix (Appendix 1)	10	10	10
Open pDIVA	1	1	1
Virus fragment	3	5	0
DEPC-treated water	6	3	9
TOTAL	20	20	20

During Gibson assembly, three enzymes were utilized: T5’ exonuclease (New England BioLabs), Phusion High-Fidelity DNA polymerase (Thermo Scientific™), and Taq DNA ligase (New England BioLabs). The reaction mix (Table 10) was prepared on ice in a sterile 200 µL tube. Vector to gene ratio was 1:3 for all fragments, but BChV P02. For BChV P02, two ratios were used, 1:5 and 1:3, to ensure successful assembly. As an indication of possible false positives, due to self-assembling of the

pDIVA vector, reaction with only linearised pDIVA without virus fragment was done. The reaction mix was incubated in a thermocycler at 50°C for 60 min.

Reaction products were either immediately transformed into *E. coli* cells or stored at -20°C.

3.3 Transformation of the virus constructs

3.3.1 Transformation into chemically competent *E. coli*

A transformation into competent *E. coli* cells allows the multiplication of the synthesised virus constructs. For this purpose, a chemically competent strain of *E. coli* DH5α from IfZ collection was used. The strain does not harbour any resistances, allowing for the selection of the successful transformants with kanamycin-enriched media (Appendix 1) since functioning pDIVA plasmid enables kanamycin resistance. Short heat application increases *E. coli* cell membrane permeability to plasmid DNA and allows transformation. All bacteria-related manipulations were done under the clean bench (Thermo Scientific™, Herasafe™), following the protocol:

1) 20 µL of Gibson assembly reaction product was added to 100 µL of competent *E. coli* cells.

2) The mixture was incubated for 30 minutes on ice.

3) Heat induction of 42°C was applied for 90 seconds.

4) The mixture was cooled down on the ice for 30 seconds.

5) 400 µL of SOC-medium (Appendix 1) was added.

6) The mixture was incubated at 37°Cand 200 rpm for 60 minutes.

7) 100 µL of cell suspension was plated on solid LB-medium with kanamycin (Appendix 1), 2 plates per construct or construct-vector ratio was used.

8) The plates were incubated at 37°C overnight.

9) The colonies were counted the next day and moved to +4°C storage.

3.3.2 Plasmid DNA extraction

For further work with the viral constructs, they needed to be isolated from *E. coli.* The procedure is based on the different denaturation pH of genomic and plasmid DNA of bacteria. The method is described by Birnboim and Doly (1979). The extraction was done following the protocol:

1) Single colonies (12 per each construct, but 24 for BChV P02) were transferred with a toothpick into 3 ml liquid LB-medium (Appendix 1) containing 3 µL of kanamycin and incubated overnight at 37°C and 200 rpm.

2) 2 ml of suspension was transferred in 2 ml tube and centrifuged for 3 minutes at 10000 x g. Supernatant was discarded, and tubes were tapped dry.

3) 200 µL of solution A (Appendix 1) was added, bacterial pellet was resuspended, then incubated at

room temperature for 15 minutes.

4) 400 µL of solution B (Appendix 1) was added, rack was carefully rotated to mix the components, and left at room temperature for 5 minutes to incubate.

5) 300 µL of solution C (Appendix 1) was added, rack was inverted to mix, incubation was done on ice for 30 minutes.

6) Centrifugation for 10 minutes at 10000 x g, 750 µL of supernatant is transferred to a new 1,5 ml tube.

7) Centrifugation for 10 minutes at 10000 x g, 650 µL of supernatant is transferred to a new labelled 1,5 ml tube.

8) 600 µL of cooled at -20°C isopropanol (Carl Roth) was added, rack was inverted to mix.

9) Centrifugation for 10 minutes at 10000 x g, supernatant was discarded, and tubes were tapped dry.

10) 200 µL of solution D (Appendix 1) was added, the rack was incubated at room temperature for 5 minutes, then carefully resuspended.

11) 400 µL of cooled at -20°C ethanol 99.5% (Carl Roth) was added, rack was inverted to mix.

12) Centrifugation for 10 minutes at 10000 x g, supernatant was discarded.

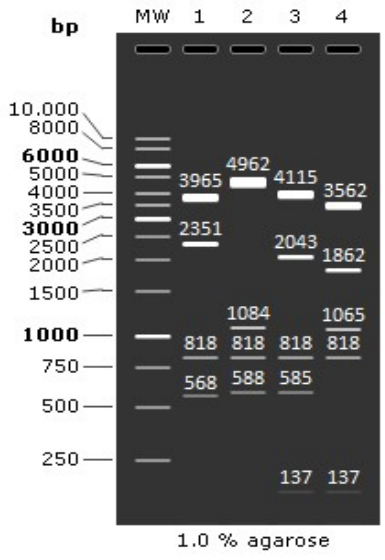

Figure 6. Expected results of analytic restriction endonuclease digestion with EcoRI and XhoI

MW: GeneRuler™ 1 kb DNA Ladder
1: pDIVA_BChV_P02
2: pDIVA_BChV_RO
3: pDIVA_BMYV_P02
4: pDIVA_BMYV_RO

Numbers over bands represent band sizes in bp.

13) DNA pellet was dried in a vacuum concentrator (Eppendorf) for 7 minutes at 45°C.

14) Dried pellets were resuspended in 100 µL RNase/TE. (1:1000-dilution).

15) The plasmids were stored at -20°C.

3.3.3 Analytic restriction endonuclease digestion

To control the quality of Gibson assembly, verifying that the plasmid DNA was synthesised as expected, is crucial. Restriction enzymes cleave the DNA at specific sites and generate a set of fragments of known sizes. By comparing the resulting fragment pattern with the expected, the identity of the assembled plasmid can be confirmed.

EcoRI 5'...G↓A A T T C...3' / 3'...C T T A A↑G...5'

XhoI 5'...C↓T C G A G...3' / 3'...G A G C T↑C...5'

EcoRI and XhoI restriction endonucleases (Fast digest Thermo Scientific™) were chosen for analytic digestion in a SnapGene program. The cleavage sites of the enzymes are illustrated above.

The expected band pattern was generated, based on the *in silico* constructs designed in SnapGene, and as can be seen (Figure 6), the enzymes allow differentiation between all four constructs.

For the analytic digestion reaction, the reagents (see Table 11) were mixed in the sterile 200 µL tube and incubated at 37°C for 15 minutes. Gel electrophoresis of the reaction products was performed, resulting band pattern was compared to the expected.

Table 11. Analytic restriction endonuclease digestion. Reaction recipe

Reagent	Volume, µL
FastDigest Green Buffer (Thermo Scientific™)	1
Plasmid	2
EcoRI, 10 U/µL (Thermo Scientific™)	1
XhoI 10 U/µL (Thermo Scientific™)	1
DEPC-treated water (Carl Roth)	10
TOTAL	15

3.3.4 Sequencing

Two plasmids from every construct, displaying a correct restriction digest pattern, were randomly selected for Sanger sequencing (Sanger et al., 1977) of the conjunction region between the virus fragment and pDIVA vector. This region was the joining site during the Gibson assembly, its integrity would indicate a successful cloning.

Two sequencing primers were designed using genome maps of the viral constructs in the SnapGene. Forward primer ARP1_35S_as corresponds to the bp numbers 3560-3579 of CaMV 35S promoter in binary vector pDIVA, and is 194 bp upstream from the 5' virus-vector conjunction. Reverse primer ARP2_HDV_s corresponds to the bp numbers 3838-3858 of HDV ribozyme and 35bp spacer sequences in pDIVA, and is 103 bp downstream from the 3' virus-vector conjunction. Primers were used in the same 10 pmol/µL working concentration.

PCR amplification of the regions intended for sequencing was performed to increase DNA concentration. The recipe is detailed in Table 12. Template DNA is an extracted plasmid (from 3.3.2) and was used undiluted.

Table 12. PCR amplification for sequencing. Reaction recipe

Reagent	µL/reaction
Phusion™ High-Fidelity PCR Master Mix (Thermo Fisher Scientific™)	10
Forward primer	1
Reverse primer	1
DEPC-trated water (Carl Roth)	7
Template DNA	1
TOTAL	20

The thermocycler program is equivalent to the program in Table 9. The temperature of the annealing step is 70°C, and the time for the elongation step is 60 seconds.

Gel electrophoresis was done after PCR, with 1% agarose gel, 20 µL reaction volume per well, 45 minutes runtime and voltage of 120V or 140V, depending on the electrophoresis chamber used. The bands were cut out of the gel, and gel extraction, as detailed in chapter 3.2.6, was performed. The volume of eluted DNA was increased afterwards from 20 to 30 µL with DEPC-treated water. Each sample was divided into two aliquots of 15 µL (for forward and reverse sequencing). 100 µL of forward and reverse primers (10 pmol/µL) were put into separate tubes and sent together with the samples. The sequencing was done via the Microsynth Seqlab.

3.3.5 Transformation into electrocompetent *A. tumefaciens*

Agrobacterium-mediated inoculation has some advantages in comparison to other inoculation methods. Reverse genetic analysis, for example, is possible and allows to investigate certain traits of interest in both the viral and plant genome.

Electrocompetent *A. tumefaciens* C58C1, from the collection of IfZ, was used as an inoculation vector for the virus constructs. *A. tumefaciens C58C1* harbours resistances to rifampicin and tetracycline. The transformation was done by short high-voltage pulse, electroporation, to increase the cell membrane permeability and allow virus constructs to enter the cell. The method is previously described in Mattanovich et al. (1989).

All steps of the protocol were conducted under a clean bench (Thermo Scientific™, Herasafe™):

1) 40 µL of electrocompetent *A. tumefaciens* C58C1 cells were thawed on ice.

2) 1 µL of plasmid (from 3.3.2) was added and mixed by pipetting.

3) 41 µL of the mix was transferred to a previously cooled at -20°C electroporation cuvette.

4) Cuvette was inserted in an electroporator (Eppendorf, Eporator) and treated with an electric pulse of 1440V

5) 400 µL of ice-cold SOB-medium (Appendix 1) was added to the cuvette and mixed by pipetting.

6) the mix was transferred to a sterile 1,5 ml tube and incubated at 28°C, 250 rpm for 3 hours.

7) 100 µL of cell suspension was plated on solid LB-medium with rifampicin, tetracycline and kanamycin (Appendix 1).

8) the plates were incubated at 28°C for 48 hours.

3.3.6 Long-term bacterial culture production

To store viral constructs in transformed *A. tumefaciens*, long-term cultures were produced. Single colonies of transformants (from 3.3.5) were picked with a toothpick and transferred to a 3 ml liquid

LB-medium with 3 µL of rifampicin, tetracycline and kanamycin (Appendix 1). The vials were incubated at 28°C and 200 rpm overnight. 500 µL of cell suspension was transferred to a sterile 1,5 ml tube, and 500 µL of 100% glycerine (Carl Roth) was added. The tubes were labelled, then shock-frozen in liquid nitrogen, and moved to a -80°C storage.

The long-term bacterial culture method was also used to replenish *A. tumefaciens*-based libraries of BMYV and BChV infectious full-length cDNA clones. For that, aliquots of existing long-term cultures were streaked on solid LB-medium with selective antibiotics (Rif, Tc, Kan), then incubated at 28°C for 48 hours. Next, scoops of grown bacterial mass, taken with sterile inoculation loop, were transferred to a 3 ml liquid LB-medium with 3 µL Rif, Tc, Kan. And after overnight incubation (28°C, 200 rpm), 500 µL of suspension was mixed with 500 µL of 100% glycerine (Carl Roth) and frozen the same way as was earlier described.

3.4 *In planta* viability evaluation of the virus constructs

3.4.1 Plant propagation

Plant propagation was done in the glass greenhouse with controlled climate conditions (24°C/14 h light, 18°C/10 h dark photoperiod). Irrigation was done daily by hand. After germination, plant seedlings of approximately 7-10 days old were transplanted into separate pots with appropriate substrates. A steamed soil was used for beets, and a 1:1 mixture of soil and peat, for spinach and *N. benthamiana*. A teaspoon of a commercial preparation of entomopathogenic nematode *Steinernema feltiae* (Katz Biotech AG) was added to each pot to suppress larvae of sciarid flies, that could have damaged young plants.

3.4.2 *Agrobacterium*-mediated inoculation

For inoculation, a suspension with *A. tumefaciens* cells was made under a clean bench (Thermo Scientific™, Herasafe™), following a protocol:

1) Viral constructs or full-length cDNA clones, taken from long-term cultures (3.3.6) were streaked on a solid LB-medium with Rif, Tc and Kan (Appendix 1) and incubated at 28°C for 48 hours.

2) Scoops of grown bacterial mass, taken with sterile inoculation loop, were transferred to a 3 ml liquid LB-medium with 3 µL Rif, Tc, Kan (Appendix 1) and incubated overnight at 28°C, 200 rpm.

3) Liquid cell culture was transferred into 10 ml plastic tubes and centrifuged at 4000 x g, 4°C for 10 minutes

4) Supernatant was removed, tubes were tapped dry.

5) Bacterial pellet was resuspended in 3 ml agroinoculation buffer (Appendix 1).

6) Optical density at 600 nm (OD600) was measured with photometer (Eppendorf BioPhotometer®).

&) OD600 was adjusted with agroinoculation buffer to 0,5 for *N. benthamiana* and to 1 for *Beta spp.*

and *T. expansa*

9) To every 10 ml of infiltration solution, 7.5 µL acetosyringone (Appendix 1) was added.

10) Inoculation solution was incubated in the dark at room temperature for 2 hours.

11) After incubation, inoculation was done with the help of a needle-free syringe. The syringe was filled with inoculation liquid, the air was pressed out and the solution was inoculated into the leaf.

12) In the case of virus constructs inoculation, equal inoculation solutions volumes of appropriate constructs and DK70 silencing suppressor, based on P19 silencing suppressor of tomato bushy stunt virus (TBSV), were mixed together before inoculation (e.g. 1ml of BChV P02 + 1ml of BMYV RO + 1ml of DK70 silencing suppressor).

3.4.3 Screening for the experimental plant host

Three different potential experimental plant hosts , namely, white beet (*B. vulgaris* subsp. *vulgaris '*Albina Vereduna'), wild beet (*B. macrocarpa*), and New Zealand spinach (*T. expansa '*G 865'), were tested for their suitability and effectiveness of polerovirus infection.

The experimental design is detailed in Table 13. Plants were infected with BMYV and BChV infectious full-length cDNA clones via *Agrobacterium*-mediated inoculation into cotyledons (3.4.2). For the detection of the systemic infection, 200 mg samples were taken from mature but not senescent leaves in the middle section of the plant 28 days post inoculation (d.p.i.). Virus detection was done by TAS-ELISA (3.5).

Table 13. Screening for the experimental plant host. Experiment design

Plant species	BMYV inoculated	BChV inoculated	Healthy control
B. *vulgaris* subsp. *vulgaris*	12 plants	12 plants	6 plants
B. macrocarpa	12 plants	12 plants	6 plants
T. expansa	10 plants	10 plants	5 plants

3.4.4 Virus constructs viability evaluation in *N. benthamiana*

N. benthamiana was chosen as an experimental plant host due to its susceptibility to polerovirus infection, fast growth rate, ease of genetic transformation, and substantial body of available research, as it is one of the most common experimental hosts in plant virology.

Within one experiment, two sets of samples were collected from a "local patch" assay and a "systemic infection" assay. A local patch assay was done to demonstrate a successful inoculation and ensure no recombinant virus was overlooked, as it is known that BChV can replicate in the infiltration patch but is not able to spread systemically in *N. benthamiana* (Wetzel et al., 2018), which could be

the case for potential recombinant viruses. On the other hand, a systemic infection assay could show that the recombinant is capable of both replication and systemic spread.

Table 14. Viability assay of the virus constructs. Experiment design

Construct combinations	Experiment 1 (assays are done on separate plants)		Experiment 2 (both assays are done on the same plant)
	Local patch assay	Systemic assay	
BMYV P02+BMYV RO+DK70	20 patches, 5 plants	20 plants	20 plants
BMYV P02+BChV RO+DK70	20 patches, 5 plants	20 plants	20 plants
BChV P02+BChV RO+DK70	20 patches, 5 plants	20 plants	20 plants
BChV P02+BMYV RO+DK70	20 patches, 5 plants	20 plants	20 plants
BMYV P02+DK70[1]	5 patches, 2 plants	5 plants	5 plants
BMYV RO+DK70	5 patches, 2 plants	-	5 plants
BChV P02+DK70	5 patches, 2 plants	-	5 plants
BChV RO+DK70	5 patches, 2 plants	-	5 plants
BMYV full-length	4 patches, 1 plant	5 plants	5 plants
BChV full-length	4 patches, 1 plant	5 plants	5 plants
Healthy, mock inoculation	-	-	5 plants
Healthy, no inoculation	4 patches, 1 plant	5 plants	5 plants

1. Systemic spread of partial virus genomes was not expected to occur, so only BMYV P02, containing RdRp motif was tested in the systemic assay in experiment 1.

Four combinations of four constructs with DK70 silencing suppressor (Table 14) were inoculated as described in 3.4.2. To control whether CaMV 35S promoter-induced expression of viral CP can generate a positive signal during detection with TAS-ELISA, separate constructs mixed with DK70 silencing suppressor were also inoculated. BMYV and BChV full-length cDNA clones were used as a positive control. Plants inoculated with agroinoculation buffer only and non-inoculated plants served as “mock inoculation” and healthy control, respectively.

Two repetitions of the experiment were done. In the first experiment, local and systemic assays were executed on different plants to ensure that sample collection of local assay would not interfere with the systemic spread of potential recombinants. During the second repetition, both assays were performed on the same plants. Mock inoculation control was added in the second experiment to

discriminate between symptoms produced with mechanical force of inoculation and with virus replication.

Sample collection for local patch assay was done 7 d.p.i, for systemic, 35 d.p.i. In both cases, 200 mg of leaf tissue was collected into a 2 ml tube with ceramic beads and frozen in liquid nitrogen. Local patch assay samples were collected from the inoculation patch, systemic, from mature but not senescent leaves in the middle section of the plant.

3.5 Virus detection with TAS-ELISA

ELISA, an enzyme-linked immunosorbent assay, is a routine method for detecting plant viruses. In ELISA, antibodies (immunoglobulin G, IgG) bound to the surface of a microplate are used to capture viral proteins (usually capsid protein). These complexes are then detected by another antibody labelled with an enzyme (e.g. alkaline phosphatase, AP) that can react with the substrate (e.g. para-Nitrophenylphosphate, pNPP), producing a signal. The signal can be measured in the photometer. The intensity of the signal is proportional to the virus concentration in the sample.

Triple antibody sandwich (TAS) ELISA uses an additional antibody (mouse antibody, Mab) that detects IgG-antigen complex and then is detected by the enzyme-linked antibody (rabbit anti-mouse AP, RAM-AP), that reacts with a substrate and produces a signal. The method is highly sensitive and allows the simultaneous processing of significant sample volumes.

A set of polyclonal antibodies: BWYV-IgG, BWYV Mab, and RAM-AP, produced by “Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH” (DSMZ), was used in this work. The antibodies can detect capsid protein ()CP of sugar beet-infecting poleroviruses, including BMYV and BChV. The analysis was done following the protocol by Leibniz-Institute DSMZ (n.d.), appropriate buffers are listed in Appendix 1:

1) Specific antibody BWYV-IgG was diluted in coating buffer 1:1000. 200µL was added to each well of the microtiter plate, except for the first column, that was left as “blank” (substrate background).

2) The plate was covered and incubated at 37 °C for 3 hours.

3) Then washed with PBS-Tween, soaked for 3 minutes. Washing was repeated two times. Plate was tapped dry.

4) 200 µL of 2% skim milk in PBS-Tween was added to each well (blocking), except “blank”.

5) The plate was covered and incubated for 30 min at 37°C

6) Blocking solution was removed, plate was tapped dry.

7) Before extraction, frozen samples were pulverised in homogeniser (Precellys 24, Bertin Technologies). Then, extraction buffer was added to the samples 1:5 (w/v). 200 µL aliquots of the test samples were added to duplicate wells. Frozen dried leaves of BChV- or BMYV-infected sugar

beets (*B. vulgaris*) were used as positive control, sample buffer, as negative.

8) The plate was covered and incubated at 4 °C overnight.

8) Next day the plate was washed three times as in step 3.

9) 200 µL of BWYV Mab diluted 1:1000 in conjugate buffer was added to each well, except “blank”.

10) The plate was covered and incubated at 37 °C for 2 hours.

11) The plate was washed three times as in step 3.

12) 200 µL of RAM-AP was diluted 1:1000 in conjugate buffer and added to each well, except “blank”.

13) The plate was covered and incubated at 37 °C for 1 hours.

14) The plate was washed three times as in step 3.

15) 200 µL aliquots of freshly prepared substrate (1mg/ml of pNPP in substrate buffer) was added to each well, including “blank”.

16) The plate was covered and incubated at 37 °C for 15-30 minutes.

17) Optical density (OD) was measured by photometer (Multiskan FC, Thermo Scientific™) at 405nm after 15 and 30 minutes.

Obtained measurement was processed as follows:

1) First, mean blank was subtracted from data of all of the measured wells.

2) After that, mean (A_{NC}) and standard deviation (SD_{NC}) of negative controls (extraction buffer) were calculated.

3) Threshold (T) value was calculated as follows:

$$T = A_{NC}+3*SD_{NC}, \quad (2)$$

4) All values, that are higher than T, were considered positive. If discrepancy between values within one technical duplicate occurred (e.g. one is lower than T, other, higher), then the mean of two values was calculated and compared to the threshold.

4 Results

4.1 Screening for the experimental plant host

Results of the screening for the suitable experimental plant host showed that only white beet (*B. vulgaris* subsp. *vulgaris* 'Albina Vereduna') was infected systemically with both BMYV and BChV. In addition, only one sample of New Zealand spinach (*T. expansa* 'G 865') was positive for systemic infection with BMYV. Number of samples tested positive is shown in Table 15, along with their mean OD at 405 nm after 20 minutes incubation.

Table 15. Screening for the experimental plant host. Results of detection with TAS-ELISA

Plant species	BMYV (OD_{405} ± SD)	BChV (OD405 ± SD)	Healthy (OD405 ± SD)
B. *vulgaris* subsp. *vulgaris*	1/12 (0.58 ± 0.20)	3/12 (1.37 ± 0.23)	0/6
B. macrocarpa	0/12	0/12	0/6
T. expansa	1/10 (0.14 ± 0.05)	0/10	0/5

Plants were also observed for visible signs of infection. However, none of the tested plants showed any significant symptoms compared to the control.

4.2 Virus constructs design

It is known, that polerovirus genome at 5' end, ORF0 to ORF2, can be translated directly, while 3' end, ORF3a to ORF5, is translated from a subgenomic RNA (sgRNA). Based on this natural feature of *Polerovirus* replication, it was decided to construct partial virus cDNA clones that contain full ORF0-ORF2 and ORF3a-ORF5 in respective viral constructs P02 and RO. Additionally, it was important to maintain RdRp motif in one of the virus constructs intact, as the viability of partial viral cDNA clones was already impeded by the division, and further interference with such an essential enzyme could have resulted in non-viable virus constructs. Furthermore, as viral CP is the antigen detected in TAS-ELISA, it was preferable not to include CP motif along with the full motif of RdRp, to avoid a possible expression of CP and detection with TAS-ELISA without actually occurring recombination. However, as per the prevailing consensus, RNA recombination during replication is facilitated by the RdRp ability to switch templates, and sequence similarity is one of the primary factors in that process.

Considering the aforementioned, an "overlap" region with high homology, facilitating recombination, was included in the RO fragment. ORF1-2 of the isolates P9-9E-W2_BMYV (Acc. No. ON924242.1) and BChV-2a (Acc. No. MH271171.1) were compared using pairwise alignment in the NCBI BLAST® tool. The highest sequence similarity, of 67% identity, was detected closer to the 3' end of the ORF1-2 of both isolates, namely, between bp number 2328-3393 of P9-9E-W2_BMYV isolate sequence and bp

number 2335-3400 of BChV-2a (Appendix 2). Thus, an overlap of around 820 bp, containing a partial ORF0-2 sequence, was added to the 5' end of RO fragments. Detailed content of every fragment with bp numbers is summarised in Table16.

Table 16. Composition of partial virus fragments

	5' UTR	ORF0-2	UTR 1	ORF3a-5	3' UTR	Total
BChV P02	1-162	163-3404	3405-3424			3424
BChV RO		2604-3404	3405-3606	3607-5610	5611-5777	3174
BMYV P02	1-155	156-3400	3401-3420			3420
BMYV RO		2594-3400	3401-3597	3598-5610	5611-5723	3130

1. UTR - untranslated region

With the sequence of the viral fragments defined, four virus constructs were produced *in silico*. Viral fragment sequences (Table 16) were added to the pDIVA sequence, thus concluding viral construct maps and enabling design of the primers, production of the analytical restriction enzyme digestion patterns, and alignment of the sequenced virus-vector conjunction regions.

4.3 Efficiency of the *E. coli* transformation

The number of colonies that grew after transformation fluctuated from a minimum 15 for BChV P02 construct with 1:3 vector-gene fragment ratio, to 416 for the same construct but with ratio of 1:5. pDIVA control showed growth only on 1 plate (2 colonies).

Table 17. *E. coli* colonies growth

Construct	BMYV P02	BMYV RO	BChV P02 (1:3)	BChV P02 (1:5)	BChV RO	pDIVA
Average number of colonies/plate[1]	80	80	15	416	19	1

1. 100 µL of cell suspension plated

4.4 Analytic restriction endonuclease digestion

Plasmid control digestion (Appendix 4) showed, that all constructs produced the expected band patterns (indicated in Table 18), BChV P02 had three defective plasmids out of 24 (## 4,5,19), BMYV P02 had one out of 12 (#5), BMYV RO and BChV RO had none. Bands with the size of 137 and 33 bp were not observed due to the small DNA concentration and size.

Table 18. Analytic restriction endonuclease digestion. Expected fragment sizes

Construct	Expected sizes:
BMYV P02	4115 bp; 2043 bp; 818 bp; 585 bp; 137 bp; 33 bp
BMYV RO	3562 bp; 1826 bp; 1065 bp; 818 bp; 137 bp; 33 bp
BChV RO	4962 bp; 1084 bp; 818 bp; 588 bp; 33 bp
BChV P02	3965 bp; 2351 bp; 818 bp; 568 bp; 33 bp

4.5 Sequencing

After assessing sequencing results (Appendix 3), the following plasmids were chosen for further work: BChV P02 # 9, BChV RO #7, BMYV P02 # 7, BMYV RO #4. Obtained sequences were aligned to construct maps using Clustal Omega. The biggest quantity of mismatches were detected in BChV P02 construct. However, we proceeded with the sequenced plasmids, choosing the ones that didn't have deletions to avoid possible frameshift.

4.6 Construct viability assessment in *N. benthamiana*

During TAS-ELISA procedures, technical controls of extraction buffer were always around 0,1 OD 405 and positive control (frozen-dried BChV or BMYV infected beet leaves) giving strong, clear signal (> 1,5 OD 405). The results of the TAS-ELISA analysis of *in planta* construct evaluation are given in Table 19.

Table 19. Results of TAS-ELISA detection of *in planta* construct viability evaluation

Construct combinations	Experiment 1		Experiment 2	
	Positive/ Total	OD 405 ± SD (threshold value)	Positive/ Total	OD 405 ± SD (threshold value)
Local patch assay				
BMYV P02+BMYV RO+DK70	20/20	4,73 ± 0,56 (0,15)	20/20	2,37 ± 0,58 (0,13)
BMYV P02+BChV RO+DK70	10/20	1,63 ± 1,34 (0,16)	20/20	0,61 ± 0,26 (0,11)
BChV P02+BChV RO+DK70	0/20		0/20	
BChV P02+BMYV RO+DK70	0/20		0/20	
BMYV P02+DK701	0/5		3/5	0,92 ± 0,35 (0,11)
BMYV RO+DK701	1/5	0,15 ± 0,12 (0,11)1	2/5	0,38 ± 0,26 (0,13)
BChV P02+DK701	0/5		0/5	
BChV RO+DK701	0/5		0/5	
BMYV full-length	4/4	3,21 ± 1,42 (0,18)	5/5	3,05 ± 0,23 (0,13)
BChV full-length	3/4	0,80 ± 0,61 (0,18)	5/5	2,03 ± 1,02 (0,08)
"Mock" inoculation	-	-	0/5	
Healthy, no inoculation	0/4		0/5	
Systemic assay				
BMYV P02+BMYV RO+DK70	5/20	0,12 ± 0,01 (0,11)	18/20	2,46 ± 1,84 (0,17)
BMYV P02+BChV RO+DK70	7/20	0,33 ± 0,57 (0,11)	2/20	0,22 ± 0,09 (0,18)
BChV P02+BChV RO+DK70	0/20		3/20	1,14 ± 0,11 (0,24)
BChV P02+BMYV RO+DK70	0/20		2/20	0,19 ± 0,05 (0,18)
BMYV P02+DK701	1/5	0,15 ± 0,12 (0,11)	2/5	2,11 ± 2,10 (0,18)
BMYV RO+DK70	-		1/5	3,06 ± 0,26 (0,17)
BChV P02+DK70	-		0/5	
BChV RO+DK70	-		0/5	
BMYV full-length	2/5	0,21 ± 0,11 (0,11)	5/5	4,74 ± 0,41 (0,17)
BChV full-length	1/5	0,16 ± 0,01 (0,11)	1/5	3,10 ± 0,41 (0,24)
"Mock" inoculation	-	-	0/5	
Healthy, no inoculation	2/5	0,12 ± 0,002 (0,11)	0/5	

1. Single construct controls for local patch assay, experiment 1 were grown 28 days later than the rest, therefore they were analysed together with samples of the systemic assay, experiment 1.

In the local patch assay of both Experiment 1 and 2, only construct combinations containing BMYV P02 part showed a signal. The same was true for systemic assay experiment 1, but not for experiment 2. Furthermore, the signal from samples was so high that it compared to full-length clone positive control.

All healthy controls, including mock inoculation, were well below the threshold, except in systemic assay experiment 1, where their values were slightly above the threshold. On the other hand, full-length infectious clone controls did not show sufficient signal in systemic assay experiment 1. Single construct controls unexpectedly showed a signal. BMYV RO single construct showed a signal in all of the experiments where it was employed, BMYV P02 single construct showed a positive signal everywhere except local patch assay, experiment 1.

The results of systemic assay experiment 2 are the most controversial and seem to be a product of contamination. Nevertheless, BChV P02 construct combinations showed a very strong signal here, but only in 10% of samples.

5 Discussion and conclusion

5.1 Screening for the experimental plant host

The screening for a suitable experimental host did not yield satisfactory results. Only white beet (*B. vulgaris* subsp. *vulgaris*) was systemically infected by both BMYV and BChV infectious full-length cDNA clones. The rates of infection, higher for BChV (3/12) and lower for BMYV (1/12), correspond to the results obtained in the literature (Wetzel et al., 2018; Stephan & Maiss, 2006). Unlike the results for BChV and *B. macrocarpa*, that seems to contradict Wetzel at al (2018) with a 90-100% infection rate.

Low rates of detected systemic infection might be attributed to failed inoculation, as very low volumes of inoculation solution were possible to press into cotyledons. An inexperienced operator might have failed to perform the procedure correctly, the influence of the environmental factors and quality of the infectious clones also cannot be dismissed.

5.2 Efficiency of the cloning procedure

The quantity of the transformed *E. coli* varied between constructs, but as low as 18 colonies of BChV RO construct was still enough to produce quality plasmid, at least, according to the analytic restriction endonuclease digestion results.

Sequencing results, on the other hand, showed few mismatches and deletions, especially in BChV P02 plasmids. The apparent explanation is that template DNA for BChV P02 synthesis is coming from cDNA, synthesised from total RNA of the systemically infected sugar beet. During consecutive reinfection, to maintain infected plant collection, or even during replication within a plant, various mutations can occur in the virus genome. RNA viruses are known to have a high mutation rate, partially due to the missing proofreading ability of their replicases (Domingo et al., 1991). Thus to create cDNA clones with predictable sequences, it would be advisable to use non-replicating virus material, including existing cDNA clone libraries. Another possibility would be to sequence more plasmid copies to choose the one with the least mismatches and to check if present mismatches influence amino acid sequence.

5.3 Construct viability evaluation in *N. benthamiana*

In planta assessment of the produced plasmids did not conclude as expected. Firstly, as viral constructs consist only of the partial virus genome, high signal intensity, even in BMYV P02+BMYV RO combinations, was surprising. Secondly, the absence of any signal from inter- and intraspecies BChV P02 combinations (except for systemic assay experiment 2, which will be discussed later), likely suggests that the construct is not viable, not that recombination is not able to occur. In the absence

of a complete construct sequence, it is impossible to say if there are no more drastic genome alterations, that might lead to lower homology even within species. On the other hand, consistent signal throughout all experiments, coming from intraspecies BMYV P02 and BChV RO construct combination, render this theory unlikely.

However, the signal from single construct controls suggests the presence of contamination, maybe with a full-length BMYV clone (as systemic assay shows it as well, and BChV does not spread systemically in *N. benthamiana*). Because it is improbable that partial virus genomes are capable of simultaneous systemic movement and the production of a considerable amount of CP. The signal in the local patch assay from RO construct could have been explained by CaMV 35S promoter, facilitating the expression of Cp without viral RdRp. However, its detection during systemic assay seems unplausible.

Even more unplausible seems the detection of BMYV P02 single constructs by TAS-ELISA. As mentioned in 4.2, the separation of RdRp-containing motifs from those of CP was a cornerstone of this work. So most likely, this is the result of contamination. And contamination is likely responsible for the detection of BChV full-length clone (both experiments) and intraspecies construct combination of BChV P02 and BChV Ro (experiment 2) in systemic assays by TAS-ELISA.

This contamination can occur in many steps, but considering two repetitions of the experiment, it likely resides in long-time cultures as this is the only material used repeatedly.

Thus, unfortunately, the results of construct viability evaluation should be considered void, and if there is a need to repeat *in planta* experiment, new *A. tumefaciens* transformation should be done, best if with additional complete sequencing of the plasmids used.

5.4 Further research suggestions

Creating a functioning artificial recombination system, especially to research properties of viral genome, is complicated by the lack of knowledge about said genome. However, in our case, analysis of natural recombination is available by Kozlowska-Makulska et al. (2015). They suggest that naturally occurring recombinants of BChV and BMYV show breakpoint in CP region of the genome. With sequence homology of one of the most highly conserved regions of polerovirus genome, that will facilitate recombination, only the problem of detection remains. The drawback of this suggestion is , off course, that when, CP motifs are incorporated into putative constructs, additional marker required to discriminate true recombinants.

5.5 Conclusions

A study and comparison of BMYV and BChV viral genomes were done during this work. Virus fragments were developed *in silico* and incorporated with binary vector pDIVA into construct plasmid maps, now available at IfZ.

The cloning procedure was employed to produce designed constructs, and after successful Gibson assembly, confirmed by analytic restriction endonuclease enzyme digestion and by sequencing of the adjacent virus-vector regions, the set of plasmids of four constructs (BChV P02, BChV RO, BMYV P02, BMYV RO) were produced and now stored at IfZ plasmid collection.

A. tumefaciens were transformed with produced plasmids, long-term cultures were made, and *Agrobacterium*-mediated inoculation of *N. benthamiana* was performed to asses construct viability *in planta*. Unfortunately, due to the failure of controls, results are considered void. It is recommended to discard produced long-time cultures to eliminate any possible contamination source (as the most realistic reason for a failure).

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Appendix 1

Buffers and solutions

Gel electrophoresis

1x TAE-buffer	4.84 g Tris	Carl Roth
	1.14 ml C2H4O2	Carl Roth
	2.0 ml 0.5 M EDTA (pH 8.0)	AppliChem
	1000 ml ultrapure water	Schütt Labortechnik

Growth media

LB-medium (solid) (autoclave)	20 g LB-medium (powder)	Carl Roth
	500 ml ultrapure water	Schütt Labortechnik
	500 µl antibiotic (after autoclaved)	
	Pure in petri dish under clean bench	
LB-medium (liquid) (autoclave)	12.5 g LB-medium (powder)	Carl Roth
	500 ml ultrapure water	
	500 µl antibiotic (after autoclaved)	
SOB (autoclave)	10 g Bacto Peptone	Carl Roth
	5 g yeast extract	Carl Roth
	0.584 g NaCl	Carl Roth
	0.186 g KCl	Carl Roth
	Dilute in 1000 ml ultrapure water	Schütt Labortechnik
SOC (autoclave)	1 ml glucose solution (2M)	Merck
	1 ml magnesium solution (2M)	AppliChem
	Fill up to 100 ml with SOB-medium	

Antibiotics

Ampicillin stock solution	150 mg ampicillin sodium salt	Carl Roth
	1 ml DEPC-treated water	Carl Roth
Kanamycin stock solution	50 mg kanamycinsulfate	Duchefa Biochemie
	1 ml DEPC-treated water	Carl Roth
Rifampicin stock solution	50 mg rifampicin	AppliChem
	1 ml dimethylsulfoxid	
Tetracycline stock solution	5 mg tetracycline-hydrochloride	AppliChem
	1 ml ethanol (99.5 %)	Carl Roth

Gibson assembly

5x-ISO-buffer	1000 µl Tris-HCl pH 7,5 (1 M)	AppliChem
	50 µl $MgCl_2$ (2 M)	AppliChem
	80 µl dNTP Mix (25 mM)	Carl Roth
	100 µl DTT (1 M)	AppliChem
	0.5 g PEG-8000	AppliChem
	100 µl NAD (100 mM)	AppliChem
	2000 µl DEPC-treated water	Carl Roth
	Freeze at -20 °C in aliquots	
Gibson Assembly mix	40 µl 5 x-ISO-buffer	
	8 µl T5'-Exonuklease (0.1 U/µl)	Epicentre, Madison, Wisconsin
	2.5 µl Phusion High-Fidelity DNA Polymerase (2 U/µl)	Thermo Scientific
	20 µl Taq DNA-Ligase (40 U/µl)	New England Biolabs
	29.5 µl DEPC-treated water	Carl Roth

Plasmid DNA isolation

Solution A (autoclave)	15 mM Tris-HCL	AppliChem
	10 mM EDTA	AppliChem
	50 mM glucose	Carl Roth
Solution B (autoclave)	200 mM NaOH	Merck
	1 % SDS	Carl Roth
Solution C (autoclave)	3 M $C_2H_3NaO_2$ (pH 4.8)	Merck
	Adjust pH with C2H4O2	Carl Roth
Solution D (autoclave)	100 mM $C_2H_3NaO_2$ (pH 7.0)	Merck
	50 mM Tris-HCl (pH 8.0)	AppliChem
RNase/TE-stock solution	10 mg RNase	AppliChem
	1 ml TE 10/01	
	Incubation 10 min at 95 °C	
	Cool down on ice	
TE 10/01	10 mM Tris,	Carl Roth
	1 mM EDTA	AppliChem
	Adjust to pH 8.0 with HCl	Merck

Agroinoculation

Agroinoculation buffer	10 mM MES	Carl Roth
	10 mM MgCl2	AppliChem
	Adjust pH to 5.6	

TAS-ELISA

Coating buffer	1.59g Na2CO3	VWR
	2.93 g NaHCO3	Merck
	Adjust pH 9.6 with NaOH	Merck
	Fill up to 1000 ml with ultrapure water	Schütt Labortechnik
Washing buffer	8.0 g NaCl	Carl Roth
	2.9 g Na_2HPO_4 x 12 H_2O	Merck
	0.2 g KH2PO4	Carl Roth
	0.2 g KCl	Carl Roth
	0.5 ml Tween 20	Carl Roth
	Adjust pH to 7.4	
	Fill up to 1000 ml with ultrapure water	Schütt Labortechnik
Sample extraction buffer	20 g polyvinylpyrrolidone	Sigma-Aldrich
	1000 ml washing buffer	
	Mix carefully	
Conjugate buffer	0.3 g albumin (chicken egg white)	Sigma-Aldrich
	100 ml sample extraction buffer	
	Mix and cool for 2-3 h in fridge	
	Add 2 g skim milk powder	AppliChem
	24 h in fridge	
Substrate buffer	97 ml diethanolamine	Carl Roth
	200 mg MgCl2	AppliChem
	200 mg NaN3	Sigma-Aldrich
	500 ml ultrapure water	Schütt Labortechnik
	Adjust to pH 9.8 with 5M HCl	Merck
	Fill up to 1000 ml with ultrapure water	Schütt Labortechnik

Appendix 2

BMYV and BChV ORF1-2 pairwise alignment

Query: Beet mild yellowing virus isolate P9-9E-W2_BMYV, complete genome Query ID: ON924242.1 Length: 3245

>Beet chlorosis virus isolate BChV-2a, complete genome Sequence ID: MH271171.1 Length: 5777
Range: 2335 to 3400

Score:316 bits(350), Expect:4e-89, Identities:721/1073(67%), Gaps:14/1073(1%), Strand: Plus/Plus

```
Query  2328  TAGAGCTCGAAGCCGGCGTAGGCGTCCCGTATGTTGCTTACGGTCGACGCACGCACAGAG  2387
              ||||  | || ||||| || || || |||||  | || |||||    || || |||||||
Sbjct  2335  TAGAATTAGACGCCGGTGTTGGGGTTCCGTACATCGCCTACGGGACTCGTACCCACAGAG  2394

Query  2388  GCTGGATTGAAGATCCAGATCTGTTGCCGGTCTTAGCTCGTTTCACCTTCGATCGATTAC  2447
               |||| |     | | |||  || | || || ||  ||||  | || ||| | ||  |||
Sbjct  2395  ACTGGGTGTTCAACCAAGAGTTGCTACCTGTGTTGACTCGACTGACTTTCAACCGCCTAC  2454

Query  2448  AGAAGTTATCGGAGATGAAATTTGAGCACATGAGTCCTGAGCAATTGGTTCAGGAAGGTC  2507
              ||||| | | |||  | || |  ||  || ||| | | || ||| |||||||  | || |
Sbjct  2455  AGAAGATGTTGGAAGTCAACTCGGACGACTTGAATGCGGAACAACTGGTTCAACACGGAC  2514

Query  2508  TGTGCGATCCAATACGGTTATTCGTAAAAGGCGAGCCACACAAACAATCCAAACTTGATG  2567
              | ||||| || ||| || |||| || || || || || ||||||   ||||| || || |
Sbjct  2515  TTTGCGACCCGATAAGGGTATTTGTTAAGGGAGAACCCCACAAAGTCTCCAAGCTGGAAG  2574

Query  2568  AGGGACGCTACCGCCTCATCATGAGTGTCTCATTGGTTGACCAACTGGTAGCCCGGGTTC  2627
              | || |||||||||||| || |||||||| ||  | || || ||  |||||||| | ||||
Sbjct  2575  AAGGTCGCTACCGCCTTATAATGAGTGTATCCCTAGTGGATCAGTTGGTAGCCAGAGTTC  2634

Query  2628  TGTTTCAAAACCAAAACAAGCGCGAGATTGCGCTCTGGAGGGCGATTCCCTCAAAACCCG  2687
              |||| ||||| || ||||| || || || |  ||||||||||  | ||||| || || |
Sbjct  2635  TGTTCCAAAATCAGAACAAACGGGAAATAGAACTCTGGAGGGCAGTGCCCTCGAAGCCGG  2694

Query  2688  GTTTCGGATTGTCCACGGACGGACAAGTCGTCGATTTCATGCAAGCATTATCGGCGCAGG  2747
              | || || || || || || |  ||  |||  || ||  |  |||  ||  |  | ||
Sbjct  2695  GATTTGGTTTATCAACCGATGACCAGATCGAAGACTTTGTTAAAGTCTTGGCTTCACAAC  2754

Query  2748  TGGGAGTGAATACCGC-TGAATTACTCCAAGATTGGAAATCCCACCTCATTCCTACAGAT  2806
              | || | | | ||| |  ||| |  || |  | |||    || |  | || || ||||||
Sbjct  2755  TTGGTGAGGA-ACCACAAGAAGTTTTCAATAACTGGTCCACCAAGTTGATACCAACAGAT  2813

Query  2807  TGCTCTGGTTTTGACTGGAGCGTTTCGGACTGGCTTCTAGAAGATGAAATGGAAGTTCGA  2866
              ||||||||||||||||||| ||  |||||||| | || |||||||| |||||||| ||
Sbjct  2814  TGCTCTGGTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCGC  2873

Query  2867  AACAGGCTTACGTTGGATATAAATGATCTAACCAGGCGACTGCGAGCTGGATGGCTTAAA  2926
              ||  |  | ||     | |  ||  |   ||| |  ||| | ||| | |  ||| | |||
Sbjct  2874  AATCGCTTGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAA  2933
```

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Query  2927  TGCCT-CGCAA-ACAGCGTCCTCTGTTTATCGGATGGAACATTGCTCTCGCAGCAAGTAC  2984
             ||| |  ||||  ||  ||| | ||  | || |||||     | || || |||  ||| |
Sbjct  2934  TGCATAAGCAATTCA--GTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGC  2991

Query  2985  CTGGTGTGCAAAAGAGTGGCAGCTACAACACCTCCTCGTCTAATTCTAGAATTCGAGTGA  3044
             |||| || || || ||||| || ||||| || || || || || ||  | ||  |||| |
Sbjct  2992  CTGGAGTTCAGAAAAGTGGAAGTTACAATACTTCTTCTTCAAACTCCCGTATCAGAGTTA  3051

Query  3045  TGGCTGCTTACCACTCCGGAGCCTCCTGGGCCATCGCCATGGGTGATGATGCCCTTGAAT  3104
             ||||||| || || | ||| || |||||||| || || ||||| ||||| ||||| ||||
Sbjct  3052  TGGCTGCATATCATTGCGGCGCTTCCTGGGCTATGGCGATGGGCGATGACGCCCTAGAAT  3111

Query  3105  CTGTAGATGCAGACCTAAGTCGATACTCATCCTTAGGCTTCAAAGTCGAGGTTTCTTCAC  3164
             ||||  ||  |  ||||||     ||   |   || || ||||||||||||||  |   ||
Sbjct  3112  CTGTTGACTCTAACCTAACAGAGTATAAAAAGTTGGGTTTCAAAGTCGAGGTAGCCAAAC  3171

Query  3165  AACTGGAATTCTGCTCTCATATTTTTGAGGAGGAGAACCTCGCCGTTCCGGTCAA--CAA  3222
             |||||||||| ||||| ||||| ||| || | |||  ||||||  | ||| | ||   ||
Sbjct  3172  AACTGGAATTTTGCTCACATATCTTTAAGAATGAGCGCCTCGCATTACCGCTGAATGTAA  3231

Query  3223  AGCTAAAATGCTTTATAAATTGATACATGGTTACGAACCGGAATGCGGTAACTTAGAAGT  3282
             ||  || ||||||||  ||  | ||  | || ||| | || ||  |||||||||| ||||
Sbjct  3232  AG--AAGATGCTTTACAAGCTAATTTACGGGTACAATCCTGATAGCGGTAACTTGGAAGC  3289

Query  3283  TCTGACGAACTATCTTGCAGCTTGTTTCTCAATTTTGAACGAGCTTAGATCTGATCCAGA  3342
               | | ||| || ||||  |||||   ||| ||  |||| ||  || | ||    | |||
Sbjct  3290  GATCAAGAATTACCTTGACGCTTGCCACTCGATCGTGAATGAAATTCG-TCACGACGAGA  3348

Query  3343  GCTCGTTGCCTCCCTCTACCA--GTGGCTGGTCCTTCCAGTGCAGCCACAAAA  3393
             ||| | | ||      ||  |  |||| ||||| ||||||| || ||||||||
Sbjct  3349  GCTTGGT-CCAGAAAATAATATCGTGGTTGGTCATTCCAGTCCAACCACAAAA  3400
```

Appendix 3

Alignment of the virus-vector junctions of the created plasmids using Clustal Omega

Sequence of junction of BChV P02 fragment and CaMV 35S promoter region of pDIVA

Number before ARP indicates plasmid number, e.g. BChV_P02_9_ARP11_FW - sequence of BChV P02 plasmid # 9.

```
CLUSTAL O(1.2.4) multiple sequence alignment


BChV_P02              AGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGA   60
BChV_P02_9_ARP11_FW   ------------------------------------------------------TCGTG    5
BChV_P02_3_ARP11_FW   ------------------------------------------------------------    0


BChV_P02              AAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA   120
BChV_P02_9_ARP11_FW   GAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA   65
BChV_P02_3_ARP11_FW   --AAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA   58
                        ***********************************************************


BChV_P02              CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG   180
BChV_P02_9_ARP11_FW   CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG   125
BChV_P02_3_ARP11_FW   CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG   118
                      ************************************************************


BChV_P02              TTCATTTCATTTGGAGAGGacaaaagaatactaggaggacagtgaatgaactttgagatt   240
BChV_P02_9_ARP11_FW   TTCATTTCATTTGGAGAGGacaaaagaatactaggaggacagtgaatgaactttgagatt   185
BChV_P02_3_ARP11_FW   TTCATTTCATTTGGAGAGGacaaaagaatactaggaggacagtgaatgaactttgagatt   178
                      ************************************************************


BChV_P02              tgcttcaaaacgaacagcgaactactagtgacgagcgaaagacacttgccactaaaagag   300
BChV_P02_9_ARP11_FW   tgcttcaaaacgaacagcgaactactagtgacgagcgaaagacacttgccactaaaagag   245
BChV_P02_3_ARP11_FW   tgcttcaaaacgaacagcgaactactagtgacgagcgaaagacacttgccactaaaagag   238
                      ************************************************************


BChV_P02              agatcattcataatcggaagatttctcacgcaaatcccccaacttttgcaccatttcaaa   360
BChV_P02_9_ARP11_FW   agatcatacataatcggaagatttctcacgcaaatcccccaacttttgcaccatttcaaa   305
BChV_P02_3_ARP11_FW   agatcatacataatcggaagatttctcacgcaaatcccccaacttttgcaccatttcaaa   298
                      ******* ***************************************************


BChV_P02              tatggacaccaagttgaacagttcttacgctctattctttttcagctccctaatcttatt   420
BChV_P02_9_ARP11_FW   tatggacaccaagttgaacagttcttacgctctattctttttcagctccctaatcttatt   365
BChV_P02_3_ARP11_FW   tatggacaccaagttgaacagttcttacgctctattctttttcagctccctaatcttatt   358
                      ************************************************************


BChV_P02              tggagctcttgggaccatggcaccatcttatatggtgacaggcccttacacgcgccgaa   480
BChV_P02_9_ARP11_FW   tggagctcttgggaccatggcaccatcttatatggtgacaggcccttacacgcgccgaa   425
BChV_P02_3_ARP11_FW   tggagctcttgggaccatggcaccatcttatatggtgacaggcccttacacgcgccgaa   418
                      ************************************************************
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BChV_P02              gatctcctcagattttcccttaccaccggatattacccaactcctactaacacaggactc   540
BChV_P02_9_ARP11_FW   gatctcctcagattttcccttaccaccggatattacccaactcctactaacacaggactc   485
BChV_P02_3_ARP11_FW   gatctcctcagattttcccttacc-ccggatattacccaactcctactaacacaggactc   477
                      ********************** ***********************************

BChV_P02              cgcgtgggactgccctcttcagaaaaagctgtcagattacagctacaaagaacttgtaat   600
BChV_P02_9_ARP11_FW   cgcgtgggactgccctcttcagaaaaagctgtcagattacagctacaaagaacttgtaat   545
BChV_P02_3_ARP11_FW   cgcgtgggactgccctcttcagaaaaagctgtcagattgcagctacaaagaacttgtaat   537
                      ************************************ ********************

BChV_P02              acacgtcttgcaaagaggctacaacgacacccagaaatacttgccacaactagcatcaat   660
BChV_P02_9_ARP11_FW   acacgtcttgcaaagaggctacaacgacacccagaaatacttgccacaactagcatcaat   605
BChV_P02_3_ARP11_FW   acacgtcttgcaaagaggctacaacgacacccagaaatacttgccacaactagcatcaat   597
                      ************************************************************

BChV_P02              ggatttcggaaagcactgggatactatctcagggcaattcacaggattgaatatcctgga   720
BChV_P02_9_ARP11_FW   ggatttcggaaagcactgggatactatctcagggcaattcacaggattgaatatcctgga   665
BChV_P02_3_ARP11_FW   ggatttcggaaagcactgggatactatctcagggcaattcacaggattgaatatcctgga   657
                      ************************************************************

BChV_P02              aagattgatgtggggcctcatacggttatggggttatgtaatctgggttgtcagctcatc   780
BChV_P02_9_ARP11_FW   aagattgatgtggggcctcatacggttatggggttatgtaatctgggttgtcagctcatc   725
BChV_P02_3_ARP11_FW   aagattgatgtggggcctcatacggttatggggttatgtaatctgggttgtcagctcatc   717
                      ************************************************************

BChV_P02              cacgatgagcttcttgatgaacaacttgagcctggctataatagtggcctcattgatggc   840
BChV_P02_9_ARP11_FW   cacgatgagcttcttgatgaacaacttgagcctggctataatagtggcctcattgatggc   785
BChV_P02_3_ARP11_FW   cacgatgagcttcttgatgaacaacttgagcctggctataatagtggcctcattgatggc   777
                      ************************************************************

BChV_P02              cttaagcgtgcttatggcacgggcagctcaattattcttcaaaatattacaactatgcct   900
BChV_P02_9_ARP11_FW   cttaagcgtgcttatggcacgggcagctcaattattcttcaaaatattacaactatgcct   845
BChV_P02_3_ARP11_FW   cttaagcgtgcttatggcacgggcagctcaattattcttcaaaatattacaactatgcct   837
                      ************************************************************

BChV_P02              acctgtgttggcggcaaggatggtgatgagcgcgttcaccacgatgaagagagtttgcat   960
BChV_P02_9_ARP11_FW   gcctgtgttggcggcaaagatggtgatgagcgcgttcaccacgatgaagagagtttgcat   905
BChV_P02_3_ARP11_FW   gcctgtgttggcggcaaagatggtgatgagcgcgttcaccacgatgaagagagtttgcat   897
                       **************** ****************************************

BChV_P02              agagagacctaaatcctatgtaaaagagtgtgcagtccgggggttcactacatgggctgt  1020
BChV_P02_9_ARP11_FW   agagagacctaaatcctatgtaaaagagtgtgcagtccgggggttcactacatgggcagt   965
BChV_P02_3_ARP11_FW   agagagacctaaatcctatgtaaaagagtgtgcagtccgggggttcactacatgggctgt   957
                      ************************************************************ **

BChV_P02              accaatgaaacccccaaagaattcgattttgctcataagtcatgacgacggttcacacgc  1080
BChV_P02_9_ARP11_FW   accaatgaaacccccaaagaattcgattttgctcataagtcatgacgacggttcacacgc  1025
BChV_P02_3_ARP11_FW   accaatgaaacccccaaagaattcgattttgctcataagtcatgacgacggttcacacgc  1017
                      ************************************************************
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BChV_P02              aggttatgcaacttgcgtgactttacacgacagacaatcaaccaccattggtttgataac   1140
BChV_P02_9_ARP11_FW   aggttatgcaacttgtgtgactctacacgacagacaatcaaccactattggtttgataac   1085
BChV_P02_3_ARP11_FW   aggttatgcaacttgtgtgactctacacgacagacaatcaaccactattggtttgataac   1077
                      *************** ****** ********************** **************

BChV_P02              ctgctcgcatgctcccatcaatgggagtgttttctcaaccgtcactggcaataagatcaa   1200
BChV_P02_9_ARP11_FW   ctgctcgcatgctcccattaatgggagtgttttctcaaccgtcactggcaataagatcaa   1145
BChV_P02_3_ARP11_FW   ctgctcgcatgctcccattaatgggagtgttttctcaaccgtcactggcaataagatcaa   1137
                      ****************** ****************************************

BChV_P02              aatggagagttttaaaactctctatgatgacgctgaaacagatgtaaagatcttatttgg   1260
BChV_P02_9_ARP11_FW   aatggagagtttaaaa-ctctttatgatgacsctgaaaccgatgt---------------   1189
BChV_P02_3_ARP11_FW   aatggagagttttaaaactctttatgatgacgctgaaacaaag-----------------   1180
                      ************ *** **** ********* *******  *
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Sequence of junction of BChV P02 fragment and HDV rbz region of pDIVA

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CLUSTAL O(1.2.4) multiple sequence alignment

BChV_P02             ccaacatcaaccgccggcaattccctaacgtgggaaggttttattgaagacatcaaggaa   2520
BChV_P02_9_ARP12_RV  ------------------------------------------------------aaggaa   6
BChV_P02_3_ARP12_RV  ------------------------------------------------------------   0


BChV_P02             gctgtaagctccttagaattagacgccggtgttggggttccgtacatcgcctacgggact   2580
BChV_P02_9_ARP12_RV  gctgtaagctccttagaattagacgccgg-gttggggttccgtacatcgcctacgggact   65
BChV_P02_3_ARP12_RV  ------------tagaattagacgccgg-gttggggttccgtacatcgcctacgggact   46
                                  *************** ****************************


BChV_P02             cgtacccacagagactgggtgttcaaccaagagttgctacctgtgttgactcgactgact   2640
BChV_P02_9_ARP12_RV  cgtacccacagagactgggtgttcaaccaagagttgctacctgtgttgactcgactgact   125
BChV_P02_3_ARP12_RV  cgtacccacagagactgggtgttcaaccaagagttgctacctgtgttgactcgactgact   106
                     ************************************************************


BChV_P02             ttcaaccgcctacagaagatgttggaagtcaactcggacgacttgaatgcggaacaactg   2700
BChV_P02_9_ARP12_RV  ttcaaccgcctacagaagatgttggaagtcaactcggacgacttgaatgcagaacaactg   185
BChV_P02_3_ARP12_RV  ttcaaccgcctacagaagatgttggaagtcaactcggacgacttgaatgcagaacaactg   166
                     ************************************************* *********


BChV_P02             gttcaacacggactttgcgacccgataagggtatttgttaagggagaaccccacaaagtc   2760
BChV_P02_9_ARP12_RV  gttcaacacggactttgcgacccgataagggtatttgttaagggagaaccccacaaagtc   245
BChV_P02_3_ARP12_RV  gttcaacacggactttgcgacccgataagggtatttgttaagggagaaccccacaaagtc   226
                     ************************************************************


BChV_P02             tccaagctggaagaaggtcgctaccgccttataatgagtgtatccctagtggatcagttg   2820
BChV_P02_9_ARP12_RV  tccaagctggaagaaggtcgctaccgccttataatgagtgtatccctagtggatcagttg   305
BChV_P02_3_ARP12_RV  tccaagctggaagaaggtcgctaccgccttataatgagtgtatccctagtggatcagttg   286
                     ************************************************************


BChV_P02             gtagccagagttctgttccaaaatcagaacaaacgggaaatagaactctggagggcagtg   2880
BChV_P02_9_ARP12_RV  gtggccagagttctgttccaaaatcagaacaaacgggaaatagaactctggagggcagtg   365
BChV_P02_3_ARP12_RV  gtggccagagttctgttccaaaatcagaacaaacgggaaatagaactctggagggcagtg   346
                     ** ***********************************************************


BChV_P02             ccctcgaagccgggatttggtttatcaaccgatgaccagatcgaagactttgttaaagtc   2940
BChV_P02_9_ARP12_RV  ccctcgaagccgggatttggtttatcaaccgatgaccagatcgaagactttgttaaagtc   425
BChV_P02_3_ARP12_RV  ccctcgaagccgggatttggtttatcaaccgatgaccagatcgaagactttgttaaagtc   406
                     ************************************************************


BChV_P02             ttggcttcacaacttggtgaggaaccacaagaagttttcaataactggtccaccaagttg   3000
BChV_P02_9_ARP12_RV  ttggcttcacaacttggtgaggaaccacaagaagttttcaataactggtccaccaagttg   485
BChV_P02_3_ARP12_RV  ttggcttcacaacttggtgaggaaccacaagaagttttcaataactggtccaccaagttg   466
                     ************************************************************
BChV_P02             ataccaacagattgctctggttttgactggagtgtagcggactggatgctcgaagatgac   3060
BChV_P02_9_ARP12_RV  ataccaacagattgctccggctttgactggagtgtagcggactggatgctcgaagatgac   545
BChV_P02_3_ARP12_RV  ataccaacagattgctccggctttgactggagtgtagcggactggatgctcgaagatgac   526
                     ***************** ** ************************************
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BChV_P02              atggaagtccgcaatcgcttgaccagaaacaacaaccacacaacaaaacgattacgatca   3120
BChV_P02_9_ARP12_RV   atggaagtccgcaatcgcttgaccagaaacaacaaccacacaacaaaacgattacgatca   605
BChV_P02_3_ARP12_RV   atggaagtccgcaatcgcttgaccagaaacaacaaccacacaacaaaacgattacgatca   586
                      ************************************************************

BChV_P02              gtgtggttaaaatgcataagcaattcagtcttatgcctttctgatgggtgccttctttca   3180
BChV_P02_9_ARP12_RV   gtgtggttaaaatgcataagcaattcagtcttatgcctttctgatgggtgccttctttca   665
BChV_P02_3_ARP12_RV   gtgtggttaaaatgcataagcaattcagtcttatgcctttctgatgggtgccttcttcca   646
                      ************************************************************ **

BChV_P02              cagagagtgcctggagttcagaaaagtggaagttacaatacttcttcttcaaactcccgt   3240
BChV_P02_9_ARP12_RV   cagagagtgcctggagttcagaaaagtggaagttacaatacttcctcttcaaactcccgt   725
BChV_P02_3_ARP12_RV   cagagagtgcctggagttcagaaaagtggaagttacaatacttcctcttcaaactcccgt   706
                      ****************************************** **************

BChV_P02              atcagagttatggctgcatatcattgcggcgcttcctgggctatggcgatgggcgatgac   3300
BChV_P02_9_ARP12_RV   atcagagttatggctgcatatcattgcggcgcttcctgggctatggcgatgggcgatgac   785
BChV_P02_3_ARP12_RV   atcagagttatggctgcatatcattgcggcgcttcctgggctatggcgatgggcgatgac   766
                      ************************************************************

BChV_P02              gccctagaatctgttgactctaacctaacagagtataaaaagttgggtttcaaagtcgag   3360
BChV_P02_9_ARP12_RV   gccctagaatctgttgactctaacctaacagagtataaaaagttgggtttcaaagtcgag   845
BChV_P02_3_ARP12_RV   gccctagaatctgttgactctaacctaacagagtataaaaagttgggtttcaaagtcgag   826
                      ************************************************************

BChV_P02              gtagccaaacaactggaattttgctcacatatctttaagaatgagcgcctcgcattaccg   3420
BChV_P02_9_ARP12_RV   gtagccaaacaactggaattttgctcacatatctttaagaatgagcgcctcgcattaccg   905
BChV_P02_3_ARP12_RV   gtagccaaacaactggaattttgctcacatatctttaagaatgagcgcctcgcattaccg   886
                      ************************************************************

BChV_P02              ctgaatgtaaagaagatgctttacaagctaatttacgggtacaatcctgatagcggtaac   3480
BChV_P02_9_ARP12_RV   ctgaatgtaaagaagatgctttacaagctaatttacgggtacaatcctgatagcggtaac   965
BChV_P02_3_ARP12_RV   ctgaatgtaaagaagatgctttacaagctaatttacgggtacaatcctgatagcggtaac   946
                      ************************************************************

BChV_P02              ttggaagcgatcaagaattaccttgacgcttgccactcgatcgtgaatgaaattcgtcac   3540
BChV_P02_9_ARP12_RV   ttggaagcgatcaagaattaccttgacgcttgccactcgatcgtgaatgaaattcgtcac   1025
BChV_P02_3_ARP12_RV   ttggaagcgatcaagaattaccttgacgcttgccactcgatcgtgaatgaaattcgtcac   1006
                      ************************************************************

BChV_P02              gacgagagcttggtccagaaaataatatcgtggttggtcattccagtccaaccacaaaac   3600
BChV_P02_9_ARP12_RV   gacgagagcttggtccagaaaataatatcgtggttggtcattccagtccaaccacaaaac   1085
BChV_P02_3_ARP12_RV   gacgagagcttggtccagaaaataatatcgtggttggtcattccagtccaaccacaaaac   1066
                      ************************************************************

BChV_P02              taattcaggaggatcaagcatacGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGAC   3660
BChV_P02_9_ARP12_RV   taattcaggaggatcaagcatacGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGAC   1145
BChV_P02_3_ARP12_RV   taattcaggaggatcaagcatacGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGAC   1126
                      ************************************************************
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BChV_P02              CTGGGCATCCGAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGAAGCTGGCCCGAG   3720
BChV_P02_9_ARP12_RV   CTGGGCATCCGAAGGAGGACGCAC-----------------------------------   1169
BChV_P02_3_ARP12_RV   CTGGGCATCCGAAGGAGGACGCACN----------------------------------   1151
                      ************************

BChV_P02              CTCGATC       3727
BChV_P02_9_ARP12_RV   -------       1169
BChV_P02_3_ARP12_RV   -------       1151
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Sequence of junction of BChV RO fragment and CaMV 35S promoter region of pDIVA

CLUSTAL O(1.2.4) multiple sequence alignment

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BChV_RO                 AGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGA 60
BChV_RO_2_ARP11_FW      ------------------------------------------------------------ 0
BChV_RO_7_ARP11_FW      ------------------------------------------------------------ 0


BChV_RO                 AAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA 120
BChV_RO_2_ARP11_FW      ----------GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA 50
BChV_RO_7_ARP11_FW      --------ACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA 52
                                **************************************************

BChV_RO                 CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG 180
BChV_RO_2_ARP11_FW      CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG 110
BChV_RO_7_ARP11_FW      CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG 112
                        ************************************************************

BChV_RO                 TTCATTTCATTTGGAGAGGtccctagtggatcagttggtagccagagttctgttccaaaa 240
BChV_RO_2_ARP11_FW      TTCATTTCATTTGGAGAGGtccctagtggatcagttggtggccagagttctgttccaaaa 170
BChV_RO_7_ARP11_FW      TTCATTTCATTTGGAGAGGtccctagtggatcagttggtggccagagttctgttccaaaa 172
                        ************************************ ********************

BChV_RO                 tcagaacaaacgggaaatagaactctggagggcagtgccctcgaagccgggatttggttt 300
BChV_RO_2_ARP11_FW      TCAGAACAAACGGGAAATAGAACTCTGGAGGGCAGTGCCCTCGAAGCCGGGATTTGGTTT 230
BChV_RO_7_ARP11_FW      TCAGAACAAACGGGAAATAGAACTCTGGAGGGCAGTGCCCTCGAAGCCGGGATTTGGTTT 232
                        ************************************************************

BChV_RO                 atcaaccgatgaccagatcgaagactttgttaaagtcttggcttcacaacttggtgagga 360
BChV_RO_2_ARP11_FW      atcaaccgatgaccagatcgaagactttgttaaagtcttggcttcacaacttggtgagga 290
BChV_RO_7_ARP11_FW      atcaaccgatgaccagatcgaagactttgttaaagtcttggcttcacaacttggtgagga 292
                        ************************************************************

BChV_RO                 accacaagaagttttcaataactggtccaccaagttgataccaacagattgctctggttt 420
BChV_RO_2_ARP11_FW      accacaagaagttttcaataactggtccaccaagttgataccaacagattgctccggctt 350
BChV_RO_7_ARP11_FW      accacaagaagttttcaataactggtccaccaagttgataccaacagattgctccggctt 352
                        ************************************************** ** **

BChV_RO                 tgactggagtgtagcggactggatgctcgaagatgacatggaagtccgcaatcgcttgac 480
BChV_RO_2_ARP11_FW      tgactggagtgtagcggactggatgctcgaagatgacatggaagtccgcaatcgcttgac 410
BChV_RO_7_ARP11_FW      tgactggagtgtagcggactggatgctcgaagatgacatggaagtccgcaatcgcttgac 412
                        ************************************************************

BChV_RO                 cagaaacaacaaccacacaacaaaacgattacgatcagtgtggttaaaatgcataagcaa 540
BChV_RO_2_ARP11_FW      cagaaacaacaaccacacaacaaaacgattacgatcagtgtggttaaaatgcataagcaa 470
BChV_RO_7_ARP11_FW      cagaaacaacaaccacacaacaaaacgattacgatcagtgtggttaaaatgcataagcaa 472
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BChV_RO              ttcagtcttatgcctttctgatgggtgccttctttcacagagagtgcctggagttcagaa 600
BChV_RO_2_ARP11_FW   ttcagtcttatgcctttctgatgggtgccttctttcacagagagtgcctggagttcagaa 530
BCHV_RO_7_ARP11_FW   ttcagtcttatgcctttctgatgggtgccttctTTCACAGAGAGTGCCTGGAGTTCAGAA 532
                     ************************************************************

BChV_RO              aagtggaagttacaatacttcttcttcaaactcccgtatcagagttatggctgcatatca 660
BChV_RO_2_ARP11_FW   aagtggaagttacaatacttcctcttcaaactcccgtatcagagttatggctgcatatca 590
BChV_RO_7_ARP11_FW   aagtggaagttacaatacttcctcttcaaactcccgtatcagagttatggctgcatatca 592
                     ********************* ************************************

BChV_RO              ttgcggcgcttcctgggctatggcgatgggcgatgacgccctagaatctgttgactctaa 720
BChV_RO_2_ARP11_FW   ttgcggcgcttcctgggctatggcgatgggcgatgacgccctagaatctgttgactctaa 650
BChV_RO_7_ARP11_FW   ttgcggcgcttcctgggctatggcgatgggcgatgacgccctagaatctgttgactctaa 652
                     ************************************************************

BChV_RO              cctaacagagtataaaaagttgggtttcaaagtcgaggtagccaaacaactggaattttg 780
BChV_RO_2_ARP11_FW   cctaacagagtataaaaagttgggtttcaaagtcgaggtagccaaacaactggaattttg 710
BChV_RO_7_ARP11_FW   cctaacagagtataaaaagttgggtttcaaagtcgaggtagccaaacaactggaattttg 712
                     ************************************************************

BChV_RO              ctcacatatctttaagaatgagcgcctcgcattaccgctgaatgtaaagaagatgcttta 840
BChV_RO_2_ARP11_FW   ctcacatatctttaggaatgagcgcctcgcattaccgctgaatgtaaagaagatgcttta 770
BChV_RO_7_ARP11_FW   ctcacatatctttaggaatgagcgcctcgcattaccgctgaatgtaaagaagatgcttta 772
                     ********** ***************************************************

BChV_RO              caagctaatttacgggtacaatcctgatagcggtaacttggaagcgatcaagaattacct 900
BChV_RO_2_ARP11_FW   caagctaatttacgggtacaatcctgatagcggtaacttggaagcgatcaagaattacct 830
BChV_RO_7_ARP11_FW   caagctaatttacgggtacaatcctgatagcggtaacttggaagcgatcaagaattacct 832
                     ************************************************************

BChV_RO              tgacgcttgccactcgatcgtgaatgaaattcgtcacgacgagagcttggtccagaaaat 960
BChV_RO_2_ARP11_FW   tgacgcttgccactcgatcgtgaatgaaattcgtcacgacgagagcttggtccagaaaat 890
BChV_RO_7_ARP11_FW   tgacgcttgccactcgatcgtgaatgaaattcgtcacgacgagagcttggtccagaaaat 892
                     ************************************************************

BChV_RO              aatatcgtggttggtcattccagtccaaccacaaaactaattcaggaggatcaagcatac 1020
BChV_RO_2_ARP11_FW   aatatcgtggttggtcattccagtccaaccacaaaactaattcaggaggatcaagcatat 950
BChV_RO_7_ARP11_FW   aatatcgtggttggtcattccagtccaaccacaaaactaattcaggaggatcaagcatat 952
                     ************************************************************

BChV_RO              aaacagccgggtaaacatcagttgcaaacaccggaagttttagtctgattacacaacaag 1080
BChV_RO_2_ARP11_FW   aaacagccgggtaaacatcagttgcaaacaccggaagttttagtctgattacacaacaag 1010
BChV_RO_7_ARP11_FW   aaacagccgggtaaacatcagttgcaaacaccggaagttttagtctgattacmcaacaag 1012
                     ************************************************** *******

BChV_RO              ccaaaatagacttcaaatttttagcaggattttcaagcggtcta-tgtcagcaatacccg 1139
BChV_RO_2_ARP11_FW   ccaaaatagacttcaaatttttagcaggattttcaagcggtctattgtcagcaatacccg 1070
BChV_RO_7_ARP11_FW   ccaaaatagacttcaaatttttagcaggattttcaagcggtctattgtcagcaatacccg 1072
                     ****************************************** ***************
```

```
BChV_RO                taacggtaattggcttgtattttgtctacctgaaaatctcccaacacgtaagatcaatcg 1199
BChV_RO_2_ARP11_FW     taacggtaattggcttgtattttgtctacctgaaaatctcccacmcgtaa-gatcgatcg 1129
BChV_RO_7_ARP11_FW     taacggtaattggcttgtattttgtctacctgaaaatctcccaacmcgtaagatcgatcg 1132
                       ****************************************      * **** ****

BChV_RO                ttaatgaatacggtcgtgggtaggagaacaatcaatggaagaagacgaccacgaaggcaa 1259
BChV_RO_2_ARP11_FW     tt-atgaatacggtcgtgggtag-------------------------------------- 1151
BChV_RO_7_ARP11_FW     ttaatgaatacggtcgtgggtaggagaacaancatgg----------------------- 1169
                       ** ********************
```

Sequence of junction of BChV RO fragment and HDV rbz region of pDIVA

CLUSTAL O(1.2.4) multiple sequence alignment

```
BChV_RO                 atgtggagataaccaacaataaggccgataatactttaaagtatggacacccggacatgg 2280
BChV_RO_7_ARP12_RV      a----tggataaccaacaataaggccgat--anactttaaagttggacacccggacatgg 86
BChV_RO_2_ARP12_RV      ------ggataaccaacaataaggccgataatactttaaagtatggacacccgg-actgg 53
                              **********************      ** **   **********   ***

BChV_RO                 aacttaatagttgtcatttcaatcaacaacaatgtctggaaagagatggagacttgactt 2340
BChV_RO_7_ARP12_RV      aacttaatagttgtcatttcaatcaacaacaatgtctggaaagagatggagacttgactt 146
BChV_RO_2_ARP12_RV      aacttaatagttgtcatttcaatcaacaacaatgtctggaaagagatggagacttgactt 113
                        ************************************************************

BChV_RO                 gtcacattaaaacaactggtgacaatgcctccttctttattgttggacctgctgtccaaa 2400
BChV_RO_7_ARP12_RV      gtcacattaaaacaactggtgacaatgcctccttctttattgttggacctgctgtccaaa 206
BChV_RO_2_ARP12_RV      gtcacattaaaacaactggtgacaatgcctccttctttattgttggacctgctgtccaaa 173
                        ************************************************************

BChV_RO                 agcaatccaaatataattacgccgtttcatacggagcttggacagatcgaatggtggaga 2460
BChV_RO_7_ARP12_RV      agcaatccaaatataattatgccgtttcatacggagcttggacagatcgaatgatggaga 266
BChV_RO_2_ARP12_RV      agcaatccaaatataattatgccgtttcatacggagcttggacagatcgaatgatggaga 233
                        ****************** ******************************** ******

BChV_RO                 tagggatgatagccatagcactagatgaacaaggctcatccggttccgcaaggacagaaa 2520
BChV_RO_7_ARP12_RV      tagggatgatagccatagcactagatgaacaaggctcatccggttccgcaaggacagaaa 326
BChV_RO_2_ARP12_RV      tagggatgatagccatagcactagatgaacaaggctcatccggttccgcaaggacagaaa 293
                        ************************************************************

BChV_RO                 gaccaaagagagttgggcactccatggcagtctcaacctgggagactataaacttaccgg 2580
BChV_RO_7_ARP12_RV      gaccaaagagagttgggcactccatggcagtctccacctgggagactataaacttaccgg 386
BChV_RO_2_ARP12_RV      gaccaaagagagttgggcactccatggcagtctccacctgggagactataaacttaccgg 353
                        ********************************** ************************

BChV_RO                 agaaggaagactccgagaaactcaaaaccggtcaaagacaagaccttaaaactcctttca 2640
BChV_RO_7_ARP12_RV      agaaggaagactccgagaaactcaaaaccggtcaaagacaagaccttaaaactcctttca 446
BChV_RO_2_ARP12_RV      agaaggaagactccgagaaactcaaaaccggtcaaagacaagaccttaaaactcctttca 413
                        ************************************************************

BChV_RO                 cgattagtggaagctccgatgtgaagggcatcgagaagagagacttgccccttcctgctg 2700
BChV_RO_7_ARP12_RV      cgattagtgaaagctccgatgtgaagggcatcgagaagagagacttgccccttcctgctg 506
BChV_RO_2_ARP12_RV      cgattagtgaaagctccgatgtgaagggcatcgagaagagagacttgccccttcctgctg 473
                        ********* ************************************************

BChV_RO                 atgaggacattcctgattttatcgggaatgatccttggtctaatgtatcaatcaggaagt 2760
BChV_RO_7_ARP12_RV      atgaggacattcctgattttatcgggaatgatccttggtctaatgtatcaatcaggaagt 566
BChV_RO_2_ARP12_RV      atgaggacattcctgattttatcgggaatgatccttggtctaatgtatcaatcaggaagt 533
                        ************************************************************
```

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BChV_RO                 tgcaagaagaagaggctatgacgtctaagagtggtcttagaccccagttgaaacctcccg 2820
BChV_RO_7_ARP12_RV      tgcaagaagaagaggctatgacgtctaagagtggtcttagaccccagttgaaacctcccg 626
BChV_RO_2_ARP12_RV      tgcaagaagaagaggctatgacgtctaagagtggtcttagaccccagttgaaacctcccg 593
                        ************************************************************

BChV_RO                 gtctcccaaaaccacaaccagttagaacgattggaaattttaatccaacgccggaattgg 2880
BChV_RO_7_ARP12_RV      gtctcccaaaaccacaaccagttagaacgattggaaattttaatccaacgccggaattgg 686
BChV_RO_2_ARP12_RV      gtctcccaaaaccacaaccagttagaacgattggaaattttaatccaacgccggaattgg 653
                        ************************************************************

BChV_RO                 ttgaatcgtggagacctgatgtgaaccccggatattccaaggaagacgtggcagccgcca 2940
BChV_RO_7_ARP12_RV      ttgaatcgtggagacctgatgtgaaccctggatattccaaggaagacgtggcagccgcca 746
BChV_RO_2_ARP12_RV      ttgaatcgtggagacctgatgtgaaccctggatattccaaggaagacgtggcagccgcca 713
                        ************************** ******************************

BChV_RO                 ctattctttatggaggttctatcaaagacggccggtctatgatcgataaacgcgataaag 3000
BChV_RO_7_ARP12_RV      ctattctttatggaggttctatcaaagacggccggtctatgatcgataaacgcgatgaag 806
BChV_RO_2_ARP12_RV      ctattctttatggaggttctatcaaagacggccggtctatgatcgataaacgcgatgaag 773
                        ************************************************************ ***

BChV_RO                 ctgtgttagacggccgtaagcattggggttcttccttagcttcctccttaacaggaggaa 3060
BChV_RO_7_ARP12_RV      ctgtgttagacggccgtaagcgttggggttcttccttagcttcctccttaacaggaggaa 866
BChV_RO_2_ARP12_RV      ctgtgttagacggccgtaagcgttggggttcttccttagcttcctccttaacaggaggaa 833
                        ********************* ************************************

BChV_RO                 cgcttaaggcctctgcaaagtcggagaagcttgctaaactcacctcgcgtgagagggcgg 3120
BChV_RO_7_ARP12_RV      cgcttaaggcctctgcaaagtcggagaagcttgctaaactcacctcgcgtgagagggcgg 926
BChV_RO_2_ARP12_RV      cgcttaaggcctctgcaaagtcggagaagcttgctaaactcacctcgcgtgagagggcgg 893
                        ************************************************************

BChV_RO                 aattcgaacgaattaaacgccagcaaggcaccacacgagcttcagaatacttggaattta 3180
BChV_RO_7_ARP12_RV      aattcgaacgaattaaacgccagcaaggcaccacacgagcttcagaatacttggaattta 986
BChV_RO_2_ARP12_RV      aattcgaacgaattaaacgccagcaaggcaccacacgagcttcagaatacttggaattta 953
                        ************************************************************

BChV_RO                 ttctgaagagcatgaaccccgactaatggttttgaagggatatagcctaacccttttctgg 3240
BChV_RO_7_ARP12_RV      ttctgaagagcatgaaccccgactaatggttttgaagggatatagcctaacccttttctgg 1046
BChV_RO_2_ARP12_RV      ttctgaagagcatgaaccccgactaatggttttgaagggatatagcctaacccttttctgg 1013
                        ************************************************************

BChV_RO                 tcctgatgaacctgtctaaatcatcaccgtcaagcccgtgacgttaaactaggaacgact 3300
BChV_RO_7_ARP12_RV      tcctgatgaacctgtctaaatcatcaccgtcaagcccgtgacgttaaactaggaacgact 1106
BChV_RO_2_ARP12_RV      tcctgatgaacctgtctaaatcatcaccgtcaagcccgtgacgttaaactaggaacgact 1073
                        ************************************************************

BChV_RO                 ccgaaaggataggcacgaatgttccccttatttaaagggttatacagaaggattccttct 3360
BChV_RO_7_ARP12_RV      ccgaaaggataggcacgaatgttccccttatttaaagggttatacagaaggattccttct 1166
BChV_RO_2_ARP12_RV      ccgaaaggataggcacgaatgttccccttatttaaagggttatacagaaggattccttct 1133
                        ************************************************************
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BChV_RO                 ggcatttcggtgtGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCC 3420
BChV_RO_7_ARP12_RV      ggcatttcggtgtGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCC 1226
BChV_RO_2_ARP12_RV      ggcatttcggtgtGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCC 1193
                        ************************************************************

BChV_RO                 GAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGAAGCTGGCCCGAGCTCGATC    3477
BChV_RO_7_ARP12_RV      GAAGGAGGACGCACNN----------------------------------------    1242
BChV_RO_2_ARP12_RV      GAAGGAGACGCAC-------------------------------------------    1206
                        *******
```

Sequence of junction of BMYV P02 fragment and CaMV 35S promoter region of pDIVA

CLUSTAL O(1.2.4) multiple sequence alignment

```
BMYV_P02              AGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGA   60
BMYV_P02_7_ARP11_FW   ------------------------------------------------------------   0
BMYV_P02_10_ARP11_FW  ------------------------------------------------------------   0

BMYV_P02              AAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA   120
BMYV_P02_7_ARP11_FW   ----------------AACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA   45
BMYV_P02_10_ARP11_FW  ----------GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA   50
                                 ******************************************

BMYV_P02              CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG   180
BMYV_P02_7_ARP11_FW   CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG   105
BMYV_P02_10_ARP11_FW  CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG   110
                      ************************************************************

BMYV_P02              TTCATTTCATTTGGAGAGGacaaaacaaaccagcgaggatctagcagtcgatgcaatttc   240
BMYV_P02_7_ARP11_FW   TTCATTTCATTTGGAGAGGacaaaagaaaccagcgaggatctagcagtcgatgcaatttc   165
BMYV_P02_10_ARP11_FW  TTCATTTCATTTGGAGAGGacaaaagaaaccagcgaggatctagcagtcgatgcaatttc   170
                      ************************ **********************************

BMYV_P02              agcttaaaacaaacagcttcacttgttcgttgaaccgaccgctaacagtttcagagcgag   300
BMYV_P02_7_ARP11_FW   agcttaaaacaaacagcttcacttgttcgttgaaccgaccgctaacagtttcagagcgag   225
BMYV_P02_10_ARP11_FW  agcttaaaacaaacagcttcacttgttcgttgaaccgaccgctaacagtttcagagcgag   230
                      ************************************************************

BMYV_P02              ttttgaacaccgcgtattttcttacgaatcacctaccgctcataacctttgagaatgaaa   360
BMYV_P02_7_ARP11_FW   ttttgaacaccgcgtattttcttacgaatcacctaccgctcataacctttgagaatgaaa   285
BMYV_P02_10_ARP11_FW  ttttgaacaccgcgtattttcttacgaatcacctaccgctcataacctttgagaatgaaa   290
                      ************************************************************

BMYV_P02              actgcattcgttctcttctcgctgctctgcctctgctgcttagtaagcagctcgaccctg   420
BMYV_P02_7_ARP11_FW   actgcattcgttctcttctcgctgctctgcctctgctgcttagtaagcagctcgaccctg   345
BMYV_P02_10_ARP11_FW  actgcattcgttctcttctcgctgctctgcctctgctgcttagtaagcagctcgaccctg   350
                      ************************************************************

BMYV_P02              ggagcctcgtttacactcccgggaaacgccagtctttacgactggccaggttccacaact   480
BMYV_P02_7_ARP11_FW   ggagcctcgtttacactcccgggaaacgccagtctttacgactggccaggttccacaact   405
BMYV_P02_10_ARP11_FW  ggagcctcgtttacactcccgggaaacgccagtctttacgactggccaggttccacaact   410
                      ************************************************************

BMYV_P02              actgcggaaccgctttgcccagcactcgcaacattgacttacgagtgcccccagaaaag   540
BMYV_P02_7_ARP11_FW   actgcggaaccgctttgcccagcactcgcaacattgacttacgagtgcccccagaaaag   465
BMYV_P02_10_ARP11_FW  actgcggaaccgctttgcccagcactcgcaacattgacttacgagtgcccccagaaaag   470
                      ************************************************************
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BMYV_P02              acgttaaaagattttaccttgcccgaaattcaggcagagatctggggaagaggctacaac   600
BMYV_P02_7_ARP11_FW   acgttaaaagattttaccttgcccgaaattcaggcagagatctggggaagaggctacaac   525
BMYV_P02_10_ARP11_FW  acgttaaaagattttaccttgcccgaaattcaggcagagatctggggaagaggctacaac   530
                      ************************************************************

BMYV_P02              gccgtagagaaattctctttcacggtgaagcagagtttaaaaagttcctttcagtatggt   660
BMYV_P02_7_ARP11_FW   gccgtagagaaattctctttcacggtgaagcagagtttaaaaagttcctttcagtatggt   585
BMYV_P02_10_ARP11_FW  gccgtagagaaattctctttcacggtgaagcagagtttaaaaagttcctttcagtatggt   590
                      ************************************************************

BMYV_P02              gtgctgaaagcgaaagaaaattacgggagagccctaagatcaacattaaaatggatcgta   720
BMYV_P02_7_ARP11_FW   gtgctgaaagcgaaagaaaattacgggagagccctaagatcaacattaaaatggatcgta   645
BMYV_P02_10_ARP11_FW  gtgctgaaagcgaaagaaaattacgggagagccctaagatcaacattaaaatggatcgta   650
                      ************************************************************

BMYV_P02              ttattatggtcttacgtgatatgggcactctcttgcaccgtctggtatttgttgaagaac   780
BMYV_P02_7_ARP11_FW   ttattatggtcttacgtgatatgggcactctcttgcaccgtctggtatttgttgaagaac   705
BMYV_P02_10_ARP11_FW  ttattatggtcttacgtgatatgggcactctcttgcaccgtctggtatttgttgaagaac   710
                      ************************************************************

BMYV_P02              tataccatagaaatacttatgctgagctcgctttttgcgttcaccacctttttggtgaag   840
BMYV_P02_7_ARP11_FW   tataccatagaaatacttatgctgagctcgctttttgcgttcaccacctttttggtgaag   765
BMYV_P02_10_ARP11_FW  tataccatagaaatacttatgctgagctcgctttttgcgttcaccacctttttggtgaag   770
                      ************************************************************

BMYV_P02              ctcgcggtatggatttttggcggttggctaacttccctggtaaatggtttatttgctctc   900
BMYV_P02_7_ARP11_FW   ctcgcggtatggatttttggcggttggctaacttccctggtaaatggtttatttgctctc   825
BMYV_P02_10_ARP11_FW  ctcgcggtatggatttttggcggttggctaacttccctggtaaatggtttatttgctctc   830
                      ************************************************************

BMYV_P02              acgaaacgtatcttgaaaactctttcatccagaaagagctacgtttgtgagcgatccgta   960
BMYV_P02_7_ARP11_FW   acgaaacgtatcttgaaaactctttcatccagaaagagctacgtttgtgagcgatccgta   885
BMYV_P02_10_ARP11_FW  acgaaacgtatcttgaaaactctttcatccagaaagagctacgtttgtgagcgatccgta   890
                      ************************************************************

BMYV_P02              aaaggtttcctcacctttaccatcaaacaaagcccgccgcgtaattgtattcttcaaata  1020
BMYV_P02_7_ARP11_FW   aaaggtttcctcacctttaccatcaaacaaagcccgccgcgtaattgtattcttcaaata   945
BMYV_P02_10_ARP11_FW  aaaggtttcctcacctttaccatcaaacaaagcccgccgcgtaattgtattcttcaaata   950
                      ************************************************************

BMYV_P02              caacatgcagacggttcccatgccggttacgcaacatgcgtaaccttattcgacgggaca  1080
BMYV_P02_7_ARP11_FW   caacatgcagacggttcccatgccggttacgcaacatgcgtaaccttattcgacgggaca  1005
BMYV_P02_10_ARP11_FW  caacatgcagacggttcccatgccggttacgcaacatgcgtaaccttattcgacgggaca  1010
                      ************************************************************

BMYV_P02              aacggattgttgactgcgcaacacgtagttgacgatttttacgaaggagacccgaaaaag  1140
BMYV_P02_7_ARP11_FW   aacggattgttgactgcgcaacacgtagttgacgatttttacgaaggagacccgaaaaag  1065
BMYV_P02_10_ARP11_FW  aacggattgttgactgcgcaacacgtagttgacgatttttacgaaggagacccgaaaaag  1070
                      ************************************************************
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BMYV_P02              acactaaaagtcgtctccacccgcaatggaaacaaaatccccttgatgaattcagagtg   1200
BMYV_P02_7_ARP11_FW   acmctaaaagtcgtctccacccgcaatggaaacaa-atccccttgatgaattcagagtg   1124
BMYV_P02_10_ARP11_FW  acmctaaaagtcgtctccacccgcaatggaaacaaaatccccttgatgaattcagagtg   1130
                      ** ******************************** **********************

BMYV_P02              acgtacacatctgagaaaagggatcaattgttgatgcatgggcccccaaactgggaagga   1260
BMYV_P02_7_ARP11_FW   acgtacmcatctgagaaaagggatcat-tgttgatgcwgggcccccaaatggga---agg   1180
BMYV_P02_10_ARP11_FW  acgtacmcatctgaaaaaagggatcaattgttgatgcngggcccccaactggga---agg   1187
                      ****** ******* **********  *********  ** **** *    *     *

BMYV_P02              gttcttgcctgcaaggcagttcacatgattccggcatcgaatgttgcaaaatcgaaagcg   1320
BMYV_P02_7_ARP11_FW   attyttgcctgcargca----------------------------------------------   1197
BMYV_P02_10_ARP11_FW  attcttgcctgcaaggcagttcmatgattccgg--------------------------   1220
                       ** ********* *
```

Sequence of junction of BMYV P02 fragment and HDV rbz region of pDIVA

CLUSTAL O(1.2.4) multiple sequence alignment

```
BMYV_P02              ccacgagaggaaacaaactgtcctggaacaattttctcgaagattttaaaacggcagttt   2520
BMYV_P02_7_ARP12_FV   --------ggaaacaaactgtcctggaac--aatttcngaagattttaaaacggcagttt   50
BMYV_P02_10_ARP12_FV  ---------ggaaacaaacttcctggaac--aattttngaagattttaaaacggcagttt   49
                             * **  **   ********    **   **********************

BMYV_P02              tctctctagagctcgaagccggcgtaggcgtcccgtatgttgcttacggtcgacgcacgc   2580
BMYV_P02_7_ARP12_FV   tcnctctagagctcgaagccggcgtaggcgtcccgtatgttgcttacggtcgacgcacgc   110
BMYV_P02_10_ARP12_FV  tctctctagagctcgaagccggcgtaggcgtcccgtatgttgcttacggtcgacgcacgc   109
                      ** ************************************************************

BMYV_P02              acagaggctggattgaagatccagatctgttgccggtcttagctcgtttcaccttcgatc   2640
BMYV_P02_7_ARP12_FV   acagaggctggattgaagatccagatctgttgccggtcttagctcgtttcaccttcgatc   170
BMYV_P02_10_ARP12_FV  acagaggctggattgaagatccagatctgttgccggtcttagctcgtttcaccttcgatc   169
                      ************************************************************

BMYV_P02              gattacagaagttatcggagatgaaatttgagcacatgagtcctgagcaattggttcagg   2700
BMYV_P02_7_ARP12_FV   gattacagaagttatcggagatgaaatttgagcacatgagtcctgagcaattggttcagg   230
BMYV_P02_10_ARP12_FV  gattacagaagttatcggagatgaaatttgagcacatgagtcctgagcaattggttcagg   229
                      ************************************************************

BMYV_P02              aaggtctgtgcgatccaatacggttattcgtaaaaggcgagccacacaaacaatccaaac   2760
BMYV_P02_7_ARP12_FV   aaggtctgtgcgatccaatacggttattcgtaaaaggcgagccacacaaacaatccaaac   290
BMYV_P02_10_ARP12_FV  aaggtctgtgcgatccaatacggttattcgtaaaaggcgagccacacaaacaatccaaac   289
                      ************************************************************

BMYV_P02              ttgatgagggacgctaccgcctcatcatgagtgtctcattggttgaccaactggtagccc   2820
BMYV_P02_7_ARP12_FV   ttgatgagggacgctaccgcctcatcatgagtgtctcattggttgaccaactggtagccc   350
BMYV_P02_10_ARP12_FV  ttgatgagggacgctaccgcctcatcatgagtgtctcattggttgaccaactggtagccc   349
                      ************************************************************

BMYV_P02              gggttctgtttcaaaaccaaaacaagcgcgagattgcgctctggagggcgattccctcaa   2880
BMYV_P02_7_ARP12_FV   gggttctgtttcaaaaccaaaacaagcgcgagattgcgctctggagggcgattccctcaa   410
BMYV_P02_10_ARP12_FV  gggttctgtttcaaaaccaaaacaagcgcgagattgcgctctggagggcgattccctcaa   409
                      ************************************************************

BMYV_P02              aacccggtttcggattgtccacggacggacaagtcgtcgatttcatgcaagcattatcgg   2940
BMYV_P02_7_ARP12_FV   aacccggtttcggattgtccacggacggacaagtcgtcgatttcatgcaagcattatcgg   470
BMYV_P02_10_ARP12_FV  aacccggtttcggattgtccacggacggacaagtcgtcgatttcatgcaagcattatcgg   469
                      ************************************************************

BMYV_P02              cgcaggtgggagtgaataccgctgaattactccaagattggaaatcccacctcattccta   3000
BMYV_P02_7_ARP12_FV   cgcaggtgggagtgaataccgctgaattactccaagattggaaatcccacctcattccta   530
BMYV_P02_10_ARP12_FV  cgcaggtgggagtgaataccgctgaattactccaagattggaaatcccacctcattccta   529
                      ************************************************************
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```
BMYV_P02              cagattgctctggttttgactggagcgtttcggactggcttctagaagatgaaatggaag   3060
BMYV_P02_7_ARP12_FV   cagattgctctggttttgactggagcgtttcggactggcttctagaagatgaaatggaag   590
BMYV_P02_10_ARP12_FV  cagattgctctggttttgactggagcgtttcggactggcttctagaagatgaaatggaag   589
                      ************************************************************

BMYV_P02              ttcgaaacaggcttacgttggatataaatgatctaaccaggcgactgcgagctggatggc   3120
BMYV_P02_7_ARP12_FV   ttcgaaacaggcttacgttggatataaatgatctaaccaggcgactgcgagctggatggc   650
BMYV_P02_10_ARP12_FV  ttcgaaacaggcttacgttggatataaatgatctaaccaggcgactgcgagctggatggc   649
                      ************************************************************

BMYV_P02              ttaaatgcctcgcaaacagcgtcctctgtttatcggatggaacattgctctcgcagcaag   3180
BMYV_P02_7_ARP12_FV   ttaaatgcctcgcaaacagcgtcctctgtttatcggatggaacattgctctcgcagcaag   710
BMYV_P02_10_ARP12_FV  ttaaatgcctcgcaaacagcgtcctctgtttatcggatggaacattgctctcgcagcaag   709
                      ************************************************************

BMYV_P02              tacctggtgtgcaaaagagtggcagctacaacacctcctcgtctaattctagaattcgag   3240
BMYV_P02_7_ARP12_FV   tacctggtgtgcaaaagagtggcagctacaacacctcctcgtctaattctagaattcgag   770
BMYV_P02_10_ARP12_FV  tacctggtgtgcaaaagagtggcagctacaacacctcctcgtctaattctagaattcgag   769
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BMYV_P02              tgatggctgcttaccactccggagcctcctgggccatcgccatgggtgatgatgcccttg   3300
BMYV_P02_7_ARP12_FV   tgatggctgcttaccactccggagcctcctgggccatcgccatgggtgatgatgcccttg   830
BMYV_P02_10_ARP12_FV  tgatggctgcttaccactccggagcctcctgggccatcgccatgggtgatgatgcccttg   829
                      ************************************************************

BMYV_P02              aatctgtagatgcagacctaagtcgatactcatccttaggcttcaaagtcgaggtttctt   3360
BMYV_P02_7_ARP12_FV   aatctgtagatgcagacctaagtcgatactcatccttaggcttcaaagtcgaggtttctt   890
BMYV_P02_10_ARP12_FV  aatctgtagatgcagacctaagtcgatactcatccttaggcttcaaagtcgaggtttctt   889
                      ************************************************************

BMYV_P02              cacaactggaattctgctctcatatttttgaggaggagaacctcgccgttccggtcaaca   3420
BMYV_P02_7_ARP12_FV   cacaactggaattctgctctcatatttttgaggaggagaacctcgccgttccggtcaaca   950
BMYV_P02_10_ARP12_FV  cacaactggaattctgctctcatatttttgaggaggagaacctcgccgttccggtcaaca   949
                      ************************************************************

BMYV_P02              aagctaaaatgctttataaattgatacatggttacgaaccggaatgcggtaacttagaag   3480
BMYV_P02_7_ARP12_FV   aagctaaaatgctttataaattgatacatggttacgaaccggaatgcggtaacttagaag   1010
BMYV_P02_10_ARP12_FV  aagctaaaatgctttataaattgatacatggttacgaaccggaatgcggtaacttagaag   1009
                      ************************************************************

BMYV_P02              ttctgacgaactatcttgcagcttgtttctcaattttgaacgagcttagatctgatccag   3540
BMYV_P02_7_ARP12_FV   ttctgacgaactatcttgcagcttgtttctcaattttgaacgagcttagatctgatccag   1070
BMYV_P02_10_ARP12_FV  ttctgacgaactatcttgcagcttgtttctcaattttgaacgagcttagatctgatccag   1069
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BMYV_P02              agctcgttgcctccctctaccagtggctggtccttccagtgcagccacaaaagatataac   3600
BMYV_P02_7_ARP12_FV   agctcgttgcctccctctaccagtggctggtccttccagtgcagccacaaaagatataac   1130
BMYV_P02_10_ARP12_FV  agctcgttgcctccctctaccagtggctggtccttccagtgcagccacaaaagatataac   1129
                      ************************************************************
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BMYV_P02              gagggataacataaacagcGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGG   3660
BMYV_P02_7_ARP12_FV   gagggataacataaacagcGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGG   1190
BMYV_P02_10_ARP12_FV  gagggataacataaacagcGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGG   1189
                      ************************************************************

BMYV_P02              GCATCCGAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGAAGCTGGCCCGAGCTCG   3720
BMYV_P02_7_ARP12_FV   GCATCCGAAGGAGGACGCAC----------------------------------------   1210
BMYV_P02_10_ARP12_FV  GCATCCGAAGGAGGACGCA----------------------------------------   1208
                      ********************
```

Sequence of junction of BMYV RO fragment and CaMV 35S promoter region of pDIVA

CLUSTAL O(1.2.4) multiple sequence alignment

```
BMYV_RO              AGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGA    60
BMYV_RO_4_ARP11_FW   ------------------------------------------------------------    0
BMYV_RO_11_ARP11_FW  ------------------------------------------------------------    0

BMYV_RO              AAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA    120
BMYV_RO_4_ARP11_FW   ----------------AACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA    45
BMYV_RO_11_ARP11_FW  ----------GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA    50
                                 ******************************************

BMYV_RO              CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG    180
BMYV_RO_4_ARP11_FW   CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG    105
BMYV_RO_11_ARP11_FW  CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAG    110
                     ************************************************************

BMYV_RO              TTCATTTCATTTGGAGAGGgtctcattggttgaccaactggtagcccgggttctgtttca    240
BMYV_RO_4_ARP11_FW   TTCATTTCATTTGGAGAGGgtctcattggttgatcaactggtagcccgggttctgtttca    162
BMYV_RO_11_ARP11_FW  TTCATTTCATTTGGAGAGGgtctcattggttgatcaactggtagcccgggttctgtttca    167
                     ********************************* ************************

BMYV_RO              aaaccaaaacaagcgcgagattgcgctctggagggcgattccctcaaaacccggtttcgg    300
BMYV_RO_4_ARP11_FW   aaaccaaaacaagcgcgagattgcgctctggagggcgattccctcaaaacccggtttcgg    222
BMYV_RO_11_ARP11_FW  aaaccaaaacaagcgcgagattgcgctctggagggcgattccctcaaaacccggtttcgg    227
                     ************************************************************

BMYV_RO              attgtccacggacggacaagtcgtcgatttcatgcaagcattatcggcgcaggtgggagt    360
BMYV_RO_4_ARP11_FW   attgtccacggacggacaagtcgtcgatttcatgcaagcattatcggcgcaggtgggagt    282
BMYV_RO_11_ARP11_FW  attgtccacggacggacaagtcgtcgatttcatgcaagcattatcggcgcaggtgggagt    287
                     ************************************************************

BMYV_RO              gaataccgctgaattactccaagattggaaatcccacctcattcctacagattgctctgg    420
BMYV_RO_4_ARP11_FW   gaataccgctgaattactccaagattggaaatcccacctcattcctacagattgctctgg    342
BMYV_RO_11_ARP11_FW  gaataccgctgaattactccaagattggaaatcccacctcattcctacagattgctctgg    347
                     ************************************************************

BMYV_RO              ttttgactggagcgtttcggactggcttctagaagatgaaatggaagttcgaaacaggct    480
BMYV_RO_4_ARP11_FW   ttttgactggagcgtttcggactggcttctagaagatgaaatggaagttcgaaacaggct    402
BMYV_RO_11_ARP11_FW  ttttgactggagcgtttcggactggcttctagaagatgaaatggaagttcgaaacaggct    407
                     ************************************************************

BMYV_RO              tacgttggatataaatgatctaaccaggcgactgcgagctggatggcttaaatgcctcgc    540
BMYV_RO_4_ARP11_FW   tacgttggatataaatgatctaaccaggcgactgcgagctggatggcttaaatgcctcgc    462
BMYV_RO_11_ARP11_FW  tacgttggatataaatgatctaaccaggcgactgcgagctggatggcttaaatgcctcgc    467
                     ************************************************************
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BMYV_RO              aaacagcgtcctctgtttatcggatggaacattgctctcgcagcaagtacctggtgtgca   600
BMYV_RO_4_ARP11_FW   aaacagcgtcctctgtttatcggatggaacattgctctcgcagcaagtacctggtgtgca   522
BMYV_RO_11_ARP11_FW  aaacagcgtcctctgtttatcggatggaacattgctctcgcagcaagtacctggtgtgca   527
                     ************************************************************

BMYV_RO              aaagagtggcagctacaacacctcctcgtctaattctagaattcgagtgatggctgctta   660
BMYV_RO_4_ARP11_FW   aaagagtggcagctacaacacctcctcgtctaattctagaattcgagtgatggctgctta   582
BMYV_RO_11_ARP11_FW  aaagagtggcagctacaacacctcctcgtctaattctagaattcgagtgatggctgctta   587
                     ************************************************************

BMYV_RO              ccactccggagcctcctgggccatcgccatgggtgatgatgcccttgaatctgtagatgc   720
BMYV_RO_4_ARP11_FW   ccactccggagcctcctgggccatcgccatgggtgatgatgcccttgaatctgtagatgc   642
BMYV_RO_11_ARP11_FW  ccactccggagcctcctgggccatcgccatgggtgatgatgcccttgaatctgtagatgc   647
                     ************************************************************

BMYV_RO              agacctaagtcgatactcatccttaggcttcaaagtcgaggtttcttcacaactggaatt   780
BMYV_RO_4_ARP11_FW   agacctaagtcgatactcatccttaggcttcaaagtcgaggtttcttcacaactggaatt   702
BMYV_RO_11_ARP11_FW  agacctaagtcgatactcatccttaggcttcaaagtcgaggtttcttcacaactggaatt   707
                     ************************************************************

BMYV_RO              ctgctctcatatttttgaggaggagaacctcgccgttccggtcaacaaagctaaaatgct   840
BMYV_RO_4_ARP11_FW   ctgctctcatatttttgaggaggagaacctcgccgttccggtcaacaaagctaaaatgct   762
BMYV_RO_11_ARP11_FW  ctgctctcatatttttgaggaggagaacctcgccgttccggtcaacaaagctaaaatgct   767
                     ************************************************************

BMYV_RO              ttataaattgatacatggttacgaaccggaatgcggtaacttagaagttctgacgaacta   900
BMYV_RO_4_ARP11_FW   ttataaattgatacatggttacgaaccggaatgcggtaacttagaagttctgacgaacta   822
BMYV_RO_11_ARP11_FW  ttataaattgatacatggttacgaaccggaatgcggtaacttagaagttctgacgaacta   827
                     ************************************************************

BMYV_RO              tcttgcagcttgtttctcaattttgaacgagcttagatctgatccagagctcgttgcctc   960
BMYV_RO_4_ARP11_FW   tcttgcagcttgtttctcaattttgaacgagcttagatctgatccagagctcgttgcctc   882
BMYV_RO_11_ARP11_FW  tcttgcagcttgtttctcaattttgaacgagcttagatctgatccagagctcgttgcctc   887
                     ************************************************************

BMYV_RO              cctctaccagtggctggtccttccagtgcagccacaaaagatataacgagggataacata  1020
BMYV_RO_4_ARP11_FW   cctctaccagtggctggtccttccagtgcagccacaaaagatataacgagggataacata   942
BMYV_RO_11_ARP11_FW  cctctaccagtggctggtccttccagtgcagccacaaaagatataacgagggataacata   947
                     ************************************************************

BMYV_RO              aacagccgggtaaacatcagttgcaaacgccggaagtttaaagtctgattacataacaag  1080
BMYV_RO_4_ARP11_FW   aacagccgggtaaacatcagttgcaaacgccggaagtttaaagtctgattacataacaag  1002
BMYV_RO_11_ARP11_FW  aacagccgggtaaacatcagttgcaaacgccggaagtttaaagtctgattacataacaag  1007
                     ************************************************************

BMYV_RO              ccaaaatagatttcaagtttttagcaggattttcaagtggtctattgtcagcaatacctg  1140
BMYV_RO_4_ARP11_FW   ccaaaatagatttcaagtttttagcaggattttcaagtggtctattgtcagcaatacctg  1062
BMYV_RO_11_ARP11_FW  ccaaaatagatttcaagtttttagcaggattttcaagtggtctattgtcagcaatacctg  1067
                     ************************************************************
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BMYV_RO              taacggtagttggcttgtattttgtctacctgaagatttcccaccacgtcagatcaatcg    1200
BMYV_RO_4_ARP11_FW   taacggtagttggcttgtattttgtctacctgaagatttcccaccacgtcagatcaatcg    1122
BMYV_RO_11_ARP11_FW  taacggtagttggcttgtattttgtctacctgaagatttcc-cccacgtcagatcaatcg    1126
                     ****************************************  ****************

BMYV_RO              ttaatgaatacggtcgtgggtaggagaacaatcaatggaagaagacgaccacgtaggcaa    1260
BMYV_RO_4_ARP11_FW   ttaatgaatacggtcgtgggtaggaga---------------------------------    1149
BMYV_RO_11_ARP11_FW  ttaatgaatacggtcgtgggtaggagaacaatcaatggaa--------------------    1166
                     ****************************
```

Sequence of junction of BMYV RO fragment and HDV rbz region of pDIVA

CLUSTAL O(1.2.4) multiple sequence alignment

```
BMYV_RO              gatggtctcattgcgtacaatgatgacctcaaggagggttggaatgtgggggtttacaac    2220
BMYV_RO_4_ARP12_RV   -----gtctcattggtacaatgatgacctcaaggagggtt-ggaattgggggtttacaac    54
BMYV_RO_11_ARP12_RV  ----------------------------caaggagggttggaat---tggggtttacaa    28
                                               ********** * *     *** **   *

BMYV_RO              aatgtggagataaccaacaataaggctgataacactttgaaatacggccatccagacatg    2280
BMYV_RO_4_ARP12_RV   aatgtggagataaccaacaataaggctgataacactttgaaatacggccatccagacatg    114
BMYV_RO_11_ARP12_RV  caatgtgggataaccaacaataaggctgataacactttgaaatacggccatccagacatg    88
                      *    * **************************************************

BMYV_RO              gaacttaatagttgtcatttcaaccaaggacaatgtctggaaagagacggagatttaact    2340
BMYV_RO_4_ARP12_RV   gaacttaatagttgtcatttcaaccaaggacaatgtctggaaagagacggagatttaact    174
BMYV_RO_11_ARP12_RV  gaacttaatagttgtcatttcaaccaaggacaatgtctggaaagagacggagatttaact    148
                     ************************************************************

BMYV_RO              tgtcacgttaagacaactggtgacaacgcctccttctttattgttggccccgctgtccag    2400
BMYV_RO_4_ARP12_RV   tgtcacgttaagacaactggtgacaacgcctccttctttatcgttggccccgctgtccag    234
BMYV_RO_11_ARP12_RV  tgtcacgttaagacaactggtgacaacgcctccttctttatcgttggccccgctgtccag    208
                     ****************************************** ******************

BMYV_RO              aaacaatccaaatataattatgccgtttcgtacggagcttggacagatcggatgatggag    2460
BMYV_RO_4_ARP12_RV   aaacaatccaaatataattatgccgtttcgtacggagcttggacagatcggatgatggag    294
BMYV_RO_11_ARP12_RV  aaacaatccaaatataattatgccgtttcgtacggagcttggacagatcggatgatggag    268
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BMYV_RO              atagggatgatagccatagcacttgatgaacaaggctcatccggttccgcaaagatagaa    2520
BMYV_RO_4_ARP12_RV   atagggatgatagccatagcacttgatgaacaaggctcatccggttccgcaaagatagaa    354
BMYV_RO_11_ARP12_RV  atagggatgatagccatagcacttgatgaacaaggctcatccggttccgcaaagatagaa    328
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BMYV_RO              agaccaaagagagttgggcactccatggcagtctccacctgggagactataaacttaccg    2580
BMYV_RO_4_ARP12_RV   agaccaaagagagttgggcactccatggcagtctccacctgggagactataaacttaccg    414
BMYV_RO_11_ARP12_RV  agaccaaagagagttgggcactccatggcagtctccacctgggagactataaacttaccg    388
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BMYV_RO              gagaaggagaactccgaggaattcaaaaccgatcaaagacaagatctcaatactcctccc    2640
BMYV_RO_4_ARP12_RV   gagaaggagaactccgaggaattcaaaaccgatcaaagacaagatctcaatactcctccc    474
BMYV_RO_11_ARP12_RV  gagaaggagaactccgaggaattcaaaaccgatcaaagacaagatctcaatactcctccc    448
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BMYV_RO              acagctggtgggagttccgatatgctggatatcgttcaaggaggcttgcctcttcctgtt    2700
BMYV_RO_4_ARP12_RV   acagctggtgggagttccgatatgctggatatcgttcaaggaggcttgcctcttcctgtt    534
BMYV_RO_11_ARP12_RV  acagctggtgggagttccgatatgctggatatcgttcaaggaggcttgcctcttcctgtt    508
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BMYV_RO              gaggaaaacatccctgattctattatggatgacccttggtccaacataccggccaagagt   2760
BMYV_RO_4_ARP12_RV   gaggaaaacatccctgattctattatggatgacccttggtccaacataccggccaagagt   594
BMYV_RO_11_ARP12_RV  gaggaaaacatccctgattctattatggatgacccttggtccaacataccggccaagagt   568
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BMYV_RO              tcgcaggaagatgaggctgtgtcatcaaagagtggttttaaaccccaattgaagcctcct   2820
BMYV_RO_4_ARP12_RV   tcgcaggaagatgaggctgtgtcatcaaagagtggttttaaaccccaattgaagcctcct   654
BMYV_RO_11_ARP12_RV  tcgcaggaagatgaggctgtgtcatcaaagagtggttttaaaccccaattgaagcctcct   628
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BMYV_RO              gggttgccgagaccacaaccagttagaacgatccgaaatttcgatccaaaccctgacttg   2880
BMYV_RO_4_ARP12_RV   gggttgccgagaccacaaccagttagaacgatccgaaatttcgatccaaaccctgacttg   714
BMYV_RO_11_ARP12_RV  gggttgccgagaccacaaccagttagaacgatccgaaatttcgatccaaaccctgacttg   688
                     ************************************************************

BMYV_RO              gttgaagcatggcgacctgatgtgaaccctggatattctaaagaagacgtggcagcagcc   2940
BMYV_RO_4_ARP12_RV   gttgaagcatggcgacctgatgtgaaccctggatattctaaagaagacgtggcagcagcc   774
BMYV_RO_11_ARP12_RV  gttgaagcatggcgacctgatgtgaaccctggatattctaaagaagacgtggcagcagcc   748
                     ************************************************************

BMYV_RO              actgtcatgtacgggggttctatcaaagagggccggtctatgattgacaagcgagataaa   3000
BMYV_RO_4_ARP12_RV   actgtcatgtacgggggttctatcaaagagggccggtctatgattgacaagcgagataaa   834
BMYV_RO_11_ARP12_RV  actgtcatgtacgggggttctatcaaagagggccggtctatgattgacaagcgagataaa   808
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BMYV_RO              gctgtgttagacggccgcaagagttggggttcttccctggcgtcctccttaacaggagga   3060
BMYV_RO_4_ARP12_RV   gctgtgttagacggccgcaagagttggggttcttccctggcgtcctccttaacaggagga   894
BMYV_RO_11_ARP12_RV  gctgtgttagacggccgcaagagttggggttcttccctggcgtcctccttaacaggagga   868
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BMYV_RO              acgcttaaggcctctgcaaagtcagagaagcttgctaaactcacctcgagtgaaagggcg   3120
BMYV_RO_4_ARP12_RV   acgcttaaggcctctgcaaagtcagagaagcttgctaaactcacctcgagtgaaagggcg   954
BMYV_RO_11_ARP12_RV  acgcttaaggcctctgcaaagtcagagaagcttgctaaactcacctcgagtgaaagggcg   928
                     ************************************************************

BMYV_RO              caattcgaacgaattaagcgccagcaaggtgccacacgagcttcagaatttttagaacga   3180
BMYV_RO_4_ARP12_RV   caattcgaacgaattaagcgccagcaaggtgccacacgagcttcagaatttttagaacga   1014
BMYV_RO_11_ARP12_RV  caattcgaacgaattaagcgccagcaaggtgccacacgagcttcagaatttttagaacga   988
                     ************************************************************

BMYV_RO              cttctggctggcacaaaccctgacccgaggtcctgatggaccttcccaaatcatcacagt   3240
BMYV_RO_4_ARP12_RV   cttctggctggcacaaaccctgacccgaggtcctgatggaccttcccaaatcatcacagt   1074
BMYV_RO_11_ARP12_RV  cttctggctggcacaaaccctgacccgaggtcctgatggaccttcccaaatcatcacagt   1048
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BMYV_RO              caagcccgtgactttaaacgtggaacgactccgataggataggcaacgagtgttttacgc   3300
BMYV_RO_4_ARP12_RV   caagcccgtgactttaaacgtggaacgactccgataggataggcaacgagtgttttacgc   1134
BMYV_RO_11_ARP12_RV  caagcccgtgactttaaacgtggaacgactccgataggataggcaacgagtgttttacgc   1108
                     ************************************************************
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BMYV_RO              tgggataactccctacggcacttcggtgtGGGTCGGCATGGCATCTCCACCTCCTCGCGG   3360
BMYV_RO_4_ARP12_RV   tgggataactccctacggcacttcggtgtGGGTCGGCATGGCATCTCCACCTCCTCGCGG   1194
BMYV_RO_11_ARP12_RV  tgggataactccctacggcacttcggtgtGGGTCGGCATGGCATCTCCACCTCCTCGCGG   1168
                     ************************************************************

BMYV_RO              TCCGACCTGGGCATCCGAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGAAGCTGG   3420
BMYV_RO_4_ARP12_RV   TCCGACCTGGGCATCCGAAGGAGGACGCA-------------------------------   1223
BMYV_RO_11_ARP12_RV  TCCGACCTGGGCATCCGAAGGAGGACGCACCNNN-------------------------   1202
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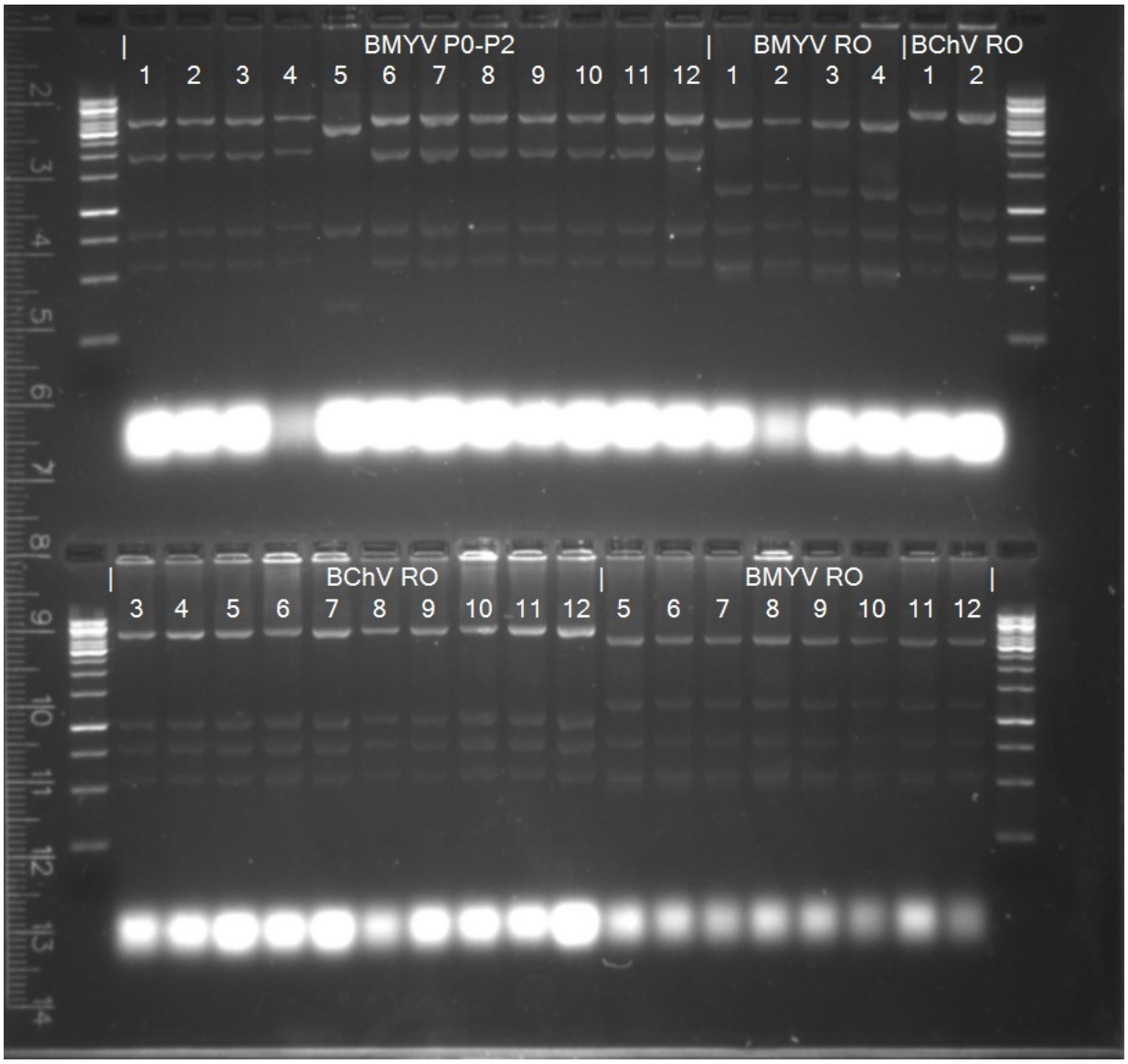
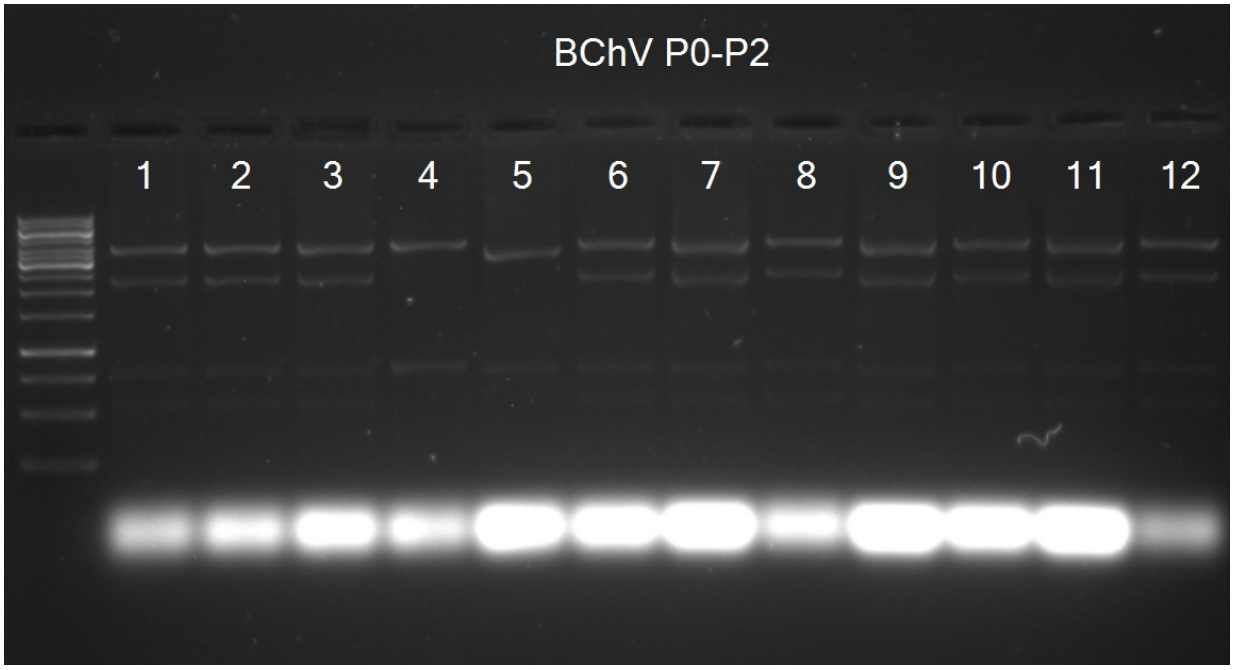
```

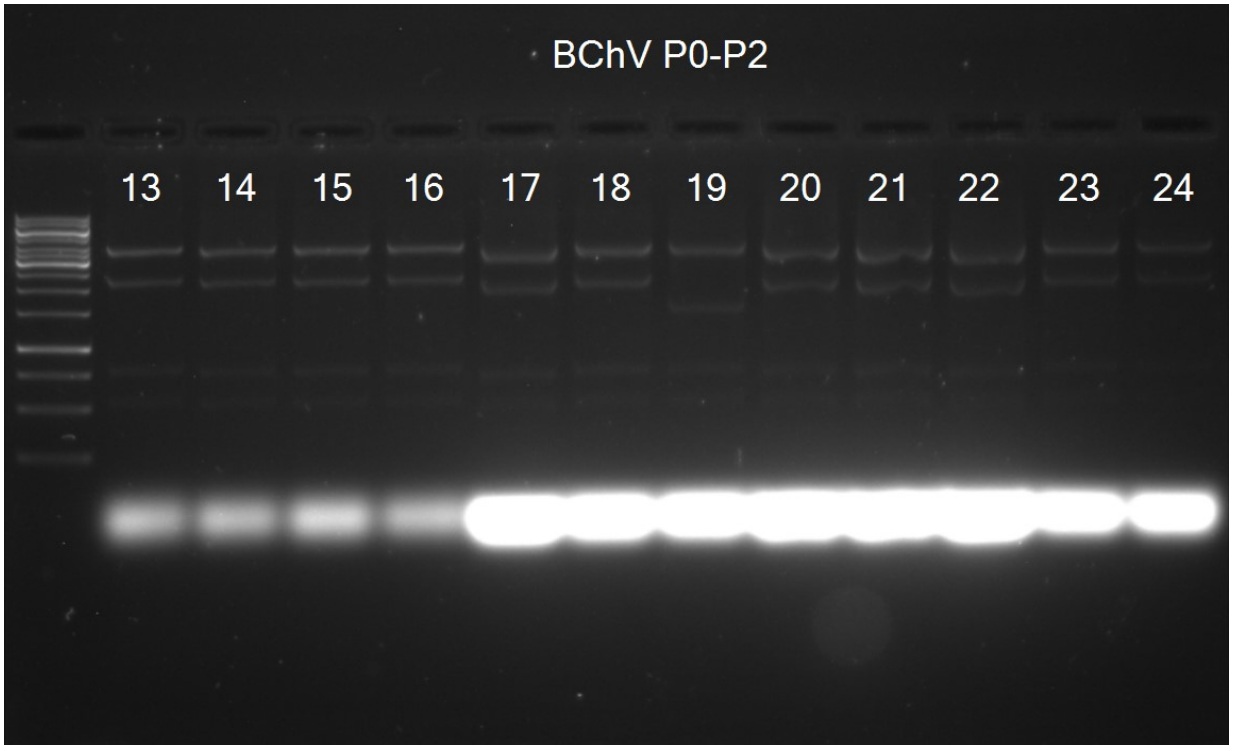
Appendix 4

Gel electrophoresis of analytical restriction endonuclease digestion



Numbers represent plasmid numbers.
Conditions:
1% agarose gel;
140 V, 45 min;
marker used Thermo Scientific™ Gene-Ruler™ 1 kb DNA Ladder
8 µL volume per well with 1 µL of plasmid DNA





Numbers represent plasmid numbers.
Conditions:
1% agarose gel;
120 V, 45 min;
marker used Thermo Scientific™ Gene-Ruler™ 1 kb DNA Ladder
8 µL volume per well with 1 µL of plasmid DNA

Acknowledgement

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My family, thank you for your absolute unconditional love. Your optimism and kindness give me strength.

And finally, my heart and soul, Taras! Your love guides me in this challenging time.

Declaration of honour

I hereby certify that I have completed the work independently without outside help and without using any tools other than those specified. I also assure that this work or parts of it have not been submitted elsewhere as proof of performance either by myself or by others. All passages that are taken literally or analogously from publications or other sources are marked as such. All secondary literature and other sources are identified and listed in the bibliography. The same applies to graphics and images as well as to all Internet sources. I assure that the written and electronic form of the work are the same.

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Göttingen, June 26, 2023

Last Name: Revva

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