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Application of reverse genetic systems to study *Beet soil-borne mosaic virus* and *Beet necrotic yellow vein virus* molecular biology, the interaction of species and their use as biotechnological tool

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1. Introduction

Beta vulgaris subsp. *vulgaris* (*B. vulgaris*) is cultivated in 35 countries worldwide (WVZ, 2017). In 2015/2016, sugar beet was produced on approximately 1.3 million hectares within the EU, whereas 334,500 hectares were cultivated in Germany (WVZ, 2017). Currently, 20% of the world supply of raw sugar is obtained from sugar beet (WVZ, 2017).

Beet yield and the extractability of sugar determine the white sugar yield, which is apart from factors like agronomic measures and environmental conditions strongly affected by pathogen infestation. Besides fungal pathogens and animal pests, plant viruses cause serious problems in sugar beet production, decreasing beet yield as well as beet quality. Sugar beet is susceptible to a number of different DNA and RNA viruses; they are vectored by nematodes, fungi or insects. Currently, about 80% of approximately 1000 recognised plant-infecting viruses possess RNA genomes e.g. *Benyviridae, Potyviridae* or *Virgaviridae*. The other 20% have a DNA genome and belong to the families of *Caulimoviridae*, *Geminiviridae* or *Nanoviridae* (Fauquet et al., 2005). Plant viruses are of economic importance as they can cause a high yield reduction of crops. Overall, estimated losses due to viral infections range between 6-7% worldwide (Oerke and Dehne, 2004). Viruses are plant pathogens which are not easy to control (Roossinck, 1997).

1.1 The disease complex of benyviruses in sugar beet

Sugar beet is subjected to different soil-borne viruses, which influence more or less the sugar yield. The most economically important sugar beet infecting virus is *Beet necrotic yellow vein virus* (BNYVV), the causative agent of rhizomania with worldwide distribution (Peltier et al., 2008). Chiba et al. (2011) hypothesised that BNYVV evolved in East Asia, because there, the greatest diversity of BNYVV isolates were found. Molecular analysis of BNYVV divided it into four distinct types: A-, B-, J- and P-type. The A-type is spread worldwide, whereas the B-type is so far limited to Central and Northern Europe, (Koenig and Lennefors, 2000). Both types consist of four RNA components and were classified into two groups based on their *CP*, *P25* and *P31* gene sequences (Schirmer et al., 2005). In contrast to this, the P- and J-type contain an additional fifth RNA component. Whereas the P-type is limited to a few sites in France, Kazakhstan and Great Britain (Harju et al., 2002; Koenig et al., 1997; Koenig and Lennefors, 2000), the J-type was detected in China and Japan. It is assumed, that the J-type was generated from a reassortment or recombination event, because it contains the *CP* gene of the B-type and other genes of the A-type (Li et al., 2008; Miyanishi et al., 1999). Schirmer et al. (2005) distinguished between P- and J-type due to sequence variability of RNA5. BNYVV is the causal agent of rhizomania, a disease which can cause yield losses of 70% and more in susceptible varieties (Peltier et al., 2008). In 1990; 15% of the sugar beet production area was BNYVV infected, 38% in 2000 and it was predicted that by 2010 56% will be infected (Richard-Molard and Cariolle, 2001). For Western Europe around 10% of sugar beet acreage was estimated to be infected in 1992 (Mannerlöf et al., 1996). The

occurrence of BNYVV is still increasing (McGrann et al., 2009), but actual estimations of the BNYVV infected area are lacking. BNYVV is controlled by growth of resistant sugar beet varieties (McGrann et al., 2009). A close relative of BNYVV is *Beet soil-borne mosaic virus* (BSBMV). The potential loss after infection with BSBMV is not yet determined, but would be justified (Heidel et al., 1997; Wisler et al., 2003), as described in the following sections. Besides BNYVV and BSBMV, other soil-borne sugar beet infecting viruses which are associated with rhizomania are *Beet soil-borne virus* (BSBV), *Beet virus Q* (BVQ) and beet oak-leaf virus (BOLV). All are transmitted by *Polymyxa betae* (*P. betae*). BSBV is distributed worldwide, restricted to the roots of sugar beet and either root or leaf symptoms are not obvious (Tamada and Asher, 2016). Therefore, speculations about the potential yield reduction exist. Koenig et al. (2000) reported about a yield reduction of up to 70%. BVQ is very similar to BSBV, but so far only found in several European countries and in Iran (Tamada and Asher, 2016). The virus was mostly detected together with BSBV or BNYVV (Meunier et al., 2003). The identity of BOLV still has to be determined. Liu and Lewellen (2008) described that it was first detected and only found in the U.S., *Rz1* and *Rz2* resistance genes do not confer resistance to BOLV and BOLV suppressed BNYVV in mixed infections. Moreover, information about the economic effect on sugar beet is limited (Liu and Lewellen, 2008). Another soil-borne sugar beet infecting virus is *Beet black scorch virus*. It was detected in sugar beets with rhizomania-like symptoms in which BNYVV remained undetected (González-Vázquez et al., 2009). The virus was first detected 2002 and is transmitted by *Olpidium brassicae* (Cao et al., 2002). The impact of an infection on sugar beet and its association with rhizomania are not clear (González-Vázquez et al., 2009).

In summary, BNYVV has the highest impact, but it can mostly be controlled by genetic resistance. The effects of the other mentioned soil-borne viruses are under evaluation, but it is speculated that they play a minor role and so far genetic resistances as a control measure are not known (Biancardi and Lewellen, 2016).

1.2 *Beet soil-borne mosaic virus* and *Beet necrotic yellow vein virus*

Both viruses are vectored by the soil-borne protist *Polymyxa betae* Keskin (a biotrophic plasmodiophoromycete) and their host range is limited to the family of *Amaranthaceae* (Heidel et al., 1997; Keskin, 1964). BSBMV was first detected 1988 in Texas, USA (Liu and Duffus, 1988). The scientists isolated BNYVV-like viruses from rhizomania infested fields in California and Texas. The isolates were serologically distinct, but morphologically similar to BNYVV. Initially, speculation emerged that BSBMV could possibly be a strain of BNYVV (Heidel and Rush, 1994). Wisler et al. (1996) reported that BSBMV isolates, because of their dissimilarities, represent a heterogeneous group which could be an indication that BSBMV might have originated in the United States. So far, BSBMV is restricted to the United States, but since 1992 found in nearly all sugar beet-growing areas of the U.S. (Colorado, California, Wyoming, Idaho, Minnesota, North Dakota and Nebraska)

(Rush and Heidel, 1995; Workneh et al., 2003). The widespread occurrence in the U.S. could be an indicator therefore that the virus has been around for a long time (Rush and Heidel, 1995). However, surveys carried out by Turina et al. (1996) in Italy and Borodynko et al. (2009) in Poland were negative for a BSBMV infection, indicating the absence of BSBMV infections in Europe. The name BSBMV was established 1993, prior it was called Texas 7 (Rush and Heidel, 1995; Wisler et al., 1994). In contrast to BSBMV, the first description of the disease rhizomania took place in Italy in 1952 (Canova, 1959). Ever since the virus is detected in numerous sugar beet-growing areas worldwide (Peltier et al., 2008). So far, BSBMV is less studied compared to BNYVV. Therefore, the following section describes both viruses, but with a particular focus on BSBMV.

1.2.1 Taxonomy and molecular biology

BSBMV is classified as a member of the genus *Benyvirus* family *Benyviridae* (International Committee on Taxonomy of Viruses). The genus *Benyvirus* was established 1997 and the family *Benyviridae* was accepted as a new family 2013 by ICTV (Gilmer et al., 2013; Rush 2003). In Colorado, Minnesota, Nebraska, Texas and Wyoming, 56 BSBMV isolates were recovered from sugar beet fields (Rush, 2003). These isolates were compared by Brewton et al. (1999) using single-stranded conformational polymorphism (SSCP) analysis. Results suggested genetic variability among BSBMV isolates. Similar to its closest relatives, BNYVV (type species of the family *Benyviridae*), *Rice stripe necrosis virus* and *Burdock mottle virus,* it possesses a multipartite single-stranded positive-sense RNA genome and is encapsidated in rigid rod shaped particles. Particles with a central core have a length of 50 to over 400nm and a width of 19nm (Heidel et al., 1997; Wisler et al., 1994). BSBMV consists of four polyadenylated, capped RNA segments. In 2001 Lee et al. published the complete nucleotide sequence of BSBMV. The genomic organisation of BSBMV (Fig.1) is identical to BNYVV and the predicted open reading frames (ORF) have an identity of 35% to 77% on nucleotide level and 23% to 92% on amino acid level (Tab.1). So far, only a functional characterisation of RNA3 and RNA4 of BSBMV is available (D'Alonzo et al., 2012; Ratti et al., 2009), but the high sequence identities of RNA1 and RNA2 between BSBMV and BNYVV suggest functional similarity (Tab.1) and a common evolutionary origin cannot be excluded (Lee et al., 2001). A preliminary consideration was that BSBMV might be a mild strain of BNYVV (Heidel and Rush, 1994). However, the molecular characterisation of the coat protein clearly showed a similarity of less than 90%, which resulted in the classification in a new species (Lee et al., 2001).

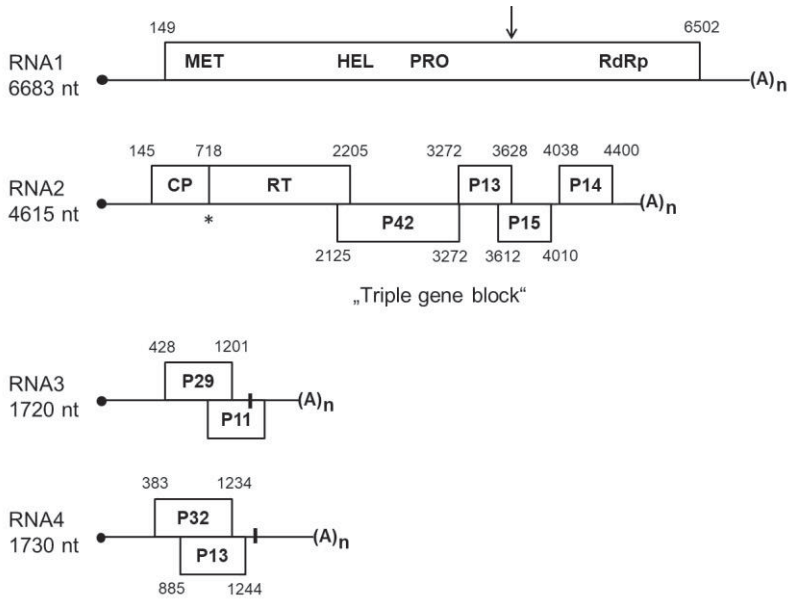

Fig. 1: Genome organisation of *Beet soil-borne mosaic virus* (BSBMV). BSBMV consists of four RNA segments, each segment possess a cap structure (filled circles) at the 5´end and a poly A-tail (A) at the 3´end. Rectangles display the open reading frames (ORF) in the genome. Inside each rectangle the names of the ORFs are indicated. Position in nucleotides (nt) of the start and stop codons are shown above the rectangles. The methyltransferase (MET), helicase (HEL) and papain-like protease (PRO) motifs and RNA-dependent RNA polymerase (RdRp) are indicated on the RNA1 (GenBank accession number AF280539). The arrow on RNA1 indicates the approximate location of the cleavage site for the polyprotein. On RNA2 (Acc. No. AF061869) an asterisk (∗) at nucleotide 718 represents the leaky UAG amber stop codon. Furthermore, RNA2 encodes the coat protein (CP), the readthrough protein (RT), the “Triple gene block” (P42, P13, P15) and P14. RNA3 (Acc. No. AF280540) encodes P29 and P11 and RNA4 (Acc. No. FJ424610) the proteins P32 and P13, respectively. The vertical line (|) represents the coremin motif, which is responsible for the long-distance movement in *Beta* species. (D’Alonzo et al., 2012; Gilmer et al., 2017; Lee et al., 2001; modified)

Table 1: BSBMV and BNYVV sequence comparison; Indicated is the total homology between BSBMV and BNYVV of the nucleotides of each RNA component as well as the weight in kilodalton (kDa) of each protein, open reading frames (ORF) annotation and percentage of ORF-homology on amino acid level for each viral protein (Lee et al., 2001, modified).

BSBMV / BNYVV	RNA1		RNA2						RNA3	RNA4
Homology	77		67						60	35
Protein weight	239 kDa		21 kDa	75 kDa	42 kDa	13 kDa	15 kDa	14 kDa	29 kDa	13 kDa
ORF-annotation	MET/HEL	RdRp	CP	RT	P42	P13	P15	P14	P29	P13
ORF-homology	80	92	56	56	74	81	65	32	23	42

Methyltransferase/Helicase (MET/HEL); RNA-dependent RNA polymerase (RdRp); Coat protein (CP); Readthrough protein (RT)

RNA1 and RNA2 carry genes with house-keeping functions. BSBMV RNA1 (6,683 nucleotides; Acc. No. AF280539) contains one ORF encoding a 239kDa polypeptide, which consists of the replication-associated enzymes: methyltransferase (MET), helicase (HEL) and RNA-dependent RNA polymerase (RdRp) (Lee et al., 2001). Moreover, it was speculated for BNYVV RNA1 that an autocatalytical cleavage site (papain-like protease) between HEL and RdRp, can cleave the polyprotein into two smaller proteins (Hehn et al., 1997).

BSBMV RNA2 (Acc. No. AF061869) is 4,615 nucleotides long and carries six ORFs, which are predicted to be involved in encapsidation, vector transmission, silencing suppression and movement. The first ORF at the 5'-end encodes a 21kDa coat protein (CP) and is terminated by a leaky UAG amber stop codon that permits expression of the 74kDa readthrough translation protein (RT). Typical CP motifs were identified for the BSBMV CP and a KTER-encoding domain was found in the RT region of BSBMV RNA2 (Lee et al., 2001). The KTER motif of BNYVV RNA2 is associated with the efficient transmission of the virus by *P. betae* (Tamada et al., 1996). Furthermore, the BNYVV RT is linked with virus assembly (Schmitt et al., 1992). The next three ORFs (P42, P13 and P15) represent the triple gene block (TGB). By a high number of viruses, of different genera, the TGB is responsible for cell-to-cell movement of the virus (Lee et al., 2001; Verchot-Lubicz et al., 2010). The last ORF, a 14 kDa cysteine-rich protein (P14), regulates RNA2 and CP accumulation and is associated with viral suppression of RNA silencing (VSR) (Chiba et al., 2013; Dunoyer et al., 2002).

RNA3 (1,720 nts; Acc. No. AF280540) of BSBMV encodes a 29 kDa protein (P29) that is involved in long-distance movement and symptom expression (Rush, 2003; Ratti et al., 2009). The function of the smaller ORF P11 on RNA3 is unknown (Gilmer et al., 2017). Ratti et al. (2009) demonstrated by heterologous complementation experiments that BSBMV RNA3 is affecting symptom expression on *Chenopodium quinoa* (*C. quinoa*). However, the sequence of P29 shows a higher homology to BNYVV RNA5 P26 as to BNYVV RNA3 P25. Rub-inoculation of BNYVV RNA1-RNA2 complemented with BSBMV RNA3 onto *C. quinoa* resulted in a phenotype similar to those when P26 was expressed; this indicates the closer relationship of BSBMV RNA3 to BNYVV RNA5 (Link et al., 2005; Ratti et al., 2009). BNYVV RNA3 encodes three proteins. The pathogenicity factor P25, is a highly variable protein between the amino acid position 67-70. Mutations within this tetrad are associated with resistance breaking abilities (Bornemann et al., 2015; Koenig et al., 2009; Schirmer et al., 2005). Moreover, P25 affects symptom expression in sugar beet roots and in the local lesion host *C. quinoa* (Commandeur et al., 1991; Jupin et al., 1992; Koenig et al., 1991; Tamada et al., 1989). Besides P25, BNYVV RNA3 encodes two smaller proteins N and P4.6 (Jupin et al., 1992). The function of P4.6 has not identified so far (Bouzoubaa et al., 1991). Whereas, N is associated with symptom expression on *Tetragonia expansa* (*T. expansa*) leaves (Jupin et al., 1992).

It was shown by heterologous expression that the P32 protein encoded by BSBMV RNA4 (1,730 nucleotides; Acc. No. FJ424610) is responsible for vector transmission by *P. betae* and influences the symptom expression in *C. quinoa* (D`Alonzo et al., 2012). Whereas, nothing is known about the putative P13, which is encoded by RNA4 (Gilmer et al., 2017). RNA4 and RNA3 of BSBMV can be transreplicated and encapsidated by BNYVV RNA1 and RNA2, complementing the corresponding functions in *trans* (D`Alonzo et al., 2012; Ratti et al., 2009). BNYVV RNA4 is 1,431 nucleotides long and encodes a 31kDa (P31) protein (Bouzoubaa et al., 1985). Like P32, P31 plays an important role in vector transmission (Tamada and Abe, 1989). Next to vector transmission is P31 also associated with a suppressor of gene silencing function (Rahim et al., 2007).
Additionally, some isolates of BNYVV (P- and J-type) containing a fifth RNA, which encodes one protein (P26) and varies in length. P26 may act in a synergistic manner with P25 and consequently enhance symptom development and symptom severity (Kiguchi et al., 1996; Koenig et al., 1997; Miyanishi et al., 1999).
Both small RNAs of BSBMV and BNYVV RNA3 and RNA5 contain a “core” nucleotide sequence, a so-called “coremin” motif, of about 20 nucleotides which are essential for systemic movement in *Beta* species (Lauber et al., 1998; Ratti et al., 2009). It was shown that the “coremin” motif stabilised the noncoding RNA3 (Peltier et al., 2012).

1.2.2 Symptom expression

In the field, sugar beets infected with BSBMV display leaf symptoms, whereas the sugar beet roots appear symptomless. In comparison, symptoms on sugar beet caused by BNYVV are distinguishable from those of BSBMV (Fig. 2). Excessive lateral root proliferation, brownish vascular bundles or wine-glass-like taproot are typical indications for a BNYVV infection. Sometimes, especially at the end of the growing season, foliar symptoms such as vein yellowing and necrosis can be observed. (Peltier et al., 2008). Rarely, root symptoms (stunting and proliferation of lateral roots) may occur due to a BSBMV infection and are comparable to those of BNYVV infected beets (Rush and Heidel, 1995). Even more, in greenhouse studies by using vortex-inoculation, BSBMV infected sugar beets had significant lower root weights as the control plants which indicates an effect on the beets. However, BSBMV infected sugar beets had a greater root weight than BNYVV infected plants (Heidel et al., 1997). In general, a high variability of leaf symptoms can be observed and is mainly influenced by environmental conditions, host plant, sugar beet cultivar and the BSBMV isolate (Rush and Heidel, 1995; Wisler et al., 1994). At the beginning of sugar beet infection, young leaves display greenish and yellowish spots that become necrotic over time. Likewise, a lightening of the veins is visible. Additionally, systemically infected sugar beet leaves display mottling or mosaic patterns and disordered growth. Sometimes systemic foliar symptoms can be similar to the yellow vein banding induced by BNYVV. In contrast, symptoms on *C. quinoa* are less variable and infection with BSBMV is mostly associated with diffuse, pale yellow

local lesions of the leaves (Rush and Heidel, 1995). However, Rush and Heidel (1995) observed that after repeated mechanical inoculations on *C. quinoa* with BSBMV, the symptom variability increased.

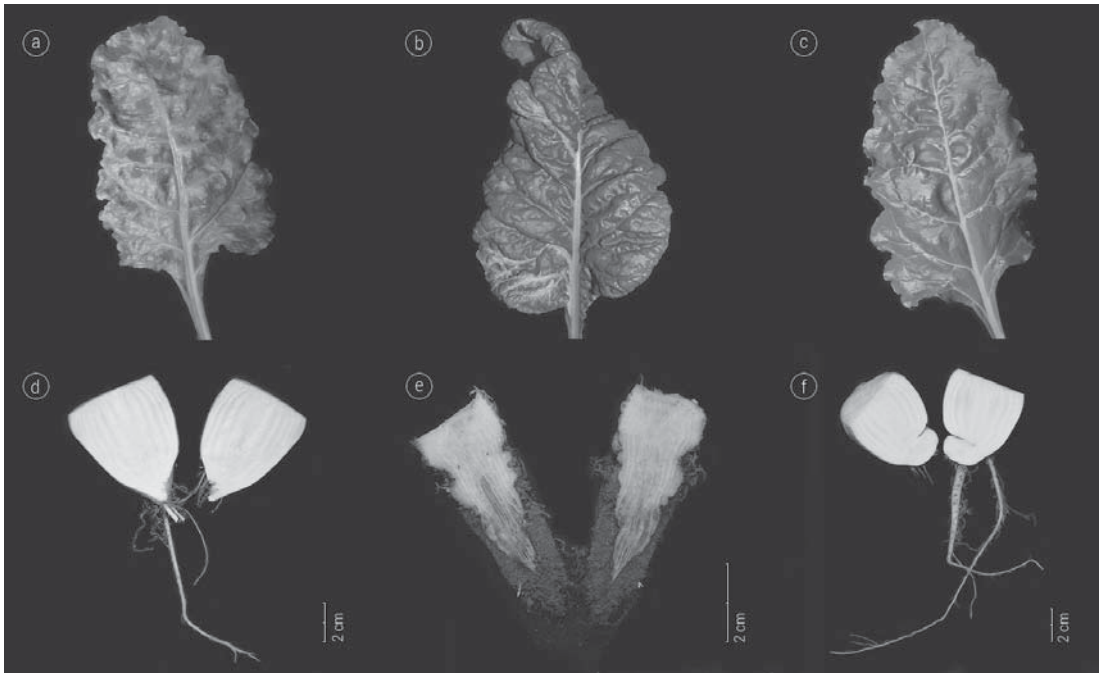

Fig. 2: Symptom expression on *Beta vulgaris* leaves (a-c, at 48 dpi) and taproots (d-f, at 84 dpi) produced after mechanical root vortex-inoculation with plant sap from *Chenopodium quinoa* local lesions infected with (a;d) wild-type BSBMV and (b;e) wild-type BNYVV compared to (c;d) healthy control. Bar represents 2 cm.

1.2.3 Economic importance and control measures of BSBMV and BNYVV in sugar beet

In contrast to BNYVV, which has a high economic importance, there is limited information available regarding the economic impact of a single BSBMV infestation in sugar beet. In infected fields, a significant reduction of fresh weight of seedlings was observed by Wisler et al. (2003). In 24 of 27 declining fields tested, BSBMV was detected without BNYVV (Wisler et al., 2003). This indicates that a negative impact on yield and sugar production is possible, but probably to a lesser degree than BNYVV (Heidel et al., 1997; Wisler et al., 2001). There is the suggestion that BSBMV has a lower virulence compared to BNYVV, but displays a higher genomic diversity (Heidel et al., 1997; Lee et al., 2001; Wisler et al., 2003).

Agronomic measures can be used to reduce the impact of a root infection through the vector *P. betae* (Tamada and Asher, 2016). The vector prefers soil temperatures around 12°C, therefore an early sowing and a rapid establishment of the plant canopy can reduce yield losses. A good soil structure and drainage is of importance, as wet conditions stimulate the release of zoospores and root infection. Furthermore, soil pH conditions and calcium content affect vector activity (Rush, 2003). No specific chemicals against *P. betae* are available and only soil fumigants can significantly reduce the pathogen (Harveson and Rush, 1994). However, due to environmental

concerns and economic considerations, a chemical control of the vector is not feasible (Draycott, 2008). Biological control measures as seed treatments with *Pseudomonas fluorescens*, *Trichoderma* spp. or *Streptomyxes* spp. to inhibit *P. betae* only have a limited efficiency (Grondona et al., 2001; Resca et al., 2001; Wang et al., 2003). Another problem are weed beets resulting from bolting beets, since they multiply the viruses in intervening crops (Draycott, 2008; Peltier et al., 2008). Additionally, infected soil particles can be distributed by wind, animals, water or agricultural machinery (Draycott, 2008). Avoiding agronomic management mistakes help to reduce distribution of the viruses. A reliable control measure to protect sugar beet production and to decrease the economic loss caused by viruses is the application of genetic resistance. So far, the cultivation of BNYVV resistant cultivars is the only way to maintain profitable sugar beet production in fields infected with BNYVV (McGrann et al., 2009). The use of BNYVV resistant varieties helps to minimise the yield losses, but not completely. In the mid-1980s the Holly and Rizor resistance were established, which are based on the *Rz1* resistance gene (Stevanato et al., 2015). Resistant plants show a reduction in virus accumulation and restricted translocation in the roots (Scholten et al., 1994), but the exact mechanism of BNYVV resistance remains unknown (Panella and Biancardi, 2016). Varieties carrying an *Rz1* resistance have been widley used (Biancardi et al., 2002), but nowadays an *Rz1* resistance-breaking ability of BNYVV A-type isolates due to specific mutations in the tetrad 67-70 of the viral pathogenicity factor P25 was reported (Bornemann et al., 2015). Over the years additional resistance genes (*Rz2-Rz5*) were discovered (Panella and Biancardi, 2016). The introduction of varities carrying double resistance (*Rz1+Rz2*) showed a phenotype of resistance in the presence of *Rz1* resistance-breaking strains (Gidner et al., 2005; Grimmer et al., 2008; Bornemann and Varrelmann, 2013). Capistrano-Gossmann et al. (2017) identified the *Rz2* gene in *Beta vulgaris* ssp. *maritima*, a crop wild relative of *B. vulgaris.* With a modified version of mapping-by-sequencing, they identified the candidate gene for *Rz2* and corroborated using RNA interference. *Rz2* encodes a protein, which contain a coiled-coil (CC) domain, a nucleotide-binding site (NB) domain, and a leucine-rich repeat (LLR). Due to yield penalty and highly variable level of resistance of combinations *Rz1* with *Rz3-Rz5*, respectively, these resistance genes have a minor importance (Gidner et al., 2005; McGrann et al., 2009). However, in contrast to BNYVV, no cultivars with resistance towards BSBMV are available (Wisler et al., 2003). *Rz1* gene that induced resistance to BNYVV, did not confer resistance to BSBMV, although a close phylogenetic relationship between the two viruses exist (Lee et al., 2001; Wisler et al., 2003).

1.2.4 Interaction between the two benyviruses BSBMV and BNYVV

In the United States, BSBMV and BNYVV occur in mixed infections, but information about their interaction is still limited and contradictory (Heidel et al., 1997; Wisler et al., 2003). During a survey in 1990-1991, Heidel and Rush (1994) found that BNYVV and BSBMV, alone or in combination, were more widespread throughout the U.S. as they initially thought. They detected BSBMV and

BNYVV more often together than each virus alone, but only in the beet. The foliar of the sugar beets were tested negative for a virus infection of BSBMV or/and BNYVV. Furthermore, they observed that BSBMV spreads more systemically in sugar beets as BNYVV. In contrast, Workneh et al. (2003) detected in samples from sugar beet fields BSBMV and BNYVV more often alone as together; 1-42% of the samples displayed mixed infections and both viruses had a similar spatial distribution within the field. Artificial mixed infection experiments showed that BSBMV interferes with BNYVV symptom expression. The BSBMV phenotype is more pronounced on *C. quinoa* and *Beta maritima* (Rush and Heidel, 1995). Whereas a greenhouse test conducted by Wisler and co-workers (2001) resulted in a faster BNYVV accumulation, compared to the BSBMV accumulation in sugar beets. Results of an enzyme-linked immunosorbent assay (ELISA) indicated that BNYVV suppressed BSBMV in sugar beets grown in naturally infested soils (Wisler et al., 2003). Moreover, they showed that the plant weight of BSBMV infected sugar beets was significantly lower compared to the healthy control. Interestingly, this effect was less pronounced when plants were mixed infected with BNYVV and BSBMV. They concluded that this might be caused by interference or competition between BSBMV and BNYVV in mixed infections and that BNYVV was able to out-compete or suppress BSBMV. Reciprocal cross protection between the two species has been observed under artificial conditions by mechanical inoculation of sugar beet seedlings; primarily BSBMV infected beets showed a lower BNYVV titer and were less diseased (Mahmood and Rush, 1999). Additionally, the authors demonstrated that cross-protection was most efficient by an interval of five to ten days between inoculations of the two viruses. Moreover, they indicated that the viral CP accumulation of the challenging virus was affected by cross-protection. According to Piccinni and Rush (2000) an infection with both viruses resulted in a higher root yield and a lower disease impact compared to a single BNYVV infection, but in a lower yield as a single BSBMV infection in a field experiment. Besides the field experiment, they also showed in a greenhouse experiment that virus infections have an effect on root dry weights and plant water use. Mixed infected sugar beets had a higher root dry weight and water use than BNYVV infected beets. Therefore, it was concluded that BSBMV reduced BNYVV symptom expression. These results are consistent with Mahmood and Rush (1999), but contradictory to Wisler et al. (2003).

An antagonistic effect of BSBMV and BNYVV in mixed infected plants cannot be ruled out (Mahmood and Rush, 1999; Rush and Heidel, 1995; Wisler et al., 2003; Workneh et al., 2003). A natural formation of reassortants between BSBMV and BNYVV is unknown (Rush, 2003). However, Ratti et al. (2009) and D'Alonzo et al (2012) showed under artificial conditions with infectious clones that a reassortment between BSBMV and BNYVV is possible. In competition experiments with BSBMV and BNYVV RNA3 in BNYVV background, the heterologous RNA3 was outcompeted and no competition between BNYVV RNA5 and RNA3s was observed. Reassortment of BNYVV RNA1-2 supplemented with BSBMV RNA3 resulted in long-distance movement in *Beta macrocarpa* (*B. macrocarpa*) (Ratti et al., 2009). In addition D'Alonzo et al. (2012) demonstrated in

B. vulgaris via agroinoculation that also RNA4 of BSBMV can be amplified by BNYVV RNA1+RNA2, complementing BNYVV RNA4 for virus transmission.

1.3 Mixed infection of RNA viruses

Mixed infection, infection of two or more viruses within a single plant, is a common phenomenon in the nature of plant viruses (Asaoka et al., 2010). A distinction of mixed infection has to be made between co-infection and super-infection. Co-infection is the infection of two or more viruses simultaneously or in a short interval of time, whereas super-infection is the invasion at different time points (Syller, 2012). Following mixed infection, different scenarios can occur and lead to a high variety of virus-virus interactions. Sometimes viruses can be detected in different cells or tissues of the host and do not interact at all, but there is the potential of a dual infection of host cells with more than one virus. In this case, the possibility exists that the viruses interact with each other (Roossinck, 2005).

1.3.1 Antagonistic interaction

In 1929, McKinney described the establishment of cross-protection (antagonistic interaction) in plants during virus infections, whereas a mild strain of a virus (protecting virus) can prevent the invasion of a more virulent strain (challenging virus). Cross-protection is more likely when the virus strains are more similar to each other and invade the plant at different time points (Roossinck, 2005). Furthermore, it is similar to the ‘vaccine’ concept in animals (Sarika et al., 2010), in which a biologically or genetically engineered antigen is used to stimulate the immune system. The competitive virus-virus interaction is also termed as ‘super-infection exclusion’ or ‘homologous interference’ and can be used as a protection mechanism in plants against viral diseases (Fulton, 1986; Syller, 2012). In practice, using the benefit of cross-protection the protecting virus has to be artificially inoculated as immunising agents, to protect the plant against the more virulent isolate. Under field conditions the system seems to be more or less practical (Syller, 2012). Over the years the phenomenon of super-infection was observed and studied on several plant +ssRNA-viruses like *Alfalfa mosaic virus* (AMV), *Barley yellow dwarf virus*, *Citrus tristeza virus* (CTV), *Plum pox virus*, *Potato virus A*, *Tobacco mosaic virus* (TMV), *Tobacco streak virus* or *Zucchini yellow mosaic virus* (Capote et al., 2006; Folimonova et al., 2010; Fulton, 1978; Hull and Plaskitt, 1970; Lecoq et al., 1991; Lee and Keremane, 2013; McKinney, 1929; Valkonen et al., 2002; Wen et al., 1991). In Taiwan an 82% higher papaya fruit yield could be achieved by controlling papaya ringspot disease through cross-protection (Wang et al., 1987). Also, CTV is widely used as protecting virus in citrus crops (Lee and Keremane, 2013). For greenhouse crops like tomatoes Fulton (1986) reported an increased yield in tomatoes between 1971-1973 due to cross-protection of 15% in the Netherlands, 6-9% in the UK and that in 1974, 70% of the cultivated Japanese tomatoes were inoculated with a protecting virus controlling tomato mosaic. Mahmood and Rush (1999) conducted a greenhouse

cross-protection study with BSBMV and BNYVV resulting in cross-protection when BSBMV was the protecting virus and BNYVV the challenging virus. Moreover, they showed that the degree of protection heavily depends on the inoculation interval. Because of the lack of an effective control management against rhizomania and especially the fact that some BNYVV isolates overcome the current traits of resistance, cross-protection is hypothesized to be useful for reducing the virulence of BNYVV. However, Fulton (1986) emphasised being cautious using the mechanism of cross-protection because the protecting virus might back-mutate to a more severe strain and spread to other non-infected hosts or might undergo a synergistic effect with other viruses. Interestingly, disease symptoms of the challenging virus can be suppressed and a resistance to super-infection of the host plant can occur (Ziebell and Carr, 2010). The authors Powell Abel et al. (1986), Ratcliff et al. (1999) and Sarika et al. (2010) assumed that a sequence-specific degradation, RNA silencing, a disassembly or an interference of replication of the challenging virus is initiated by the protecting virus. Furthermore, the antagonistic action is possibly generated by the competition for the same host factors after multiple infections of plant cells (Roossinck, 2005). Tatineni and French (2016) identified that viral proteins are responsible for super-infection exclusion. Besides super-infection exclusion, co-infection was attributed with a prevention of multiple infections with several related viral genomes and resulting in a separate distribution. Experiments on cellular level were performed by using fluorescent labelled full-length clones (Dietrich and Maiss, 2003; Gutiérrez et al., 2015; Julve et al., 2013; Takahashi et al., 2007; Tatineni and French 2016). Thus, the exclusion can be studied on cellular level and can be visualised by means of confocal laser scanning microscopy (CLSM). It should be mentioned; that co-infection results in a higher degree of fitness compared to a single-infection. Co-infections give more opportunities for competition and thus generate a higher level of viral fitness. Whereas super-infection did not increase the viral fitness level of RNA viruses, because the protecting virus has the advantage for exploiting the limiting resources and do not has to compete for them (Miralles et al., 2001). The exclusion mechanism indicates a spatial distribution of related viruses following mixed infection and generates a specific bottleneck (Roossinck, 2005). The exclusion mechanism seems to be strongly driven by the degree of relationship. As exemplified by Folimonova et al. (2010) super-infection exclusion of CTV occurred only between isolates of the same strain and not between isolates of different strains. As a result, spatial separation could lead to decreasing fitness of the population due to the reduction of competition and the lacking opportunity to displace unfit variants (Syller, 2012; Syller, 2014). Moreover, it results in a strong reduction of recombination and reassortment (Elena et al., 2011). This demonstrates that viral similarity seems to be essential for competition between viruses (Roossinck, 2005).

1.3.2 Facilitative interaction

A facilitative interaction (cooperation) of viruses during mixed infection is also possible. An interaction of viruses can be beneficial by sharing genetic information or gene products, the viruses are in symbiosis (Roossinck, 2005). Moreover, a mixed infection of a host plant can result in synergism. Synergism is mainly observed by unrelated viruses, but rarely reported for closely related viruses (Syller, 2012). Normally, due to synergistic interaction, at least one of the viral partners benefits by an increase in virus accumulation and symptom expression (Hull, 2009). As it was already described in 1955 for *Potato virus Y* (genus: *Potyvirus*) and *Potato virus X* (PVX; genus: *Potexvirus*) in tobacco plants, the interaction of the two increased the titre of PVX and disease symptoms compared with single infections (Rochow and Ross, 1955). Generally, potyvirus-associated synergistic interactions are the best studied synergism between plant viruses (García-Cano et al., 2006). Surprisingly, in doubly infected plants the potyvirus accumulation is almost not affected and the non-potyvirus titre increases (Calvert and Ghabrial, 1983; Goldberg and Brakke, 1987; Rochow and Ross, 1955; Vance, 1991). Wintermantel (2005) showed a triple synergism of three sugar beet infecting viruses (*Beet yellows virus*, *Beet western yellows virus* (BWYV) and *Beet mosaic virus*), which lead to an increased titer of all three viruses. Intriguingly, the mechanism seems to be host and host cultivar dependent, but mostly viral products and mechanisms involved remain unresolved (García-Cano et al., 2006; González-Jara et al., 2004; Tatineni et al., 2010; Wintermantel et al. 2008). Only a few hypotheses are described in the literature. A pathogenicity enhancer, which is encoded by the potyvirus *Tobacco etch virus*, transactivates replication of *Cucumber mosaic virus* (CMV) and TMV, respectively (Pruss et al. 1997). Other scientists described that the RNA silencing machinery is involved in the synergistic effect by decreasing the targeting of the other virus (Voinnet et al., 1999). Also virus-virus interactions in mixed infection could result in synergisms due to a complementation of a movement deficiency (Ryang et al., 2004; Savenkov and Valkonen, 2001). Another scenario called ‘helper dependence’ is defined as an event in which a virus promotes the transmission of another virus by vectors. It is well characterised for umbraviruses, which are not aphid transmissible without a co-infection of a virus from the family *Luteoviridae* (Syller, 2012). It should be emphasised that an enhancement of virus pathogenicity as a result of facilitative interaction can end up in unpredictable pathological consequences (Tatineni et al., 2010). Moreover, various growth parameters could be affected by viral synergism and could lead to plant death (García-Cano et al., 2006).

Overall, in most cases facilitative and antagonistic interactions between viruses in mixed infected plants have been partially studied and the mechanisms behind remain unknown (García-Cano et al., 2006; Syller, 2012).

1.4 Variation in RNA viruses

Plant viruses have disparate strategies to ensure variation in their genomes. RNA viruses are more susceptible to genetic variation than DNA viruses (Roossinck, 1997). Consequently, RNA viruses can adapt to environmental changes more rapidly, caused by their genetic variability (Castro et al., 2005). Thus, in the past two decades, nearly 50 new RNA viruses within the families of e.g. *Arenaviridae, Bunyaviridae, Filoviridae, Flaviviridae, Myxoviridae, Picornaviridae*, and *Retroviridae* have been detected (Domingo and Holland, 1997). Alterations in the environment and the genetic plasticity of RNA viruses favour the emergence of new viruses. Three basic mechanisms are associated with the enormous genetic variability, epidemiology, emergence and rapid evolution of viruses. A high mutation rate, recombination and reassortment (pseudorecombination) are responsible for diversity and the forces of virus adaptation to new niches or environmental challenges (Roossinck, 1997). Sometimes these mechanisms are closely linked but can also be independent (Asaoka et al., 2010). Short replication times, relatively small genome size and a high multiplicity of viruses favour a dynamic mutant population (Aaziz and Tepfer, 1999). Furthermore, a specific genetic structure within a definite environment known as a quasi species can be formed caused by the continuous subjection of a viral population to genetic variation, competition and selection (Domingo and Holland, 1997). The authors defined quasi species as “complex, dynamic distributions of nonidentical but related replicons”. It was speculated that BNYVV variants probably coexist as quasi species in the field and that this could be a reason for advantageous traits (Chiba et al., 2011). Beside the “quasi species theory”, another strategy that could probably explain genetic variation is the “bottleneck theory”. A viral genome can contain a mutation, which can consequently lead to a lower or higher fitness of the progeny. Mostly progeny with a lower fitness do not survive in the population. Progeny with higher fitness survive the bottleneck (a selection event), can multiply in the host, could replace the ancestor and a new virus population could emerge (Domingo and Holland, 1997; Zwart and Elena, 2015). A genetic bottleneck can occur at different stages of infection, but it is expected that it mainly occurs during early infection. Generally, bottlenecks could be disadvantageous due to narrow bottlenecks which reduce genetic variation in a virus population, but they could also be advantageous for viruses due to their effects on evolution by a stronger selection (Zwart and Elena, 2015).

1.4.1 Mutation

Mutation seems to be the major source of generating new virus species (Hull, 2009). It is estimated for RNA viruses that between 0.01 and 2 mutations per genome and generation are possible. Whereas the rate of spontaneous mutations for plant RNA viruses are less studied, they are estimated to occupying the lower side of the range (Tromas and Elena, 2010). Mutation, e.g. base substitutions, additions or deletions, within the viral genome is influenced by different factors like environmental stress (e.g. physical or chemical damage) or polymerase errors during elongation.

Especially RNA-dependent RNA polymerases (RdRp) are notoriously error-prone, thus RNA viruses are subjected to a higher mutation rate within their genomes as DNA viruses (Barr and Fearns, 2010; Choi, 2012). Little is known about the factors influencing the fidelity of viral RdRp, but it is often described as a low-fidelity enzyme (Castro et al., 2005). The main strategy to reduce mutation can be achieved by proofreading a 3'-5' exonuclease activity. The exonuclease recognises misincorporated nucleotides and removes the offending nucleotide. This proofreading-repair function is lacking in RdRps (Choi, 2012; Duffy et al., 2008; Elena and Sanjuán, 2005; Roossinck, 1997). Subsequently, RNA viruses mutate faster than DNA viruses, because DNA-dependent DNA polymerase (DdDp) has this proofreading capability. Moreover, as reviewed by Barr and Fearns (2010), the fidelity of RdRp can differ between RNA viruses. Consequently, RNA viruses with longer genomes probably have a greater fidelity (Barr and Fearns, 2010). Besides posttranscriptional mismatches and polymerase errors, short repeats in genes caused by replication slippage can introduce mutation (Hancock et al., 1995). Another common reason for mutation in DNA is due to oxidative damage, which is less studied for RNA (Rhee et al., 1995).

1.4.2 Recombination

The phenomenon of recombination is being recognised as an increasing event in viral biology and virus evolution. A phylogenetic survey of recombination frequency of +ssRNA plant viruses demonstrated that 12 of the 36 analysed RNA virus species displayed evidence of recombination (Takács et al. 2014). Recombination is defined as a formation of chimeric molecules within a single genomic segment from parental genomes of mixed origin (Simon-Loriere and Holmes, 2011). In the early 1960s Hirst (1962) and Ledinko (1963) detected the first RNA recombinations in poliovirus. RNA viruses have a high genetic variability due to their low fidelity RdRp, especially the ability for template-switching as well as replicase jumping supports the formation of recombinants (Castro et al., 2005; Cheng et al., 2002; Choi et al., 2016; Hancock et al., 1995). Template switching of the RdRp is the most widely accepted model of RNA recombination. Via breaking and joining of different parental sequences, RNA molecules with mixed ancestry can be generated too (Chetverin, 1999). A distinction is made between 'homologous RNA recombination' and 'non-homologous RNA recombination', whereas homologous RNA recombination occurs more frequently than non-homologous RNA recombination (Chetverin, 1999). Non-homologous is an exchange between genetically dissimilar genomic region and homologous recombination is an exchange between two closely similar parental sequences (Worobey and Holmes, 1999). RNA recombination probably has the function to repair mutation errors in essential viral genes (Aaziz and Tepfer, 1999). For example two partially deleted RNA3 variants of cowpea chlorotic mottle virus (CCMV) were repaired by homologous recombination, thus an intact RNA3 sequence was generated (Allison et al., 1990).

1.4.3 Reassortment

Reassortment (pseudorecombination) occurs naturally in plant viruses with segmented genomes (Domingo and Holland, 1997). It favours the creation of advantageous genotypes due to exchange of entire genomic segments between viruses (Simon-Loriere and Holmes, 2011). The number of potential reassortments increases with number of segments per virus and biological compatibility of the progeny (Briese et al., 2013). Iroegbu and Pringle (1981) and Rodriguez et al. (1998) demonstrated that compatible viral components promote the generation of reassortment between closely related viruses. Furthermore, it has been postulated by Fernández-Cuartero et al. (1994) that a recombination can occur within a reassortant virus. The first description of a naturally occurred reassortment was between two tobravirus isolates (Robinson et al., 1987). Initially it was thought that only closely related strains of virus species can form viable reassortants, but lately examples of reassortants between distinct viruses or virus strains have been described in the literature. For example reassortants of genetically distinct strains of the *Soil-borne wheat mosaic virus* or of CMV as well as reassortants between two distinct geminiviruses *Tomato mottle virus* and *Bean dwarf mosaic virus* or between *African cassava mosaic virus* and *Indian cassava mosaic virus* (Miyanishi et al., 2002; Phan et al., 2014; Frischmuth et al., 1993; Gilbertson et al., 1993). It was shown under articifal conditions for BSBMV, that RNA3 and RNA4 can be trans-replicated and trans-encapsidated by BNYVV and can complement the corresponding cognate RNA functions *in trans* (D'Alonzo et al., 2012; Ratti et al., 2009). So far, whether the BSBMV machinery can replicate and encapsidate BNYVV, has not been investigated.

Recombination and reassortment could only arise during a colonisation of the same cell and symbiosis between the parental viruses. Another requirement is that the specified RdRps recognise sequences within the untranslated regions (UTRs), which initiate the replication process (Gilmer et al., 1993). The important point is that all three mechanisms can introduce radical changes within the virus genome that may lead to highly infectious or pathogenic progeny viruses and facilitate the formation of new families or genera (Briese et al., 2013).

1.5 Reverse genetic system: Infectious cDNA full-length clones of RNA-viruses

Since the mid -1980s, reverse genetic approaches are widely used tools in the field of Plant Virology. Reverse genetic approaches work "backwards" analaysing the phenotypic consequences induced by modification of a target gene. In contrast to forward genetics, which starts with the selection of a biological process, the experimental procedure of reverse genetic begins from a protein or DNA for which no genetic information is known or is of particular interest (Alonso and Ecker, 2006). Thus, the aim is to characterise and to understand gene function, consequently various reverse genetic approaches have been developed (Alonso and Ecker, 2006; Hardy et al., 2010). For the development of a reverse genetic system to study RNA viruses, the construction of an infectious complementary DNA (cDNA) clone is a powerful tool. A genetically uniform source of

inoculum is provided by infectious cDNA clones. The RNA virus genome is maintained as a cDNA template in a bacterial plasmid, facilitating *in vitro* propagation, storage and genetic manipulation (Brewer et al., 2018). For a direct inoculation of plants, these plasmids can be used for the generation of *in vitro* transcripts or can be transformed into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) for agroinfiltration (syn. agroinoculation or agroinfection). First attempts to produce viral full-length clones were based on double-stranded DNA (dsDNA) viruses, because the DNA genome was much easier to manipulate as an RNA genome (Gronenborn et al., 1981; Nagyová and Šubr, 2007). Just a few years later, after commercial availability of enzymes for reverse transcription and *in vitro* transcription became standard, single-stranded RNA viruses became more important as viral full-length clones (Ahlquist et al., 1984; Peyret and Lomonossoff, 2015). The first examples of a successful application of infectious transcripts from cloned cDNA are *Brome mosaic virus* (BMV), CCMV, TMV and *Cowpea mosaic virus* (Ahlquist et al., 1984; Allison et al., 1988; Dawson et al., 1986; Meshi et al., 1986; Vos et al., 1988). In 1988 Ziegler-Graff et al. produced biologically active transcripts of BNYVV RNA3 and RNA4, whereas in 1989 Quillet et al. produced infectious transcripts of BNYVV RNA1 and RNA2. The production of full-length clones on the basis of RNA viruses became a widespread tool for virologist after the successful infection of a suitable host plant with an infectious cDNA clone of BMV.

1.5.1 Regulatory elements and construction of infectious cDNA full-length clones

The first step in the generation of an infectious cDNA clone from an RNA virus is the reverse transcription of the entire RNA sequence, followed by the amplification of the cDNA by polymerase chain reaction (PCR) and finally the cloning of the dsDNA into a suitable plasmid.

The success of the generation of such a clone depends on several factors: cDNA synthesis, cloning strategy, design of sequences bordering the viral insert, presence of nonviral nucleotides at the 5’-end and 3’-end and choice of vector (Boyer and Haenni, 1994). An exact 5’-end, without the presence of nonviral nucleotides, is generated due to promoters of the bacteriophages T7, T3 and SP6 or by the *Cauliflower mosaic virus* (CaMV) 35S promoter (Guilley et al., 1982; Odell et al., 1985). In concern of the applied promotor a distinction has to be made according the transcription place: *in vitro* versus *in vivo* transcription. The *in vitro* transcription is driven by promoters of the bacteriophages T7, T3 or SP6 under artificial conditions. Most *in vitro* transcripts are not infectious without a cap structure, because a protection of RNA degradation is lacking. Thus, a 5’-cap analog has to be added to the *in vitro* transcript (Yoon et al., 2002; Lee et al., 2011). The generated infectious transcripts can be transferred into host cells by different apporaches. In contrast cDNA clones under control of a CaMV promoter, are transcribed directly in the nucleus of the host cell. The *in vivo* transcription of infectious cDNA occurs in the cell nucleus (Nagyová and Šubr, 2007). The CaMV 35S promoter is a strong constitutive promoter without tissue specifity, which found the broadest application (Guilley et al., 1982; Nagyová and Šubr 2007). Both applications have their

advantages and disadvantages. An advantage of *in vitro* transcription is that the construct does not need to be introduced into the nucleus; however, the construct is very sensitive to enzymatic degradation (Nagyová and Šubr, 2007). Independence from the viral replication process and less RNA degradation are the advantages of *in vivo* transcription compared to *in vitro* transcription (Van Bokhoven et al., 1993, Boyer and Haenni, 1994). However, Gleba et al. (2007) reported about disadvantages such as ineffective transcription due to the transformation and export problems of the viral transcripts into the cytoplasm. Likewise, AU-rich sequences can be recognised as introns and consequently undergo the process of splicing in the nucleus (Nagyová and Šubr 2007). An exact termination at the 3'-end can be achieved e.g. by integration of a poly(A) signal and a ribozyme sequence. Essential for processing exact viral transcripts is e.g. the self-cleaving activity of the *Hepatitis delta virus* (HDV) ribozyme (Kapral et al., 2014). Turpen et al. (1993) observed that a ribozyme sequence instead of a transcription terminator sequence increased infectivity two-fold by a cDNA clone delivered via *Agrobacterium*. Thus, the integration of the viral cDNA under control of the CaMV 35S promotor and the ribozyme enables an exact synthesis of a viral full-length RNA. The first cDNA clone of a plant pathogenic RNA virus was BMV (Ahlquist and Janda, 1984; Ahlquist et al., 1984). A modified Λ bacteriophage promoter and a T7 bacteriophage promoter allowed for the first time RNA synthesis to initiate exactly at the 5'-end of each BMV sequence (Ahlquist et al., 1984; Ahlquist et al., 1987). In 1988, Yamaya et al. constructed a TMV clone, which was the first infectious clone under control of a CaMV 35S promoter. Moreover, to prevent loss of infectivity they modified the promoter in a way that the end coincides with the 5'-end of the virus RNA. Extensions on the 3'-end did not prevent, but decrease infectivity (Meshi et al., 1986; Yamaya et al., 1988).

Besides the choice of different regulatory elements, various cloning strategies are applied for the generation of infectious full-length cDNA clones. Since the discovery of ligase and restriction enzymes, multiple approaches of constructing full-length cDNA clones have been adapted. One of the first strategies was the approach of conventional/classical cloning by using DNA ligase and restriction endonucleases. For this, gene fragments of the known viral sequence are assembled *in vitro* and ligated into plasmids using restriction enzymes. The restriction-ligation assembly technique is time consuming and cannot assemble every sequence, because sequences of restriction enzymes used as recognition sites are prohibited within the target cDNA (Ellis et al., 2011). Later on, ligation independent strategies, which do not need specific sequences for ligation or site-specific recombination, have been developed. Aslanidis and de Jong (1990) reported about a method that does not require restriction enzymes or T4 DNA ligase (ligation-independent cloning; LIC). Primers for PCR amplification contain additionally a sequence of 12 nucleotides. Single-stranded ends are created due to T4 Polymerase in the presence of dGTP (insert) and dCTP (vector). The 5'-overhanging ends from the PCR product and the vector are complementary. Thus, a formation of dimeric circles is possible without the use of DNA ligase. Another strategy is

sequence and ligase independent cloning (SLIC), an improved method of LIC (Li and Elledge, 2007). Another approach is circular polymerase extension cloning (CPEC; Quan and Tian, 2009). Several inserts can be assembled with any vector using a single polymerase. There are overlapping sequences at both ends of the insert and the vector. After denaturation, annealing and hybridisation of the insert and the vector, they mutually use each other as templates to form a complete double-stranded plasmid. Recently, one-step isothermal reaction methods have been invented. Cooper (2014) used thermostable DNA polymerase and ligase in a one-step isothermal reaction to generate a TMV full-length clone. Another method, which resembles the overlap-extension assembly used by Cooper (2014), but includes the use of an exonuclease is Gibson assembly (GA). GA is a one-step isothermal *in-vitro* recombination method for assembling multiple overlapping DNA molecules by using three enzymes (Fig. 3).

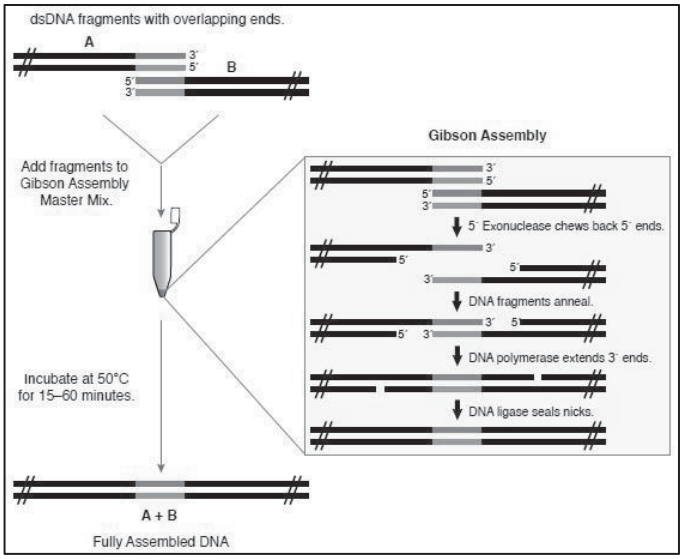

Fig. 3: Overview of the one-step isothermal in-vitro recombination approach according to Gibson et al., (2009). Two adjacent DNA fragments (A and B) with the same terminal sequence overlaps (coloured) are assembled in a one-step isothermal reaction. T5 exonuclease removes from the 5'-ends of the linear double-stranded DNA nucleotides to form overhanging 3'-ends. The overhangs hybridise in the next step before a DNA polymerase extends 3'-ends and a Taq (bacterium: *Thermus aquaticus*) DNA ligase seals the nicks. The final product of the Gibson assembly is a complete dsDNA (Figure from Gibson Assembly ® Master Mix - Instruction Manual Page 2, New England BioLabs).

Firstly, a T5 exonuclease removes nucleotides at the 5'-end of each DNA fragment to create single-stranded 3'-end overhangs. The single-stranded DNA (ssDNA) overhangs anneal specifically and the thermostable DNA polymerase extends the 3'-ends. Lastly, a thermostable DNA ligase seals the nicks (Gibson et al., 2009). Due to complementary ends more than one fragment can be assembled in one reaction. Blawid and Nagata (2015) applied the approach of GA for the first time for the generation of an infectious full-length clone of tomato blistering mosaic virus. A second report followed by Bordat et al. (2015), they constructed an infectious full-length

clone of lettuce mosaic virus using GA. The authors speculated that the efficiency of annealing the single overlapping fragments during GA is depending on the choice of primers which create the overlapping regions. During the reaction, there is a risk that secondary structures will be formed, which may hinder the reaction. However, today various commercial kits (GATEWAY (Invitrogen, USA); In-Fusion® HD Cloning kit (Clontech, USA) or GeneArt Seamless Cloning and Assembly kit (Life technologies, USA) have replaced traditional construction methods of infectious full-length cDNA clones (Tuo et al., 2015).

Bacterial plasmids can serve as vector for the full-length cDNA. The viral genomes are cloned in plasmids for multiplication of obtained constructs in a preparative scale. Toxicity of particular viral sequences for the bacteria can lead to instability of cloned cDNA in *Escherichia coli* (Miyanishi et al., 2002). The reason behind is not understood, but it can maybe eliminated by the substitution of *E. coli* strain or cloning vector (Boyer and Haenni, 1994). Many useful and versatile vectors have been constructed. The most commonly used transformation vectors are binary vectors which have the ability to replicate in *E.coli* and *Agrobacterium tumefaciens* (*A. tumefaciens*) (Xiang et al., 1999). Binary vectors allow efficient infection of dicotyledonous plants by agroinfiltration and have been continually improved (Komori et al., 2007; Peyret and Lomonossoff, 2015). First binary vectors for plant transformation were built by Hoekema et al. (1983) and Bevan (1984), who manipulated the tumor-inducing (Ti) plasmid of *A. tumefaciens*, resulting in a disarmed strain. The natural DNA transfer systems of *Agrobacterium* has been exploited through genetic engineering by replacing the native transfer DNA (T-DNA), which is responsible for the pathogenic phenotype, by any DNA sequence and consequently transferred a modified T-DNA region into the plant genome (Bevan, 1984; Murai et al., 2013). Normally, “armed” *A. tumefaciens* induces tumorous growths or crown galls on dicotyledonous plants (Bevan, 1984). Decisive is the fact that the T-DNA and the *vir* region can reside on separate plasmids. The T-DNA is provided on the vector and the *vir* gene functions by the disarmed *Agrobacterium* strain (Hellens et al., 2000). Besides the artificial T-DNA, which has to be delimited by the right border (RB) and the left border (LB) sequences, a binary vector consists of a vector backbone. The vector backbone consists of a replicon for propagation in *Agrobacterium* and *E. coli* and as selectable marker an antibiotic resistance gene for the bacteria. Additionally to regulatory elements, the T-DNA region may contain multiple cloning sites, a reporter gene (e.g. fluorescent protein), selectable marker gene for plants and other genes of interest (Komori et al., 2007). Besides binary vectors, so called “super binary vector” exist. They carry extra copies of the *virB, virC* and *virG* genes which have the ability to increase the virulence of strains of *A. tumefaciens* (Frame et al., 2002; Komari, 1990). After the full-length cDNA has been successfully cloned into a vector and propagated in competent *E. coli* cells, the recombinant plasmids are used to transform competent *A. tumefaciens* cells by electroporation for artificial plant infection.

1.5.2 Inoculation techniques and application of infectious cDNA full-length clones

To infect host plants, RNA transcripts from full-length cDNA clones are produced *in vitro* or *in vivo*. Infection testing of transformed agrobacterium can be performed with little effort by means of agroinfiltration methods (Yoon et al., 2002; Nagyová and Šubr, 2007). This is the most common and important infection method, which was invented 1986 by Grimsley and co-workers. For the first time Leiser et al. (1992) demonstrated that agroinfection is applicable for single-stranded RNA (ssRNA) viruses, too. It is mainly used for viruses, which are difficult to transfer mechanically and also suitable for insect transmitted viruses. In the case of agroinoculation, plants are inoculated with viral genomes by using disarmed agrobacterium strains. For this plant transformation methodology two bacteria, *Agrobacterium rhizogenes* (*A. rhizogenes*) and *A. tumefaciens*, are described in the literature (Mankin et al., 2007). *A. rhizogenes* is inducing a hairy-root (rhizogenic) reaction in host plants (Mankin et al., 2007; Young et al., 2001). The plant pathogen *A. tumefaciens* can naturally transfer its DNA to 90 families of dicotyledonous plants, causing disease in over 600 species (Zupan et al., 2002). The infection mechanism is similar for both and is described using the example of *A. tumefaciens.* The T-DNA is located on the Ti plasmid of *A. tumefaciens* and enters the plant cell during infection. It is transferred to the plant genome via the bacterial *virulence* machinery and followed by integration into the plant genome. The plant cell is manipulated for the benefit of the agrobacterium (Bevan, 1984; Grimsley et al., 1986; Leiser et al., 1992). The induction of the *vir* genes (virulence region of the Ti plasmid) is the basis for a successful T-DNA transfer and integration. This procedure is probably dependent on the activation of phenolic substances such as wound signals or acetosyringone (Alt-Moerbe et al., 1988; Alt-Moerbe et al., 1989; Stachel et al., 1985). For a successful expression of the viral proteins, it is important that the cDNA is transferred into the cell nuclei for transcription into an mRNA and that the resulting mRNA is transported into the cytoplasm for translation (Nagyová und Šubr, 2007). It cannot be excluded that an agroinfection is also possible by transcription of T-DNA and by synthesis of viral intermediates before integration into the plant genome (Grimsley, 1995). After infection of the agrobacterium, virus genome can enter the host organism and the viral life cycle can begin. Additionally, variations of agroinoculation methods find application as macroinjection into the plant stem, leaf infiltration with a needleless syringe, through artificial created wounds (Nagyová und Šubr, 2007), sonication-assisted *Agrobacterium*-mediated transformation (SAAT) (Bakshi et al., 2011; Beranová et al., 2008), vacuum-infiltration with *Agrobacterium* suspension (Kapila et al., 1997), picking and stabbing the colonies with a toothpick into the leaf (Lu et al., 2003a), spraying with *A. tumefaciens* suspensions (Liu et al., 2002) or vortex-inoculation of plant roots with *A. tumefaciens* suspensions (Delbianco et al., 2013). A novel method is agroinoculation by drenching the soil adjacent to the plant root (Ryu et al., 2004) and “root absorption” by dipping the root into an *A. tumefaciens* solution containing the viral vector (Yang et al., 2008).

To bypass problems associated with *A. tumefaciens*, e.g. to infect monocotyledonous plants, a plant infection using *in vitro* transcripts are an alternative. Klein et al. (1987) used high-velocity microprojectiles to deliver nucleic acids of TMV into living plant cells of *Allium cepa*. By this mechanical method of biolistic transformation foreign DNA/RNA is bounded to colloidal gold or tungsten particles and delivered into the plant tissue with a gene gun. A physical transfection method is the microinjection, in which the desired nucleic acid is injected directly into an isolated cell (protoplast), embryo or meristem culture using a fine pipette (Nagyová und Šubr, 2007; Schnorf et al., 1991). Schnorf et al. (1991) used this approach to regenerated kanamycin resistant plants or Angell and Baulcombe (1995) to study the replication and cell-to-cell movement of PVX in protoplasts. A further transfection method is electroporation. By using short, high-voltage electrical fields, cell membranes become temporarily partially permeable for the recombinant nucleic acid (Van Wert and Saunders, 1992). Another route of transfection is the mechanical inoculation of *in vitro* transcripts to celite-dusted leaves as performed by Quillet et al. (1989) or D'Alonzo et al. (2012).

Infectious full-length cDNA clones have a wide range of applications in Plant Virology. Thus, plant viruses are an important research tool due to the ability to replicate and manipulate plant viral genomes in the form of cDNA clones. Infectious clones provide the possibility for disease resistance screening of different host genotypes (Brewer et al., 2018). For example Kuria et al. (2017) used infectious clones of cassava mosaic virus to screen cassava germplasm for resistance, because the virus is a major threat in Africa. An infectious clone of tomato chlorotic mottle virus was used to analyse the resistant mechanism of the host plant after viral infection (Carmo et al., 2017). By protein analysis the authors could develop a hypothetical model that shows how a resistant host cell responds to an infection. Thus, the clones could contribute to the understanding of resistance mechanism and of host response at a molecular level. In addition, prior inoculation of the clones the viral genome can be modified. By genomic modifications by induced targeted *in-vitro* mutagenesis (deletions, insertions or substitutions), and the formation of recombinants or reassortants (pseudorecombinants), it is possible to gain a deeper insight into virus genome organisation and genetic expression, replication as well as functions of viral proteins and of the UTR (Boyer and Haenni, 1994; Nagyová and Šubr, 2007). Ziegler-Graff et al. (1988) showed for example that extraneous 5' non-viral nucleotide sequences can diminish the biological activity of BNYVV RNA3 transcripts. Whereas, Quillet et al. (1989) discovered by using *in vitro* transcripts of BNYVV, that a coat protein mutation influenced the RNA packaging and interfered with long distance movement in spinach. Bleykasten-Grosshans et al. (1997) and Lauber et al. (2001) used *in vitro* transcripts of BNYVV and a replicon derived from BNYVV RNA3 to analyse the functions of TGB in the local lesion host *C. quinoa*. Moreover, Lauber et al. (2001) introduced point mutations in P13 and P15 and used an elaborate "replicon screening" approach to identify mutants, which could confer resistance against BNYVV in transgenic sugar beets. Deletion

mutants of BNYVV RNA2 *in vitro* transcripts were generated by Schmitt et al. (1992) to demonstrate the effect of the 75kDa readthrough protein on virus accumulation. Recently, Harper et al. (2016) studied the effect of substituting genes (P65 and P61) of CTV isolates on the efficiency of transmission by the brown citrus aphid. The introduction of two specific mutations into the coat protein of pepino mosaic virus clones conferred resistance breaking abilities in *Solanum lycopersicum* (Duff-Farrier et al., 2015). However, Martin and Rybicki (2002) used a highly pathogenic maize streak virus isolate and less pathogenic isolates to identify pathogenicity determinants by testing individual genome constituents. Ratti et al. (2009) and D'Alonzo et al. (2012) demonstrated by formation of reassortants (BNYVV/BSBMV) the function of BSBMV P29 and P32. Furthermore, infectious cDNA clones are the basis for the creation of a viral vector (Boyer and Haenni, 1994; Nagyová and Šubr, 2007). For example track viral movement, to study function of specific viral proteins and its interaction with host proteins the viral genomes can be labelled with fluorescent markers (see 1.6.1). Additionally, viral vectors can be used to trigger silencing of host genes (see 1.6.2).

1.6 Plant virus-based vectors

Viral genomes are an effective tool to express foreign nucleic acid sequences in plants and therefore viral vectors can help to understand virus biology and the antiviral defence mechanisms in plants. Viral vectors can be used as tools for gene discovery or for the production of recombinant proteins (Hammond, 2005). Moreover, the fact that viruses manipulate the biosynthetic apparatus of their hosts for their own replication and multiply within a short time make them suitable as research tool (Nagyová and Šubr, 2007). Pogue et al. (2002) reviewed that "the ability of plant viruses to produce recombinant proteins rapidly and inexpensively opens the door for new agricultural applications". Before RNA viruses can be used as viral vectors, infectious cDNA full-length clones have to be generated for manipulation (Hammond, 2005). As described above (1.5.1) the generation of infectious cDNA clones depends on several factors and can prevent the use as expression vector. Likewise, the size of the insert as well as its complexity and instability can be a limiting factor of viral vectors and may lead to the loss of infectivity (Hammond, 2005; Peyret and Lomonossoff, 2015). It is necessary to find a suitable cloning site within the viral genome where any nucleic acid sequences can be stably integrated, does not adversely affect virus replication and is successfully expressed in the host plant (Nagyová and Šubr, 2007). The first plant viral vectors were derived from CaMV, a virus with a dsDNA that replicates through an RNA intermediate (Brisson et al., 1984; Gronenborn et al., 1981). The authors replaced non-essential viral genes by different foreign genes, which were successfully integrated and expressed in the host plant. Furthermore, the use of Geminiviruses (ssDNA genome), whereby the non-essential coat protein was easily manipulated, showed some difficulties. They have a limited host range, undergo rapid deletion and the integrated foreign sequence should not exceed the size

of the coat protein. Otherwise, the virus does not spread systemically in the infected host anymore (Hayes et al., 1989). Aronson et al. (2002) demonstrated that nanoviruses (ssDNA) are suitable as viral vectors too, but they have a limited host range and infect mainly legumes. As described, DNA viruses do not seem to have the same potential as viral vectors due to their size constraints movement compared to RNA viruses. First viral vectors based on RNA were derived from BMV, after Ahlquist et al. generated 1984 the first infectious cDNA clone of the RNA virus (French et al., 1986). Nowadays, TRV-derived verctors are widely used. Different strategies have been used to express a gene of interest. Mostly, foreign genes are expressed by duplicating the subgenomic (sg) RNA promoter, by replacement of non-essential viral genes or cloning in frame within a viral ORF (Hammond, 2005; Pogue et al., 2002). Despite their utility, all viruses have their own limitations, gene expression strategies and advantages (Lindbo et al., 2001; Pogue et al., 2002).

1.6.1 Fluorescence labelling of viral vectors

Viral vectors can be used as expression vectors for fluorescent markers to study protein localisation, transport pathways, protein-protein interaction, virus-virus interaction and tracking gene expression in infected plants (Brandizzi et al., 2002). The first discovered fluorescent protein was the green fluorescent protein (GFP), which was cloned from the jellyfish *Aequorea victoria* (Chalfie, 1995). The second one was DsRed, a red fluorescent protein isolated from *Discosoma* sp. (Held et al., 2008). Both are molecular markers, finding wide use in observing the distribution of viruses directly in living cells and tissue (Ckurshumova et al., 2011; Dietrich and Maiss, 2002; Thiel et al., 2012). Over the years the markers have been optimized to enhance various properties (e.g. enhanced solubility, accelerated chromophore formation) and spectral variants have been developed (Brandizzi et al., 2002). Hence, in the last 25 years several variants of fluorescent markers covering nearly the entire visible spectrum were established (Held et al., 2008). For instance, Campbell et al. (2002) constructed a monomer of DsRed (monomeric red fluorescent protein mRFP), which is described as having faster maturation, does not built aggregates and has beneficial excitation and emission peaks compared to the original DsRed. Due to its spectral properties mRFP is ideal for dual colouring in combination with GFP (Campbell et al., 2002). Shaner et al. (2004) created derivatives from mRFP like mCherry, mOrange or mBanana, which improved the availability of different colours (red, orange, yellow). Fluorescent tagging with wildtype GFP resulted in a low fluorescence yield in plants, because of the cytotoxicity of GFP aggregates (Crameri et al., 1996). Therefore, a large collection of GFP derivatives has been generated (Davis and Vierstra, 1998). For example, GFP was modified by Davis and Vierstra (1998) into a more soluble and brighter version called soluble-modified GFP (smGFP). Moreover, the authors used the smGFP template to create a soluble-modified red-shifted GFP (smRS-GFP) and a soluble-modified blue-fluorescent protein (smBFP) with the benefit of distinct spectral properties. Multiple fluorescent images can be recorded with CLSM. It should be noted that for

faithful multi-imaging with CLSM, the fluorescent proteins should have an appropriate combination of excitation and emission wavelengths (Brandizzi et al., 2002). Optical imaging based on CLSM provides advantages by visualising proteins and organelles in living cells viewing separate overlapping spectra and minimizing plant autofluorescence (Held et al., 2008). For instance, Dietrich and Maiss (2003) characterised the distribution of different fluorescent labelled potyviral populations, as well as in mixed infection with a fluorescent labelled potexvirus. Takahashi et al. (2007) labelled *Apple latent spherical virus* (ALSV) to study the distribution of identical virus populations and for mixed infection studies with ALSV they used a labelled *Bean yellow mosaic virus* (BYMV). Fluorescent protein-tagged CTV was used to investigate the function of p33 in the mechanism of super-infection exclusion (Bergua et al., 2014). Remarkably, Delbianco et al. (2013) used a BNYVV RNA5 based replicon expressing GFP to demonstrated efficient expression of BNYVV RNA1-4 agroclones in *Nicotiana benthamiana* (*N. benthamiana*) and *B. macrocarpa*. However, there exists a potential risk that fluorescent labelling could lead to a misfolding, loss or alteration of function or masking of specific signals of target proteins (Brandizzi et al., 2002). Furthermore, the fusion or insertion of fluorescent proteins can result in a misguiding to other cellular compartments compared to the non-tagged wild-type (Brandizzi et al., 2002).

1.6.2 Mechanism of virus-induced gene silencing (VIGS)

Virus-induced gene silencing (VIGS) is a tool to study plant gene functions by applying virus-based vectors for posttranscriptional gene silencing (PTGS). PTGS relies on a natural sequence-specific RNA degradation activity and is an important mechanism in plant defence against pathogens (Lu et al., 2003). It protects the genome by silencing transposons, repetitive elements and sources of aberrant transcripts and by suppressing invading viruses (Voinnet, 2001; Becker and Lange, 2010). The silencing mechanism is triggered by the double-stranded RNA (dsRNA), a special conserved molecule of the pathogen. Especially in plants this mechanism can acts on RNA and DNA levels (Fátyol et al., 2015). VIGS exploits this mechanism of RNA-mediated antiviral defence (Baulcombe, 1999).

During replication the viral RdRp produces dsRNA from the ssRNA viral transcripts. Thus, RNA viruses replicate through dsRNA replicative intermediates, which are a key activator of the innate immune response against viral infection (Weber et al., 2006; Becker and Lange, 2010). Subsequently, the dsRNA is recognised, bound and cut by Dicer-like enzymes into 21-25nt long double-stranded small interfering RNAs (siRNA) (Ramegowda et al., 2014). Dicer-like enzymes are endoribonucleases which are analogous to RNase III-like enzymes (Kalantidis et al., 2008; Melnyk et al., 2011). The main executors of antiviral defence are the Argonaute proteins, which are highly specialised binding modules. They are the direct binding partners of the siRNAs and activate RNA-induced silencing complex (RISC) (Meister, 2013). The siRNA associates and is separated into a single-strand by the RISC. RISC binds to the specific single-stranded mRNA transcripts,

which is complementary to the siRNA (Ramegowda et al., 2014). This procedure is responsible for the cleavage and blocking of translation of the target mRNA. It leads to a repression of genes which contain sequences identical or highly similar to the initiate dsRNA. The virus-derived silencing signal can move from cell to cell through plasmodesmata and also systemically spread through the phloem system of the plant (Becker and Lange, 2010; Kalantidis et al., 2008). This long-range silencing signal is probably based on RNA molecules (24nt long siRNAs), which have to exceed a specific threshold level for a systemic spread (Kalantidis et al., 2008; Melnyk et al., 2011). Whereas the short-range silencing signal is associated with 21nt long siRNAs (Kalantidis et al., 2008).

Generally, silencing efficiency can be influenced by different factors like shown by Smith et al. (2000). They demonstrated for PVY that PTGS was nearly 100% efficiency if the gene constructs encoding intron-spliced RNA with a hairpin structure, 7% efficiency with a sense and 4% efficiency with an antisense gene construct, respectively. Furthermore, silencing efficiency is influence by sequence lengths (from 23nt up to 1.5kb), identity to the target sequence and thermodynamic properties of the siRNA (Burch-Smith et al., 2004). PTGS can silence any transcript with 23nt identity to the targeted sequence (Burch-Smith et al., 2004; Robertson 2004). However, partial sequence identity of the siRNA to a non-target mRNA can also result in off-target silencing (Senthil-Kumar and Mysore, 2011). Furthermore, the virus-host combinations and interaction influence the extent of silencing and severity of viral symptoms (Robertson, 2004). Replication cycle of the virus and environmental conditions, especially temperature may negatively affect the efficiency of VIGS (Burch-Smith et al., 2004; Senthil-Kumar and Mysore, 2011). Additionally, Pignatta et al. (2007) showed that the sequence of the insert might influence the efficiency and stability of silencing, too. Viruses like BSBMV, BNYVV, TRV and TMV encode viral suppressors of RNA silencing (VSR), which can affect the silencing efficiency by downregulatiing DICER activity and as a consequence the formation primary siRNAs (Chiba et al., 2013; Zhang et al., 2005). A further disadvantage is the duration and appearance of the silencing effect, which is species-specific and mostly remains transient (Becker and Lange, 2010). Limitations of the VIGS-technology could be the alteration of the physiology of the plant due to the infection of a viral vector, a not homogenous silencing effect, varying levels of silencing between single plants and experiments (Burch-Smith et al., 2004). However, through VIGS a loss-of-function genotype can be generate within 3-4 weeks after inoculation. Moreover, it avoids plant transformation and genotype-specific effects. Even a rapid comparison of gene function between species is possible (Burch-Smith et al., 2004; Lu et al., 2003a). VIGS has the advantage to silence several distinct genes by using vectors harbouring two different target gene fragments as demonstrated by Peele et al. (2001) and Turnage et al. (2002). This allows to study and to link a gene of unknown function with a gene, which has a visible and predictable phenotype after silencing.

1.6.2.1 Application of VIGS

The described silencing mechanism can be targeted against endogenous plant genes, by using standard binary Ti-plasmid derived viral vectors carrying elements derived from plant host genes (Lu et al., 2003). Therefore, short homologous gene fragments of a targeted plant gene are cloned into a viral vector. After artificial virus introduction into a host cell and triggering the plant defence machinery, a silencing effect is expected. Here, agroinfiltration is the most commonly used inoculation technique and suitable for high throughput (Robertson, 2004). Until now VIGS is mostly applied in *N. benthamiana*, but further plant species for VIGS application increase with the development of new virus vectors (Lu et al., 2003; Becker and Lange, 2010; Wang et al., 2016). Over the years VIGS vectors based on e.g. TRV, PVX, ALSV or CMV were generated (Ramegowda et al., 2014; Kawai et al., 2016; Wang et al., 2016). Kumagai et al., (1995) demonstrated for the first time that an RNA virus (TMV) can be applied as silencing vector. The authors used a part of the sequence from the *phytoene desaturase* (*pds*) to demonstrate silencing effect in *N. benthamiana*. Basically, TRV-based VIGS vectors are widely used, because of their advantages to infect a wide host range, produce mild virus symptoms in infected plants and spread systemically throughout the entire plant including meristematic tissue (Ramegowda et al., 2014; Ratcliff et al., 2001; Yang et al., 2008). TRV was applied e.g. in *Petunia hybrida, Gerbera hybrida, Solanum lycopersicum* and several more (Broderick and Jones, 2014; Deng et al., 2012; Ekengren et al., 2003). So far, VIGS based on a sugar beet infecting RNA virus has not been reported for sugar beet (family of *Amaranthaceae*). Golenberg et al. (2009) developed a silencing vector derived from the geminivirus *Beet curly top virus* (DNA virus) targeting the ribulose bisphosphate carboxylase and transketolase in *S. oleracea* (belonging to the family of *Amaranthaceae*), but no experiment in sugar beet was conducted. In 2012 and 2015 Hatlestad and co-workers, developed a silencing system based on a GATEWAY (Invitrogen,USA) vector *pTRV2-Gateway* (Karimi et al., 2002; Liu et al., 2002) for *Agrobacterium*-mediated transformation to silence successfully the gene encoding cytochrome P450 and BvMYB1 protein to study the red betalain pathway in beets. In contrast to the availability of approximately 37 VIGS vectors in dicotyledonous plants, only a few VIGS vectors in monocotyledonous are available (Wang et al., 2016). Holzberg at al. (2002) used a barley stripe mosaic virus (BSMV) vector to induce for the first time silencing in the monocot host barley. Wang et al. (2016) developed a VIGS vector, which efficiently silenced maize genes in a short time.

The variability of silencing efficiency can be sorted out when using target genes, which induce a predicted visual phenotype (Robertson, 2004). As reliable visual markers glutamate 1-semialdehyde aminotransferase (GSA), PDS, magnesium chelatase subunit I (ChlI), magnesium chelatase subunit H (ChlH) and phosphoribosylanthranilate isomerase (PAI) find application to study the silencing dynamics of endogenous genes (Robertson, 2004). Silencing of the endogenous *pds* gene, which causes photobleaching of the silenced regions, is a common positive

control for VIGS efficiency. The *pds* gene plays a key role in the carotenoid biosynthetic pathway and catalyses the conversion of phytoene to lycopene. Carotinoides are essential of the photosynthetic machinery and especially protect chlorophyll molecules. Consequently, a decline of endogenous PDS mRNA leads to photooxidative damage and therefore to photobleaching (Kumagai et al., 1995; Lopez et al., 2008; Ruiz et al., 1998). Another widely used VIGS control is the multicomponent enzyme magnesium chelatase, which can be divided into three subunits. It is required for chlorophyll production by catalysing the insertion of magnesium into protoporphyrin IX (Walker and Willows, 1997). A silencing turns green plant tissue into white-yellow tissue (Hiriart et al., 2002). GSA, a sensitive visible marker, is involved in synthesising chlorophyll molecules and a suppression results in a variety of chlorophyll deficiencies mainly along the leaf veins (Höfgen et al., 1994). A striking PAI activity, which functions in the tryptophan biosynthetic pathway, leads to a blue fluorescence plant phenotype under UV light (Jeddeloh et al., 1998). Once a visual marker has been effectively used to cause a VIGS phenotype it is possible to target other nuclear genes with the viral vector (Liu et al., 2002).

VIGS technology is widely used to silence genes which are involved in development and disease resistance as well as in metabolic pathways. For instance Lu et al. (2003a) and Ekengren et al. (2003) studied with the help of VIGS *Pto*-mediated resistance against *Pseudomonas syringae*. Yoshioka et al. (2003) utilized VIGS to determine the previously unknown function of *N. benthamiana* respiratory burst oxidase homologs (*Nbrboh* genes) and indicated that *NbrbohA* and *NbrbohB* are required for resistance to *Phytophthora*. The role of several kinases in defence against TMV in *N. benthamiana* has been study with VIGS-technology (Burch-Smith et al., 2004). Liu et al. (2002) used a TRV-based vector for a successful suppression of *ctr1* (constitutive triple response1) gene in tomato and *N. benthamiana* that leads to a constitutive expression of ethylene-regulated genes. Burton et al. (2000) used a PVX-based VIGS vector to analyse cellulose synthase genes (*CesA*).

2. Research objectives

BSBMV is a very close relative of BNYVV. Little is known about their interaction, so far no reassortants or recombinants between both viruses have been detected. To gain deeper insights into the similarities and differences of biology and epidemiology of BSBMV and BNYVV, as well as to study their interaction infectious full-length cDNA clones will represent a new tool for benyvirus studies. Therefore, the first aim of this thesis was to generate infectious full-length cDNA clones of BSBMV under control of the CaMV 35S promotor by using the approach of a one-step isothermal *in vitro* recombination assembly named Gibson assembly (manuscript I). Infectious full-length cDNA clones of BNYVV A-type using the same strategy were constructed and kindly provided by the project partners Prof. Dr. Edgar Maiss and co-workers. The functionality of the recombinant virus had to be demonstrated by displaying a comparable symptom expression to the wild-type, displaying characteristic virus particles, transmission by *P. betae*, demonstrating replication of all components and systemic infection after agroinoculation of host plants. A second focus was to study whether the two viruses can form reassortants. Thus, artificially formed reassortants of BSBMV and BNYVV A-type shall be argoinoculated into different host plants. The exchangeability of the genomic RNAs shall be tested and the phenotypes shall be compared with the wild-types.

Fluorescence labelling of BSBMV and BNYVV could offer for the first time a detailed analysis of the distribution and colonisation strategy in mixed infection experiments and allows to draw conclusions about BSBMV and BNYVV reassortants and true recombinants in natural infections. Thus, another aim (manuscript II) was to develop a strategy of fluorescence labelling and to find a suitable marker, without losing the functionality of the recombinant virus. The labelled full-length clones shall be compared to the wild-type isolates. Moreover, it was aimed to characterise the distribution of differently labelled BSBMV and BNYVV A-type clones and artificial reassortants as well as the distribution of a benyvirus with taxonomically distinct viruses after co-infections and super-infections in *N. benthamania* plants. The experiments should be evaluated using confocal laser scanning microscopy.

Finally, a virus-induced gene-silencing system based on BSBMV and BNYVV A-type should be developed (manuscript III). An initial step should be to find a suitable insertion site for target genes within the viral genome and to test BSBMV and BNYVV based VIGS system on the model plant *N. benthamiana*. Firstly, genes which lead to an easily observable phenotype should be tested as targets for VIGS. Silencing efficiencies shall be confirmed using quantitative reverse-transcription real-time polymerase chain reaction (qRT-PCR).

3. Manuscript I

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Biological properties of *Beet soil-borne mosaic virus* and *Beet necrotic yellow vein virus* cDNA clones produced by isothermal *in vitro* recombination: insights for reassortant appearance

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ABSTRACT

Two members of the *Benyviridae* family and genus *Benyvirus*, *Beet soil-borne mosaic virus* (BSBMV) and *Beet necrotic yellow vein virus* (BNYVV), possess identical genome organization, host range and high sequence similarity; they infect *Beta vulgaris* with variable symptom expression. In the US, mixed infections are described with limited information about viral interactions. Vectors suitable for agroinoculation of all genome components of both viruses were constructed by isothermal *in vitro* recombination. All 35S promoter-driven cDNA clones allowed production of recombinant viruses competent for *Nicotiana benthamiana* and *Beta macrocarpa* systemic infection and *Polymyxa betae* transmission and were compared to available BNYVV B-type clone. BNYVV and BSBMV RNA1+2 reassortants were viable and spread long-distance in *N. benthamiana* with symptoms dependent on the BNYVV type. Small genomic RNAs were exchangeable and systemically infected *B. macrocarpa*. These infectious clones represent a powerful tool for the identification of specific molecular host-pathogen determinants.

KEYWORDS: *Beet soil-borne mosaic virus, Beet necrotic yellow vein virus*, *Benyviridae*, Gibson assembly, infectious cDNA full-length clones, *Polymyxa betae,* reassortment

1. Introduction

Beet necrotic yellow vein virus (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) are both members of the genus *Benyvirus* in the family *Benyviridae* with BNYVV representing the type species (Gilmer and Ratti, 2012, 2017). Both virus species mainly infect plants of the family *Amaranthaceae* (Heidel et al., 1997). BNYVV is known as the causative agent of rhizomania with worldwide distribution in nearly all sugar beet-growing areas (Peltier et al., 2008). In contrast, BSBMV is currently restricted to the United States (Heidel et al., 1997; Lee et al., 2001). BNYVV and BSBMV are both vectored by the soil-borne Plasmodiophoromycete *Polymyxa betae* Keskin where viral particles persist in the protozoa resting spores and therefore in the soil for decades (Keskin 1964; Tamada and Kondo 2013). Although representing closely related species sharing a similar host range (Heidel et al., 1997) and vector species, the symptoms in the natural host sugar beet (*Beta vulgaris*) differ considerably. In the field BSBMV infected sugar beet roots appear symptomless, whereas leaves displays light yellow vein banding, mottling or mosaic patterns and growth disorders (Heidel and Rush, 1994; Rush and Heidel, 1995). In contrast BNYVV infections are mainly confined to the root system that displays extensive proliferation of necrotizing secondary rootlets, a stunted tap root and a brownish discolouration of the vascular system. The leaves in upright position only rarely show symptoms like vein yellowing and necrotic leaf tissue (reviewed in Peltier et al., 2008). The impact of BNYVV on root weight is higher for BNYVV than BSBMV after mechanical inoculation (Heidel et al., 1997) and corresponds to higher yield losses with BNYVV compared to BSBMV (Wisler et al., 2003). Remarkably, *Rz1* gene used for rhizomania

control has no effect on BSBMV infection (Wisler et al., 2003). Among the three major BNYVV subgroups (namely A, B and P), B-type is so far limited to Central and Northern Europe, whereas A-type is present worldwide (Koenig and Lennefors, 2000). Specific P25 amino acid residue variations, required for *Rz1* resistance-breaking abilities in sugar beet have only been detected in A-type isolates (Bornemann et al., 2015; Koenig et al., 2008; Koenig et al., 2009; Liu and Lewellen, 2007; Pferdmenges et al., 2008). Geographic genetic variability in BSBMV has not been analysed yet.

Benyviruses represent multipartite single-stranded positive-sense RNA viruses and consist of four capped, polyadenylated RNA segments that are separately encapsidated in rod-shaped particles (reviewed in Peltier et al., 2008). Some isolates of BNYVV possess an additional fifth RNA species (Tamada et al., 1996). While BNYVV and BSBMV display a similar genome organisation, sufficient sequences differences allow classification as distinct species (Lee et al., 2001; Gilmer and Ratti, 2012, 2017). RNA1 and RNA2 carry genes required for replication, movement, silencing suppression, packaging and vector transmission (Lee et al., 2001; Peltier et al., 2008). BNYVV RNA1+2 segments alone are sufficient for systemic infection in the experimental host *Nicotiana benthamiana* (Rahim et al., 2007). The single 237K open reading frame (ORF) on RNA1 produces a polypeptide possessing methyltransferase (MetT), helicase (Hel), papain-like protease (Prot) and RNA-dependent RNA polymerase (RdRp) signatures. The six ORFs of RNA2 encode the coat protein (CP) which leaky UAG stop codon allows the translation of the readthrough protein (RT) associated with vector transmission (Tamada and Kusume, 1991). The next three overlapping ORFs form a cluster named triple gene block (TGB1-3) essential for cell-to-cell movement (Gilmer et al., 1992, Verchot-Lubicz et al., 2010). The 3'-proximal ORF encodes for a 14 kDa cysteine-rich protein with viral suppressor of RNA silencing (VSR) activity (Chiba et al., 2013; Dunoyer et al., 2002). Nearly all the molecular biology of Benyvirus RNA1 and RNA2 has been investigated on BNYVV. However, the high sequence similarity of the different proteins encoded by the viruses suggests functional similarity of BSBMV (Lee et al., 2001). BNYVV RNA3 has been described to be involved in viral pathogenicity and required for long distance movement in *Beta macrocarpa* (Lauber et al., 1998; Peltier et al., 2012, Flobinus et al., 2016). It encodes the P25 protein responsible for virus pathogenicity and the rhizomania disease phenotype in sugar beets (Chiba et al., 2008; Koenig et al., 1991). BSBMV RNA3 is also involved in long-distance movement and encodes a P29 protein that shows 23% amino acid similarity compared to BNYVV P25 and a much higher (43%) similarity to BNYVV RNA5-encoded P26 (Ratti et al., 2009). Both RNA4-encoded BNYVV P31 and BSBMV P32 proteins are responsible for vector transmission (D'Alonzo et al., 2012; Tamada and Abe, 1989). Sequence similarity suggests that P32 might be involved in symptom expression and suppression of RNA silencing that has been evidenced for P31 only in *N. benthamiana* roots (Rahim et al., 2007). BSBMV smaller RNAs are replicated and encapsidated by the BNYVV housekeeping machinery and complement the corresponding cognate RNA functions

in *trans* (D'Alonzo et al., 2012; Ratti et al., 2009). The opposite situation of BNYVV smaller RNA replication by BSBMV RNA1+2 has not been reported yet.

To understand functional differences in molecular biology, pathogenicity mechanisms, symptom expression as well as interaction with the host and between viral species, a reverse genetic system represents a prerequisite. For BNYVV B-type, infectious cDNA clones for agroinoculation for RNA1-4 are available (Delbianco et al., 2013); however, A-type and BSBMV cDNA clones infectious clones were lacking. Initial construction of BNYVV B-type infectious clone for generation of infectious *in vitro* transcripts of RNA2 (Quillet et al., 1989), was associated with stability or toxicity problems in *Escherichia coli*. Therefore Delbianco et al. (2013) successfully transformed ligated plasmids from reamplified BNYVV B-type cDNA and binary expression vectors into *A. tumefaciens*.

The aim of this study was to generate infectious BSBMV and BNYVV A-type cDNA clones. In order to avoid possible cloning problems, the standard restriction enzyme based cloning was replaced with a one-step isothermal *in vitro* recombination assembly named Gibson assembly (GA) (Gibson et al., 2009). Recently, this method was applied for the first time for the generation of an infectious full-length clone of tomato blistering mosaic virus (ToBMV) (Blawid and Nagata, 2015). The clones obtained were characterized for their ability to reproduce the entire viral cycle including systemic infection, symptom expression in different host plants and vector transmission to demonstrate major functionality of the virus encoded proteins. We applied this approach on BSBMV and A-type BNYVV to extend the availability of cDNA clones and study the biological properties of standardized isolates and artificial reassortants. Viral accumulation, symptom expression and long-distance movement were assayed in *N. benthamiana* and *B. macrocarpa* to demonstrate the exchangeability of genome components between species.

2. Materials and methods

2.1. Virus and plant material

A BSBMV isolate (BSBMV-CA) from California USA, originally isolated by H.-Y. Liu (United States Department of Agriculture, Salinas, CA) and a BNYVV A-type isolate BNYVV-Yu2 (Kruse et al., 1994), (Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany PV-0649) originated from former Yugoslavia were provided by DSMZ. As BNYVV-Yu2 did not allow RNA4 amplification, roots from sugar beet plants grown in BNYVV A-type containing soil from Rovigo (Italy) were used as source for RNA4.

The benyviruses hosts *C. quinoa* (local lesion)*, B. macrocarpa* Guss., *B. vulgaris ssp. vulgaris* (*B. vulgaris*) susceptible genotype (without *Rz1* or *Rz2* resistance) and *N. benthamiana* served as host plants for the experimental work under greenhouse conditions (24°C/14h 18°C/10h).

2.2. Virus detection

Plant total RNA extracts were prepared using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For each genome fragment of BNYVV and BSBMV, respectively, specific primers were developed (Table S1, see Supporting Information) to allow RT-PCR detection. The cDNA synthesis was performed using RevertAid H Minus Reverse transcriptase (Thermo Fisher Scientific) and specific antisense primers. The PCR reaction was conducted with Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer´s instruction. PCR products were visualised following agarose gel electrophoresis.

BNYVV genomic RNA detection by Northern hybridization was performed as previously described (Link et al., 2005; Schmidlin et al., 2005) while BSBMV RNAs 1 and 2 were detected using ^{32}P labeled RNA probes corresponding to position 4747-6549 of RNA1 and 2311-3774 of RNA2.

Additionally, a specific enzyme-linked immunosorbent assay (ELISA) of infected *N. benthamiana* leaves was performed to determine the virus content as previously described (Pferdmenges et al., 2008).

2.3. BNYVV B-type full-length clones

The BNYVV B-type full-length clones (RNA1-4) for agroinoculation have been described previously (Delbianco et al., 2013).

2.4. Generation of BSBMV and BNYVV A-type full-length clones

For generation of full-length cDNA clones of both benyvirus species, total RNA preparations (RNeasy Plant Mini Kit, Qiagen) and dsRNA preparations (Darissa et al., 2010) from *C. quinoa* virus-induced local lesions were produced. Gibson assembly was applied as *in vitro* recombination method for the cloning of full-length cDNA of BSBMV and BNYVV A-type RNA1-4 into a small binary vector. For the full-length clones construction the plasmid pDIVA was used (Acc. No. KX665539), which is based on the mini binary vector pCB (Xiang et al., 1999), supplemented with a cauliflower mosaic virus (CaMV) 35S promoter followed by a hepatitis delta virus (HDV) ribozyme and the polyadenylation signal of CaMV. All fragments (viral cDNA inserts and vector fragments) were generated by PCR amplification using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) following the manufacturer´s instructions. The vector plasmid was linearized by means of PCR amplification with a sense primer annealing to the 5´-end of the HDV ribozyme and an antisense primer annealing to the exact end of 35S promoter sequence. For successful GA the following sequence overlaps were generated during PCR. The 5´-end of each viral genome fragment was supplied with 25 nucleotides overlap to the exact 35S end. If multiple viral cDNA fragments had to be produced, a ca. 30-50 nt overlap between fragments was generated. The 3´-viral cDNA end was supplied with 18 nt overlap to the 5´-end of the HDV ribozyme sequence. The following Genbank nucleotide sequences represented the basis for the primer design: BSBMV

RNA1 (6 683 nt, NC_003506.1), RNA2 (4 615 nt, NC_003503.1), RNA3 (1 720 nt, NC_003507.1), RNA4 (1 730 nt, FJ424610.2), BNYVV RNA1 (6 746 nt, NC_003514.1), RNA2 (4 609 nt, NC_003515.1), RNA3 (1 774 nt, NC_003516.1), RNA4 (1 465 nt, NC_003517.1). Viral cDNA was generated with the appropriate 3´-end antisense primer for each genome fragment (including the overlap) with RevertAid H Minus Reverse Transcriptase. Primers for amplification of the different viral genome components are displayed in Table S1 (see Supporting Information). To generate full-length cDNA clones BSBMV RNA1 (6 683 nt) was converted into cDNA and PCR amplified in three overlapping products with size ranging from 2 255 to 2 275 nt. Two overlapping PCR fragments (from 908 to 3 879 nt) were generated for BSBMV RNA2 (4 615 nt), A-type BNYVV RNA1 (6 746 nt), RNA2 (4 609 nt), RNA3 (1 774 nt), whereas the smaller BSBMV RNAs 3 (1 720 nt), 4 (1 730 nt) and BNYVV RNA4 (1 465 nt) were RT-PCR amplified in one fragment each. All PCR products were gel-purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The GA was performed as described by Gibson et al. (2009).

In vitro recombination products were transformed into chemical competent *E. coli* cells (strains DH5α or NM522) (Inoue et al., 1990). Viral inserts were sequenced (Eurofins MWG Operon, Ebersberg, Germany and Seqlab, Goettingen, Germany) with specific primers. Resulting sequences were assembled with the Molecular Evolutionary Genetics Analysis (Tamura et al., 2013) software. Clustal Omega of the European Molecular Biology Laboratory-European Bioinformatics Institute was used to create a multiple sequence alignment and to check for sequence similarity with NCBI published genome sequences (http://www.ebi.ac.uk/Tools/msa/clustalo/). Subsequently all plasmids were electroporated into *Rhizobium radiobacter* (syn. Agrobacterium tumefaciens) strain GV2260 (pGV2260). Plant infection was performed by means of agroinoculation according to Voinnet et al. (1998) with an OD_{600} = 0.5 for *B. macrocarpa* infection at BBCH12 stage. Fourteen-day-old *N. benthamiana* were agroinfiltrated with an OD_{600} of 0.1 to avoid necrosis induction with more concentrated suspensions. *A. tumefaciens* cultures carrying the different clones were mixed in equal amounts.

Leaves of *C. quinoa* were mechanically rub-inoculated with plant sap (1/5 diluted in 0.05 M phosphate buffer) from systemically agroinfected *N. benthamiana* plants. Besides agroinoculation, vortex-inoculation was used to infect *B. vulgaris* seedlings (BBCH 10) as described by Bornemann and Varrelmann (2011).

2.5. Electron microscopy

To obtain evidence for particle formation, transmission electron microscopy (TEM) was performed. *N. benthamiana* leaf tissue, systemically infected with BSBMV and BNYVV RNA1-4 respectively, initiated from agroinoculation of lower leaves was used for preparation of plant sap that was applied for TEM specimen preparation and visualisation (Milne and Lesemann, 1984). Particle

decoration with specific antisera was performed at Julius-Kühn-Institute, Institute for Epidemiology and Pathogen Diagnostics (Braunschweig, Germany).

2.6. Polymyxa betae transmission

The BSBMV and BNYVV full-length clones derived from RNA1-4, respectively, were used for agroinoculation of *N. benthamiana* and leaf tissue sap was used for mechanical inoculation of 42 sugar beet seedlings (BNYVV-susceptible sugar beet cultivar KWS03). As controls served *C. quinoa* local lesions of the Californian BSBMV isolate and of the BNYVV A-type isolate. Seven plants per pot (six pots in total) were planted into virus-free field soil from a sugar beet field in Reutershof, (Brandenburg, Germany) that contained *P. betae* according to Bornemann and Varrelmann (2011). After growth for five weeks, plants and roots were removed and new seedlings were planted into the virus loaded soil for another period of five weeks. Finally, lateral roots were harvested and virus infection was assayed by means of RT-PCR with specific primers for RNA3 and RNA4.

3. Results

3.1. Generation of full-length cDNA clones of BSBMV and BNYVV A-type for agroinoculation

Following GA *in vitro* recombination into pDIVA (Acc. No. KX665539), clone identification, sequencing and *in silico* assembly of the complete viral cDNA inserts, the following BSBMV genome sizes (excluding polyA-tail) were obtained: BSBMV RNA1 6 674 nt, RNA2 4 615 nt, RNA3 1 720 nt and RNA4 1 729 nt. For BNYVV A-type the different RNA components displayed the following lengths: RNA1 6 746 nt, RNA2 4 588 nt, RNA3 1 775 nt and RNA4 1 470 nt. Viral sequences were submitted to Genbank (Acc. No. KX352033, KX352170, KX352171, KX352034, KX665536, KX665537, KX665538 and MF476800).

Sequence comparisons with published sequences of BSBMV isolates MRM06 (originating from Texas, USA; D'Alonzo et al., unpublished; D´Alonzo et al., 2012; Ratti et al., 2009) and EA (originating from Colorado, USA; Lee et al., 2001) were performed (Table S2 and Table S3, see Supporting Information). Sequence similarity of isolate BSBMV-CA at nucleotide level over all RNA components was closer to isolate MRM06 than EA (Table S2). The amino acid sequence similarities between isolate BSBMV-CA and MRM06 was striking for all ORF except for the RNA3 encoded P29 and RNA4 encoded 32K protein, respectively (Table S3).

The BNYVV Yu2 sequence obtained here represents the second complete A-type genome sequence in addition to the Japanese A-type isolate (BNYVV-S) (Saito *et al.*, 1996). We determined the relatedness of these two geographically distant A-type strains and compared them to European B-type and P-type strains, confirming the closer relationship between the two A-type isolates compared to the P-type isolate (Table S4, Table S5).

3.2. Proof of infectivity

To obtain evidence about infectivity comparable to wild-type isolates, each cDNA clone was transformed into *R. radiobacter* (GV2260) and agrobacterial clones harbouring RNA1-4 cDNA of each species were mixed and agroinfiltrated into leaf tissue of known host plants *N. benthamiana*, *Chenopodium quinoa*, *B. macrocarpa* and *B. vulgaris* (Young et al., 2001). In *N. benthamiana* BSBMV RNA1-4 cDNA clones produced systemic symptoms of chlorotic vein banding, yellow blotches and leaf crinkling 12 to 16 days post-infiltration (dpi) and necrosis at 22 dpi (Fig. 1a).BNYVV A-type RNA1-4 cDNA clones led to systemic infection with deviating symptoms consisting of light yellow chlorosis that appeared delayed at 20-22 dpi (Fig. 1b).

The local lesion host *C. quinoa* turned out to be resistant towards agroinoculation. Therefore, sap from systemically infected tissues of agroinoculated *N. benthamiana* was used for rub-inoculations. Both viruses induced typical specific local lesions at 7-10 dpi with BSBMV lesions developing to necrotic spots quite rapidly comparable to wild-type viruses. Agroinoculation of *B. macrocarpa* leaves with cDNA clones (RNA1-4) of both viruses initially resulted in local lesion formation at 6-9 dpi inside the infiltrated patch. Systemic spread and symptoms development occurred at 25-33 dpi (BSBMV) and 19-22 dpi (BNYVV), respectively (Fig. 6, c, h, see below). BSBMV or BNYVV agroinoculation of *B. vulgaris* leaf tissue resulted in local lesions formation inside the infiltrated patch at 13 and 18 dpi, respectively. Although lesions increased in size and slow spreading to leaf veins was observed, systemic spread associated with virus symptoms in newly emerging leaves was not observed with any of the two viruses. Additionally, variation of agroinoculation methods like vacuum-infiltration, root-dipping or vortex-inoculation of *B. vulgaris* roots with agrobacterium suspensions did not result in development of systemic infection. Finally, vortex-inoculation of 12 days old seedlings was applied according to Bornemann and Varrelmann (2011) using sap from *C. quinoa* local lesions. Following this approach, first systemic viral symptoms were observed at 35 dpi with cDNA derived BSBMV, 26 dpi with wild-type BSBMV, 30 dpi with cDNA derived BNYVV and 26 dpi with wild-type BNYVV (Fig. 2). Both cDNA clones induced wild-type like symptoms (Fig. 2a-d). ELISA based detection of viral accumulation in *B. vulgaris* lateral roots resulted in mean absorbance (A_{405nm}) values that were similar between cDNA derived and wild-type virus (0.40 vs 0.44 for BSBMV and 1.18 vs 1.46 for BNYVV). RT-PCR with specific primers allowed the detection and validation of the replication of all BSBMV or BNYVV RNA-components in total RNA extracts from systemically infected *N. benthamiana*, *B. macrocarpa* (agroinoculated) and *B. vulgaris* (vortex-inoculated) according to infiltrated or inoculated combinations.

3.3. Electron microscopy

By means of TEM from BSBMV and BNYVV, respectively, cDNA clone infected *N. benthamiana*, rod shaped viral particles of varying lengths displaying a central core were observed from both samples (Fig. 3). Clear decoration with specific antisera was observed supporting virus identity.

3.4. Polymyxa betae transmission of recombinant viruses

Furthermore, we verified the ability of BSBMV and BNYVV cDNA clones to be vectored by *P. betae.* After mechanical vortex inoculation of sugar beet and subsequent transplanting into the vector-containing soil, composite root samples from all source plants in each pot were ELISA-tested. BSBMV and BNYVV were detected in five out of six and six out of six pots, respectively (data not shown). Bait plants were subsequently grown in the loaded soil. RT–PCR confirmed the efficient transmission of BSBMV (4/6) and A-type BNYVV (4/7).

3.5. Viability of different BNYVV and BSBMV RNA1+2 reassortants in N. benthamiana

We aimed to test for viability of RNA1+2 reassortants of the two viral species in *N. benthamiana* to assess the possibility for the production of reassortants during natural infection. As A- (BNa) and B-type (BNb) BNYVV differ substantially, we used both cDNA clones to produce BNYVV/BSBMV reassortants. To simplify the designation, we summarized the viral species by two capital followed if required by a lower case designating A- or B-type such as exemplified: BNa1BS2 corresponds to A-type BNYVV RNA1 inoculated in the presence of BSBMV RNA2, while BS1BN2b stands for BSBMV RNA1 inoculated in the presence of B-type BNYVV RNA2.

N. benthamiana symptoms produced by the inoculation of BS12 and BNa12 were undistinguishable from those produced after the inoculation of the all set of BSBMV and BNYVV RNAs suggesting that the smaller genomic RNA species did not significantly affect the systemic movement and symptom induction (compare Fig. 1a and 1b to Fig. 4a and 4b). BNa1BS2, BNb1BS2 and BS1BNb2 combinations were able to systemically infect *N. benthamiana* (9/9; 7/9 and 9/9 plants inoculated) (Fig. 4c, 4f, 4g and Table 1). However, symptoms appeared delayed and were less pronounced when compared to the natural RNA1+2 combination of each species. BNb1BS2 did not result in visible symptoms while BS1BNb2 displayed severe necrosis 21-23 dpi (Fig. 4 and Table 1). BNa12, BNb12 and BS12 infections appeared systemic 12-16 dpi while reassortants containing A-type BNYVV RNA species produced mild symptoms 16-23 dpi (BNa1BS2) and 23-29 dpi (BS1BNa2). Only one plant was systemically infected with BS1BNa2 and displayed faint systemic symptoms 23-29 dpi including rare occurrence of mild yellow veins. BNa1BS2 symptoms were similar but less pronounced to BS12. High ELISA values (29 dpi) were obtained for all plants displaying systemic symptoms as well as some symptomless plants inoculated by BNb12 and BN1bBS2 combinations (Table 1). Symptomless plants inoculated with BS1BNa2 produced ELISA values similar to the healthy control. BS12, BNa1BS2 and BNbBS2 were ELISA positive when assayed with BSBMV specific antiserum and BNa12, BNb12, BS1BNa2 and BS1BNb2 were detected with BNYVV specific antiserum.

3.6. Influence of different RNA components on local lesion expression in C. quinoa

Sap from systemic leaves of *N. benthamiana* inoculated with BNa12, BNb12, BS12, BNa1BS2, BS1BNa2, BNb1BS2 and BS1BNb2 was applied for rub-inoculation of *C. quinoa* leaves and lesions appeared at 7 dpi (Fig. S1). BNa12 and BNb12 lesions were faint whereas BS12 lesions quickly developed necrosis. Lesions produced by reassortants BNa1BS2 (Fig. S1c) were comparable to BS12 whereas BNb1BS2 (Fig. S1f) displayed a more faint and BS1BNb2 (Fig. S1g) showed necrotic appearance. Interestingly, BS1BNa2 sap from infiltrated leaves applied for rub-inoculation of *C. quinoa* leaves did not produce local lesions (Fig. S1d). As already suspected under *3.5* and shown in Fig. 4 for systemic movement in *N. benthamiana* there was also a consistent specific RNA component effect on local lesion phenotype formation in *C. quinoa*.

3.7. Influence of different RNA components on viral RNA accumulation in C. quinoa

To evidence the effective replication of reassortants and find indications for an RNA species effect, lesions derived from BS12, BNb12 and corresponding reassortant infection were individually (5 each) collected for RNA extraction and northern blot analysis using RNA species specific probes (Flobinus et al., 2016). Signal processing using ImageJ software (Schneider et al., 2012) was used to estimate the accumulation of viral RNAs within each local lesion using RNA loading (ribosomal RNAs) normalization. We evidenced a higher accumulation of both genomic RNAs in BNb1BS2 reassortant when compared to BS1BNb2 and BNb12 or BS12 combinations (Fig. 5), confirming the specific RNA composition effect described above.

3.8. Long distance movement function of BSBMV and BNYVV RNA3 in cis and in trans

In the USA, natural mixed infections occur between BNYVV A-type and BSBMV. As BSBMV RNA3 can substitute BNYVV RNA3 in BNYVV long-distance movement in *B. macrocarpa*, we focused on this combination for further experiments. As expected, agroinoculation of *B. macrocarpa* with BSBMV and BNYVV RNA1-2, respectively, did not lead to systemic infection (0/10 plants inoculated) (Fig. 6a, f, Tab. 2). Agroinoculation of primary leaves resulted in yellowing at 4-6 dpi and necrosis development at 12-18 dpi with no phenotypic differences between species and no such effect was observed when empty binary vector was used (data not shown). Systemic symptoms produced by wild-type BNYVV RNA1-3 (BNa1-3) were more pronounced and occurred rapidly (9/10 plants, 19-22 dpi) when compared to BSBMV RNA1-3 (BS1-3; 5/10 plants, 25-33 dpi). BSBMV infection induced yellow blotches and bands (Fig. 6b, c) while BNYVV mainly provoked vein yellowing (Fig. 6g, h). Systemic infection using BSBMV RNA1-2+BNYVV RNA3 reassortants (BS12+BNa3, Fig. 6d) was visible 19-25 dpi with symptoms comparable to those provoked by BS1-3. The systemic infection produced by BNYVV RNA1-2+BSBMV RNA3 reassortants (BNa12+BS3, Fig 6i) appeared delayed (33-39 dpi) with symptoms similar to those formed by BS1-3 (Fig. 6b) and BS1-4 (Fig. 6c). When RNA4 was added to the three genomic species

combinations, no differences were observed on the phenotype or infection kinetics (Fig. 6e, j and Table 2). In all treatments, presence or absence of individual viral RNAs in systemically infected *B. macrocarpa* tissue was assayed with specific primers by RT-PCR and corresponded to the input (data not shown).

4. Discussion

This study describes successful application of the GA for the generation of agroinfectious cDNA clones of two multipartite RNA viruses and to our knowledge it is the first example that describes successful assembly of three cDNA fragments in the case of BSBMV RNA1 (6 674 nt total cDNA) and two fragments for BNYVV RNA1 (6 746 nt) without any detectable functional errors. Identical properties were found when cDNA clones and natural isolates were compared.

Agroinoculation represents a quick and easy method to infect plants with cDNA clones of viruses (Nagyová and Šubr, 2007). However, our study illustrated also some limitations of agroinoculation that failed in *C. quinoa* leaves and *B. vulgaris* roots. Flores Solís et al., (2003) and Komari (1990) reported difficulties to transform *C. quinoa* by *A. tumefaciens*. Agroinoculation failure in sugar beet roots could be explained by a general lower efficiency of *A. tumefaciens* to transform root cells (Grevelding et al., 1993) and particularly by the sugar beets ability being recalcitrant to transformation (Krens et al., 1996; Wozniak, 1999), combined with a synergistic antiviral defence. Further work is required to bypass this issue, possibly by using *A. rhizogenes* described to efficiently transform sugar beet roots (Cai et al., 1997; Pavli et al., 2010). For such purpose, disarmed *A. rhizogenes* described by Mankin et al. (2007) will be required to alleviate undesired hairy roots phenotypic effect. Meanwhile, root inoculation with sap issued from agroinfected tissues provided an alternative inoculation method that successfully produced specific symptoms with viral accumulation assays similar to those observed in naturally infected plants (Heidel et al., 1997; Peltier et al., 2008).

This work also describes for the first time a direct comparison of BSBMV and BNYVV effects on several host plants including the natural host sugar beet. Specific symptoms observed on *N. benthamiana*, *B. macrocarpa* and *B. vulgaris* reflect the interspecies genetic variability observed and reproduced with the artificial clones. A-type BNYVV RNA1+2 and BSBMV RNA1+2 derived from cDNA clones are sufficient for long-distance movement in *N. benthamiana* confirming previous observations with BNYVV (Rahim et al., 2007). Such behaviour underlines the functional similarities of the two large genomic RNAs for both species. Our work also confirmed the involvement of both BSBMV and BNYVV RNA3 in virus long distance movement in *Beta* species (Lauber et al., 1998; Ratti et al., 2009, Peltier et al., 2012) as well as for the viral long distance movement of the reassortants produced (Table 2). The same observation applied as well for the RNA4 species for their involvement in vector transmission. While some RNA segment exchanges were performed in our study, all chimeric combinations were not assessed. However, taking

advantage of infectious cDNA agroclones availability, there might be no limit for segment exchanges and recombinant production. Gene-exchange recombinants or mutants using A- and B-type BNYVV as well as BSBMV (not targeted by *Rz1*) will provide more precise information about the molecular basis of rhizomania resistance recognition by *Rz1* and/or *Rz2* as well as *Rz1* resistance breaking BNYVV strains (Bornemann and Varrelmann, 2013 and 2011; Koenig et al., 2009).

Benyvirus (BSBMV and BNYVV) RNA1 and 2 chimeric combinations analysed in *N. benthamiana* gave a first clear hint for the involvement of genome segments in symptom development mainly determined by the RNA2 species that encode the most divergent proteins. Interestingly, BS1BN2b reassortant was efficiently amplified and moved long distance in *N. benthamiana* whereas the same combination using A-type BNYVV did not led to reproducible and significant infection. Some of the combinations tested revealed the possible fitness penalty of reassortants compared to wild-type isolates. These combinations require further extensive analyses that were not the first objective of this study. An open question remains about the phenotypes observed in host plants where some reassortants symptoms appeared more severe than wild-type viruses or did not produced progeny in some hosts. One could expect a lethal effect of some combinations for infected cells or necrotic phenotype that would restrict the viability of the reassorted virus. So far, detection of reassorted BSBMV/BNYVV in sugar beet has never been described in the USA. To investigate, if the two species are able to co-infect the same cell, labeling of individual genome components including RNA2 is required. Preliminary experiments using replicons derived from BSBMV and BNYVV RNA3 species already evidenced the exclusion of the smaller RNA species (Ratti et al., 2009). Interestingly, under natural mixed infections (Rush and Heidel, 1995), BSBMV infection is lowered by BNYVV (Wisler et al., 2003) and cross protection has been described (Mahmood and Rush, 1999). If true recombinants occur in natural infections, leading to new virus genotypes with different properties and abilities to cause damage and disease needs to be investigated.

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Author Contributions

MV, EM, CR and DG conceived the study and experiments; EM and HM designed and constructed pDIVA and generated the BNYVV (A-type) cDNA clones including infectivity tests; ML generated the BSBMV cDNA clones, performed infectivity tests, vector transmission and reassortant analysis; KRP performed the TEM experiments; MDA performed the BNYVV (B-type)/BSBMV reassortant

experiments; SL performed the BNYVV (A-type)/BSBMV reassortant experiments on *C. quinoa*; MDA and CR performed the Northern blot experiments; ML, MV, CR and DG wrote the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version.

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Figures and Figures Legends

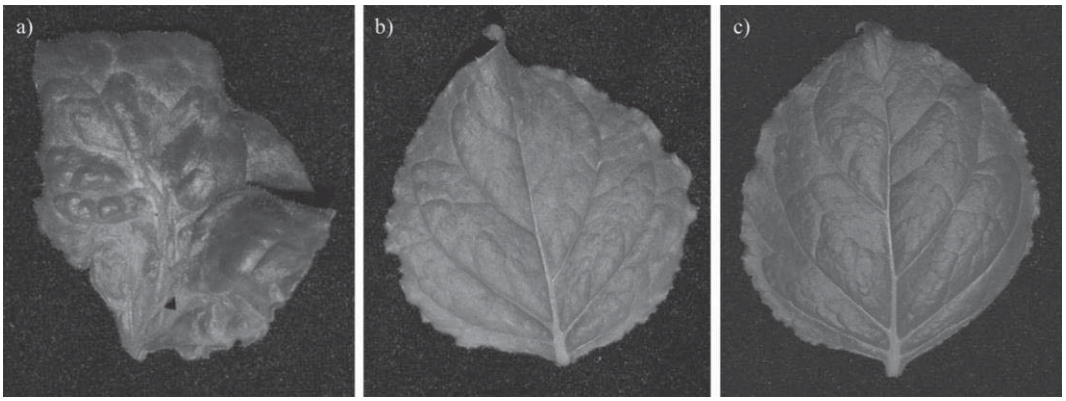

Fig. 1 Symptom expression on *Nicotiana benthamiana* leaves obtained after agroinoculation of (a) BSBMV cDNA clones RNA1-4 and (b) BNYVV cDNA clones RNA1-4 compared to (c) mock-inoculated healthy control at 22 dpi.

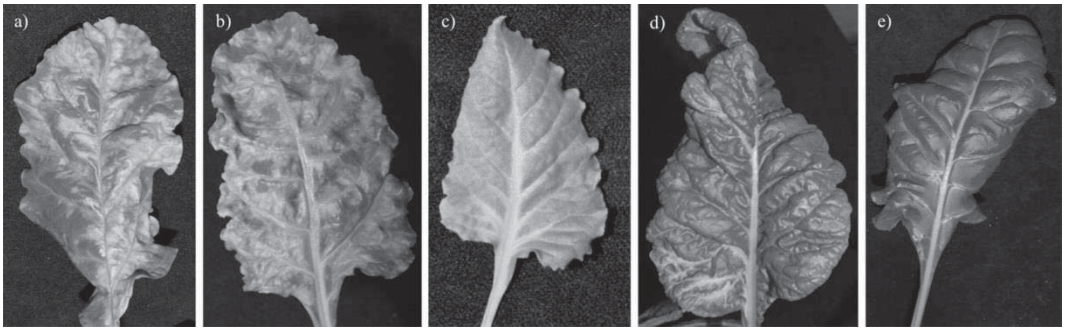

Fig. 2 Systemic symptom expression on *Beta vulgaris* leaves produced after mechanical root vortex-inoculation with plant sap from *C. quinoa* local lesions infected with (a) BSBMV cDNA clones RNA1-4, (b) wild-type BSBMV, (c) BNYVV cDNA clones RNA1-4 and (d) wild-type BNYVV compared to (e) healthy control at 48 dpi.

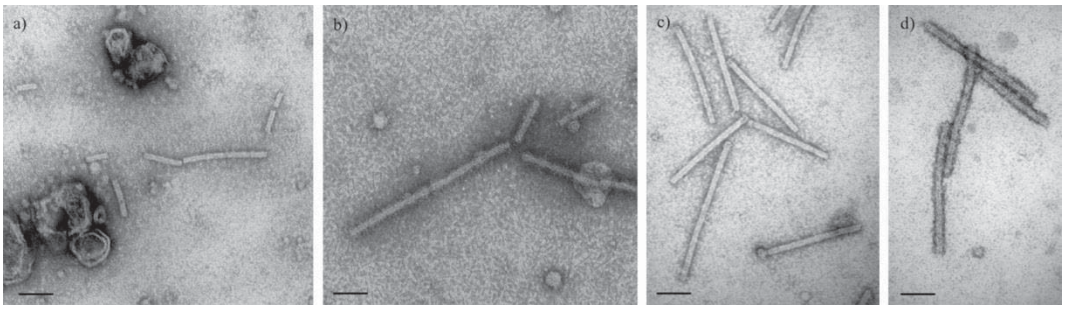

Fig. 3 (a) Transmission electron microscopy (TEM) of *Beet soil-borne mosaic virus* (BSBMV) and (c) *Beet necrotic yellow vein virus* (BNYVV) particles from systemically infected *N. benthamiana* leaf tissue and (b) BSBMV and (d) BNYVV particles, respectively, decorated with virus specific antisera. Bar represents 100nm.

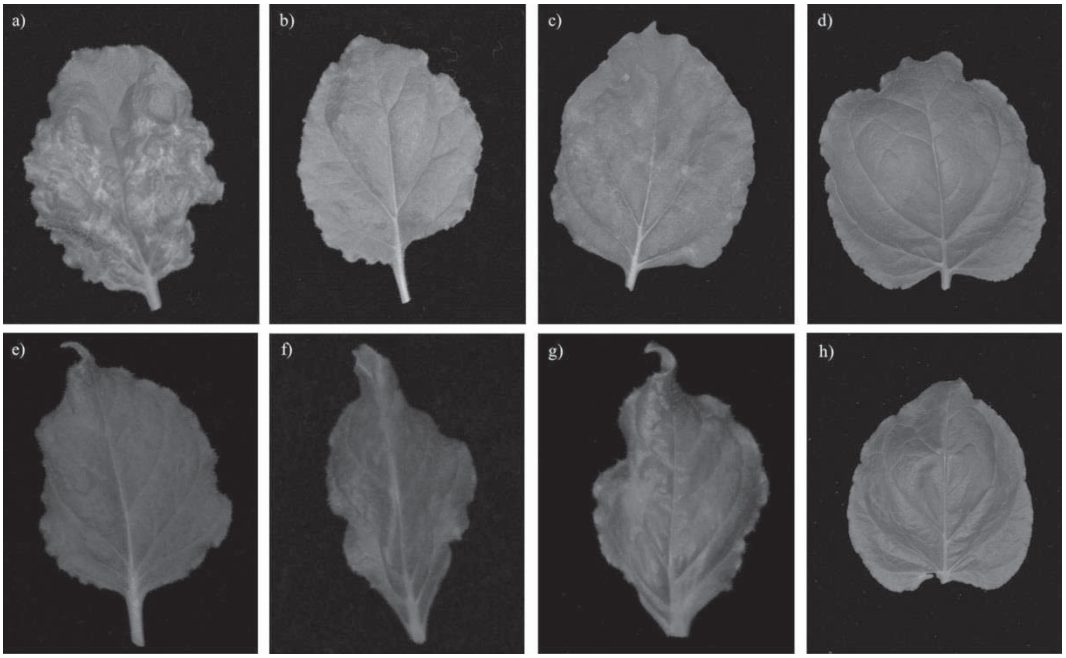

Fig. 4 Systemic symptom expression on *N. benthamiana* upper leaves at 17 days post agroinoculation of (a) BSBMV cDNA clones RNA1-2 (BS12), (b) BNYVV A-type cDNA clones RNA1-2 (BNa12) and pseudorecombinants consisting of (c) BNYVV RNA1+BSBMV RNA2 cDNA clones (BNa1BS2) and (d) BSBMV RNA1+BNYVV RNA2 cDNA clones (BS1BNa2), (e) BNYVV B-type cDNA clones RNA1-2 (BNb12) and pseudorecombinants consisting of (f) BNYVV B-type RNA1+BSBMV RNA2 cDNA clones (BNb1BS2) and (g) BSBMV RNA1+BNYVV B-type RNA2 cDNA clones (BS1BNb2) compared to (h) healthy control.

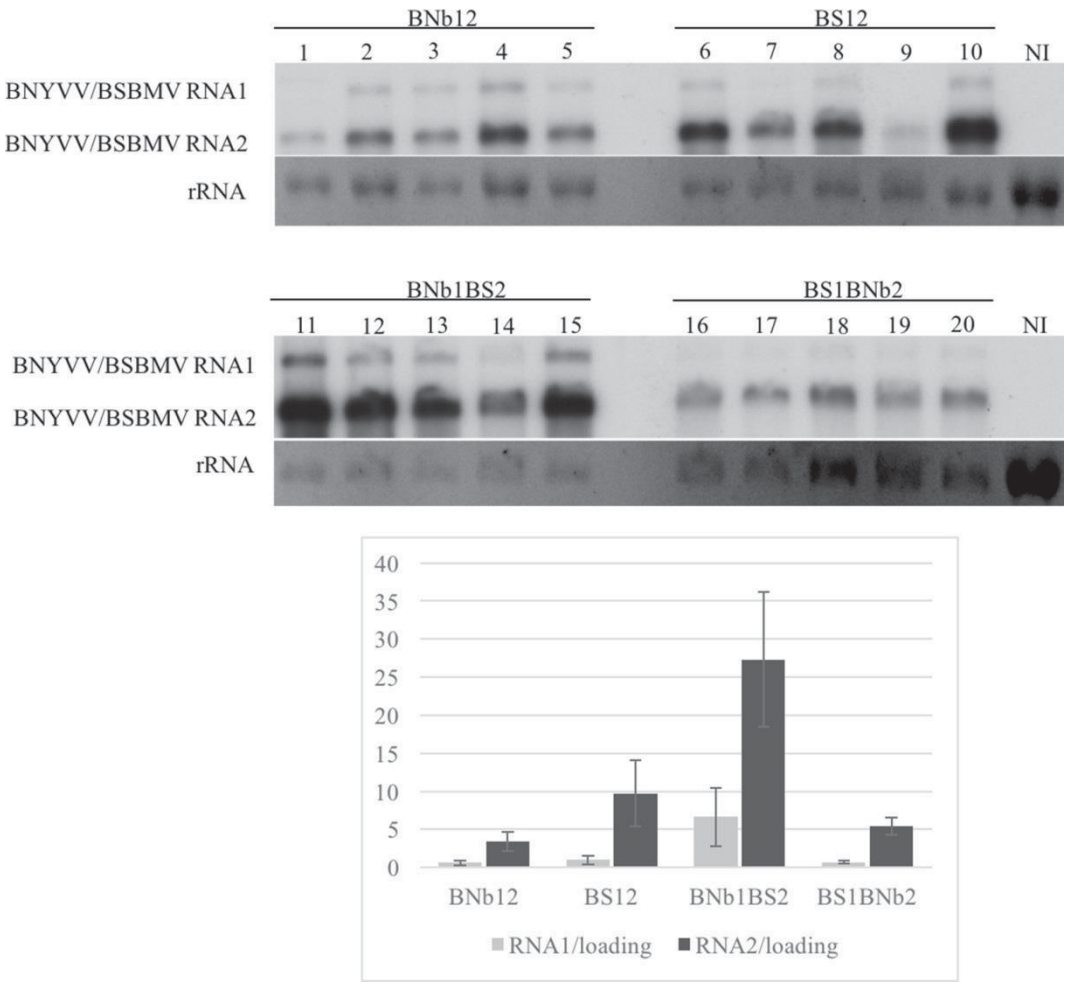

Fig. 5 Northern blot analysis of RNA extracted from local lesions of *C. quinoa* described in Fig. S1. BNYVV and BSBMV RNAs 1 and 2 were detected using specific ^{32}P labeled RNA probes while ribosomal RNAs (rRNA) have been used as loading control. Blotting image has been analyzed by the ImageJ software to quantify the viral RNAs accumulation. Each bar in the presented graph indicates accumulation of BNYVV or BSBMV RNA1 and 2 normalized to the RNA loading (rRNAs)

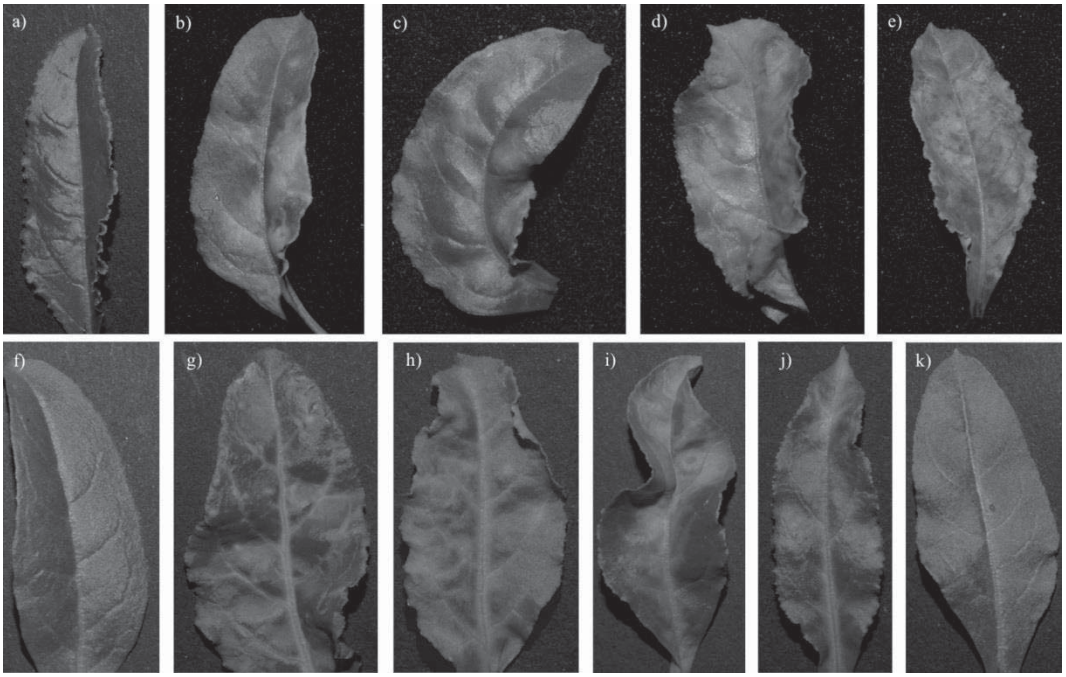

Fig. 6 Systemic symptom expression in *Beta macrocarpa* obtained after agroinoculation of cDNA clones a) BSBMV RNA1+2, b) BSBMV RNA1-3, c) BSBMV RNA1-4, d) BSBMV RNA1+2 plus BNYVV RNA3, e) BSBMV RNA1-3 plus BNYVV RNA4, f) BNYVV RNA1+2, g) BNYVV RNA1-3, h) BNYVV RNA1-4, i) BNYVV RNA1+2 plus BSBMV RNA3, j) BNYVV RNA1-3 plus BSBMV RNA4, compared to k) healthy control at 43 dpi. BNYVV genome components are A-type derived.

Tables

Table 1 Occurrence of local and systemic symptoms in *Nicotiana benthamiana,* systemic infection rate and ELISA values after agroinoculation of BSBMV and BNYVV RNA1+2 (A or B-type) full-length clones compared to RNA1+2 BSBMV/BNYVV pseudorecombinants (see text for nomenclature). * only one plant was infected

Full-length clone	Symptom appearance (dpi)		Efficiency of systemic movement (%)	ELISA values (average)	Systemic symptoms
	Local	Systemic			
BS12	4-6	12-16	100 (9/9)	0.41	Chlorotic veins, leaf crinkling
BNa12	4-6	12-16	100 (9/9)	0.91	Light yellow chlorosis
BNb12	4-6	12-16	100 (9/9)	0.94	Mild yellowing/No symptoms
BNa1BS2	4-6	16-23	100 (9/9)	0.43	Chlorotic veins, leaf crinkling
BS1BNa2	4-6	23-29	11,1 (1/9)	0.83*	Faint leaf crinkling
BNb1BS2	6-7	26-28	77.7 (7/9)	0.40	Mild yellowing/No symptoms
BS1BNb2	6-7	21-23	100 (9/9)	0.86	Necrotic veins, leaf crinkling

Table 2 Development of local and systemic infection in *Beta macrocarpa* after agroinoculation of BSBMV and A-type BNYVV cDNA clones with different RNA composition compared to BSBMV and BNYVV pseudorecombinants.

Full-length clone	Symptoms occurrence (dpi)		Efficiency of systemic movement (%)
	Local	Systemic	
BS12	6-9	-	0 (0/10)
BS1-3	6-9	25-33	50 (5/10)
BS1-4	6-9	25-33	30 (3/10)
BS12BNa3	6-9	19-25	80 (8/10)
BS1-3BNa4	6-9	22-26	70 (7/10)
BNa12	6-9	-	0 (0/10)
BNa1-3	6-9	19-22	90 (9/10)
BNa1-4	6-9	19-22	90 (9/10)
BNa12+BS3	6-9	33-39	80 (8/10)
BNa1-3BS4	6-9	19-22	100 (10/10)

Supporting Information

Table S2 Oligonucleotides used for viral full-length cDNA cloning

genome component and primer name	**sequence (5'- 3')**
BSBMV RNA1	
RNA1-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTTCCCATTCGCCATCATTG
RNA1-low1	CGATCTGACCAAGTGATACCCTT
RNA1-up2	TGTTGGAGAAGTTGATGAAG
RNA1-low2	CATAATAGTAGCCTCCAAAA
RNA1-up3	GCTGATAGTGGTGTGTCTCCAAC
RNA1-low3	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTTATATCAATA
BNYVV RNA1	
RNA1-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCGATTCTTCCCATTC
RNA1-low1	GTGTAGGAATTTTCTGATGTACACCTATTAAC
RNA1-up2	GTTAATAGGTGTACATCAGAAAATTCCTACAC
RNA1-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTTATATCAATATAC

BSBMV RNA2	
RNA2-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTAATTATTATCTCCATTG
RNA2-low1	GAAGACACGTCTAATCTTTCTACTA
RNA2-up2	CGGCAATTAAGTTGGATATAGTAG
RNA2-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTCAATAAACT
BNYVV RNA2	
RNA2-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTAACTATTATCTCC
RNA2-low1	CATTTATACCCATCCTCTACTAGTGTTTTCTC
RNA2-up2	GAGAAAACACTAGTAGAGGATGGGTATAAATG
RNA2-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTCAATATACTG
BSBMV RNA3	
RNA3-up	AGGAAGTTCATTTCATTTGGAGAGGAAATTTAAATCTATCACCACATT
RNA3-low	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTCTTCAATAT
BNYVV RNA3	
RNA3-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAATTTACCATTACATATTG
RNA3-low1	CGAGGGAAATTTGTTGCATTAGGC
RNA3-up2	GCCTAATGCAACAAATTTCCCTCG
RNA3-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTGTCAATATACTGAC
BNYVV RNA4	
RNA4-up	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAACTCAAAAATATAA
RNA4-low	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTAATAAACTG
pDIVA vector amplification	
35S-as	CCTCTCCAAATGAAATGAACTTCCTTATATAG
HDV-s	GGGTCGGCATGGCATCTCCACCTCCTC

Underlined sequences represent the pDIVA vector sequences required for Gibson assembly.

Table S2 Overall nucleotide sequence similarity in percentage (%) of BSBMV-CA genome components RNA1-4 compared to BSBMV isolate EA (NC_003506.1, NC_003503.1, NC_003507.1) and MRM06 (JF513082.1, JF513083.1, EU410955.1, FJ424610.2)

genome components	RNA1	RNA2	RNA3	RNA4
MRM06	99.79	100	99.77	99.48
EA	99.34	99.85	99.88	-*

*Because EA RNA4 sequence represents a deletion variant, it was omitted from the sequence comparison

Table S3 Sequence similarity of the virus encoded proteins on amino acid level of different BSBMV isolates in percentage (%): isolate CA compared to EA and MRM06

Protein	239K	21K	75K	42K	13K	15K	14K	29K	32K
MRM06	99.95	100	100	100	100	100	100	99.22	99.48
EA	97.68	100	99.71	100	100	100	100	100	-

Table S4 Overall nucleotide sequence similarity in percentage (%) of BNYVV A-type isolate Yu2 genome components RNA1-3 and RNA4 from Italian isolate compared to isolates S (NC_003514.1, NC_003515.1, NC_003516.1 NC_003517.1), F2/13 (X05147.1, X04197, M36894, M36896.1) and Pithiviers (HM126464.1, HM117903, DQ682454, DQ682453)

genome components	RNA1	RNA2	RNA3	RNA4
S	99.35	98.69	98.76	95.84
Pithiviers	99.41	98.65	98.37	97.07
F2/13	98.43	95.82	97.35	99.85

Table S5 Sequence similarity of the virus encoded proteins on amino acid level of different BNYVV isolates in percentage (%): A-type isolate Yu2 (RNA1-3) and Italian RNA4 compared to Japanese A-type isolate S, B-type F2/13 and P-type Pithiviers

Protein	237K	21K	75K	42K	13K	15K	14K	25K	31K
S	99.38	100	98.39	99.74	98.31	98.48	95.42	94.52	96.81
Pithiviers	99.67	98.94	97.66	99.74	98.31	97.73	100	95.89	96.10
F2/13	98.91	97.87	95.76	99.74	96.61	96.97	94.49	95.43	99.65

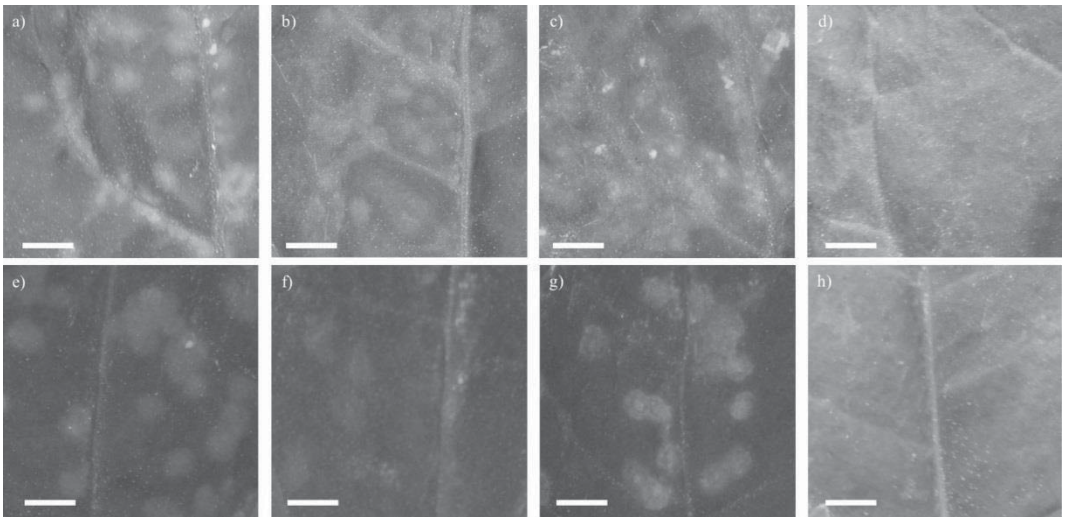

Fig. S1. Local lesions in *C. quinoa* inoculated leaves (7 dpi) obtained by rub-inoculation of sap from *N. benthamiana* leaves infiltrated by (a) BS12, (b) BNa12, (c) BNa1BS2, (d) BS1BNa2, (e) BNb12, (f) BNb1BS2, (g) BS1BNb2, (h) healthy; bars represent 5 mm.

4. Manuscript II

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Fluorescent labelling of Beet necrotic yellow vein virus and Beet soil-borne mosaic virus for co-infection and super-infection experiments in *Nicotiana benthamiana*

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ABSTRACT

Infectious full-length clones of Beet necrotic yellow vein virus (BNYVV) and Beet soil-borne mosaic virus (BSBMV), both genus *Benyvirus*, were used for fluorescent labelling with the objective to study their interaction in co-infection and super-infection experiments. Fluorescent labelling was achieved by replacing a part of the RNA2 encoded coat protein read-through domain with either GFP or mRFP fluorescent marker proteins. This resulted in a translational fusion made of the coat and the fluorescent protein. The labelled viruses were infectious, replicated and moved systemically in *Nicotiana benthamiana*, producing wild-type like symptoms. Virus particles could be observed by electron microscopy demonstrating that the viral read-through domain is dispensable for particle formation. Co-infection experiments revealed a spatial separation of differentially labelled populations of the same as well as different *Benyvirus* species after *N*. *benthamiana* agroinoculation. Identical observations were obtained when Tobacco rattle virus (TRV) was differentially labelled and used for co-infection. In contrast, co-infections of BSBMV with Potato virus X (PVX) or TRV resulted in many co-infected cells without spatial separation. Microprojectile co-bombardment of *N*. *benthamiana* leaves revealed that two differently labelled populations of the same virus co-infected only a few cells before starting to separate. In super-infection experiments in *N*. *benthamiana*, BSBMV and BNYVV were unable to establish a secondary infection in plants that were previously infected with BNYVV or BSBMV. Taken together, this is the first work describing the interaction between two economically important Benyviruses using fluorescence labelled full-length clones.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) belong to the genus *Benyvirus* in the family *Benyviridae* with BNYVV representing the type species [1]. BNYVV is the causal agent of rhizomania, a disease with world-wide distribution in nearly all sugar beet growing areas [2], whereas BSBMV is yet restricted to the United States [3, 4]. Both viruses are transmitted by the soil-borne plasmodiophoromycete *Polymyxa betae*, which produces resting spores that allow the virus to persist in soil for decades [5-6]. Although both viruses are closely related species, symptoms caused in the natural host sugar beet differ considerably. Roots infected with BSBMV appear asymptomatic, whereas light yellow vein banding, mottling, mosaic patterns and slight distortions can be observed on the leaves. In contrast, BNYVV infections are mainly restricted to the root system with characteristic necrosis of vascular veins and massive root proliferation. Foliar symptoms comprise vein yellowing and yellow chlorotic spots. As BNYVV and BSBMV share the same host-range and vector species, mixed infections in sugar beet plants have been identified in various commercial fields [7]. Moreover, reassortment experiments revealed that BSBMV small RNAs (RNA3 and 4) can even be trans-replicated and -encapsidated by BNYVV [8-9].

Mixed infections of related or unrelated viruses can occur after co- or super-infection, depending on the interval between two viruses infecting the same host plant [10-11]. Co-infection is used when two viruses infect a host plant simultaneously [12] leading to an antagonistic or synergistic interaction. On cellular level, two viral populations remain spatially separated (co-infection exclusion) with only a few mixed infected cells when they interact in an antagonistic manner. This exclusion mechanism has been described for a broad range of plant viruses (12-16). However, co-infection can also lead to synergistic interaction with many mixed infected cells without spatial separation when two distantly related viruses are co-infected [12]. The term super-infection is applied when a host plant, which has been previously systemically infected by a primary virus, is subsequently infected with a secondary virus. Synergistic interaction after super-infection occurs between more unrelated viruses, leading to enhanced symptom development and virus replication [13, 17-18]. Moreover, two viruses interacting in a synergistic manner are able to replicate within the same cells [13]. In contrast, related viruses tend to interact in an antagonistic manner which is also referred to as super-infection exclusion. In this case, the infection with a primary virus prevents a subsequent infection with a secondary virus. Until now, relatively little is known about the interaction between BNYVV and BSBMV after co-and super-infection. Wisler *et al.* [19] showed at the whole organism level a suppression of BSBMV by BNYVV in mixed infections of greenhouse-grown sugar beet. However, whether both viruses also remain spatially separated at the cellular level is unknown.

Previous studies, investigating the interaction between viruses used fluorescent labelled full-length clones [12-16, 20-21]. With this approach, the distribution of differentially labelled populations from one or two viruses can be easily visualised using confocal laser scanning microscopy (CLSM). This requires flexible viral genomes that allow the integration of additional coding sequences and expression of fluorescent proteins. BNYVV and BSBMV consist of four single-stranded positive-sense RNAs with a similar genome organisation [2, 3]. Some isolates of BNYVV possess an additional fifth RNA species. RNA1 of both viruses harbour one open reading frame (ORF) responsible for replication of viral RNAs. The first 5´-ORF on RNA2 encodes the 21 kDa major viral coat protein (CP) and terminates with an amber stop codon (UAG) which can undergo suppression leading to a 75 kDa coat protein readthrough (CP-RT) protein referred to as P75. The 54 kDa RT domain of CP-RT following the CP sequence is required for transmission by the fungal vector *P. betae* [22]. The next three overlapping ORFs, named triple gene block (TGB1-3), are responsible for cell-to-cell movement [23] and the last ORF encodes the viral suppressor of RNA silencing [24]. BNYVV RNA3 is involved in long distance movement [25-26] and encodes the pathogenicity factor P25 [27-28]. Similarly, BSBMV RNA3 is also involved in long distance movement and encodes a P29 protein that is probably responsible for virus pathogenicity [8]. The RNA4-encoded BNYVV P31 and BSBMV P32 proteins are responsible for vector transmission [9, 29].

In previous studies, fluorescent labelling of BNYVV and BSBMV was achieved by co-infection with viral replicons based on RNA3 [8] or RNA5 [30]. Erhardt *et al*. [31] integrated the GFP gene into the RNA2 of BNYVV by replacing a part of the RT domain of the P75. Based on previous results, it was assumed that CP-RT is required for efficient virus assembly [32]. However, in this study, labelling of BNYVV and BSBMV full-length clones was achieved by a replacement of the RT domain with different fluorescent proteins, leaving 249 nucleotides of the RT domain upstream of the TGB to act as a subgenomic promoter. The generated clones were tested for systemic infection and symptom expression. The influence of fluorescent protein fusions on particle formation was also investigated. Co- and super-infection experiments with BNYVV and BSBMV as well as two unrelated viruses, namely PVX and *Tobacco rattle virus* (TRV), were conducted in *N*. *benthamiana*. Based on these results, the interaction between two closely related Benyviruses was revealed at cellular and whole-organism level.

RESULTS

Effect of fluorescent labelling on infectivity of full-length clones

Different strategies were developed to identify a suitable position in the genome of BNYVV as well as BSBMV to introduce genes of different fluorescence markers. A deletion of the RT ORF plus marker gene expression under control of the duplicated subgenomic (sg) promotor of TGB1 (P42) failed to give detectable replication (data not shown). Similarly, clones with an insertion between TGB3 (P15) and P14, including doubling of the P14 sg promoter, were not infectious. The replacement of the P29 ORF on RNA3 and RNA4 encoded P32 ORF produced local fluorescence but systemic fluorescence was not observed. Finally, only the replacement of the RT part of the CP-RT by ORFs from fluorescence markers allowed fluorescence labelling of BNYVV/BSBMV. This was achieved by retaining the leaky stop codon and the first two codons of the RT domain followed by the fluorescence marker, two stop codons and the putative sg promotor for the 42 kDa TGB1 protein (Fig. 1b).

Agroinoculation of *N*. *benthamiana* was performed to verify that fluorescent labelling of BNYVV and BSBMV does not negatively interfere with replication, encapsidation and systemic movement. All inoculations were done with RNA1 of BNYVV or BSBMV and the corresponding labelled RNA2 cDNA clone. BSBMV constructs carrying either mRFP or GFP produced systemic symptoms, including chlorotic vein banding, yellow blotches, leaf crinkling and necrosis 16-20 days post inoculation (dpi) (Fig. 2a-b). Similarly, BNYVV carrying either mRFP or GFP also caused systemic infection with symptoms consisting of light yellow chlorosis (Fig. 2c-d). Both viruses displayed no differences in symptom onset and severity compared to the wild type (data not shown).

The virus derived expression of the fluorescent proteins was detected by means of CLSM in leaf tissue displaying symptoms of a systemic infection. A clear homogeneous mRFP expression was observed in plants infected with BSBMV-mRFP (Fig. 2e) and BNYVV-mRFP (Fig. 2g). In contrast,

the fluorescence of GFP expressed by BNYVV or BSBMV was unevenly distributed in small bright clusters (Fig. 2f, h). A similar pattern was observed when the GFP gene was replaced by GFPuv (data not shown). It was assumed that the cluster formation is due to a poor solubility of the fluorescent proteins. Therefore, an alanine to lysine mutation at amino acid position 206 was introduced into the coding sequence with the objective to increase protein solubility [33]. The resulting constructs led to a better fluorescence distribution; however, several small bright clusters remained (data not shown).

Effect of fluorescent labelling on particle formation of BNYVV and BSBMV

Since both CP and CP-RT are components of wild-type virus particles, it was questioned if fluorescent labelling interferes with particle formation. To investigate this, particle composition was studied exemplary for BSBMV by means of transmission electron microscopy (TEM). An RNA2 clone of BSBMV, in which the RT was deleted and the leaky stop codon TAG mutated to TGA, did serve as control (Fig 1c). The presence of rod shaped virus particles in systemically infected *N. benthamiana* leaf tissue could be confirmed for all BSBMV-derived constructs (Fig. 3a-d). No significant differences in particle diameter were observed (20.32 – 21.38nm). Virus particles from BSBMV were also treated with 10 nm colloidal gold-labelled GFP antibodies to localise the GFP protein on the particle surface. A decoration with gold particles was observed, indicating that the fusion protein is incorporated into particles (Fig. 3e, f). No decoration was observed in case of wild type particles.

Co-infection exclusion of differentially labelled viruses

Co-infection exclusion of differentially labelled populations from one or two virus species was studied in *N. benthamiana* systemically infected tissue following agroinoculation. Primary infections were initiated in separate leaves and virus distribution was visualised with CLSM in upper non-inoculated leaves after the expression of systemic symptoms. Depending on the combination of virus populations, the areas of mixed fluorescence varied from marginal overlapping with a single layer to large clusters of cells. When two differentially labelled clones of BSBMV (BSBMV-GFP + BSBMV-mRFP) were co-inoculated, the presence of both virus populations could be confirmed by a clear fluorescence of the two reporter proteins (Fig. 4a-d). Merged images showed that both virus populations colonised distinct areas in systemically infected leaves, clearly indicative for a spatial separation. High resolution imaging of the border separating both viral populations showed that mixed fluorescence (yellow) was restricted to a few cells. This separation effect was also observed in leaves systemically infected by BSBMV-mRFP and BNYVV-GFP (Fig. 4e-h). Thus all combinations of differentially labelled populations from one or two virus species belonging to the genus *Benyvirus* led to a spatial separation. To prove whether the exclusion effect can also be triggered by a viral reassortant, BNYVV-RNA1 and BSBMV-RNA2-mRFP was co-inoculated with

BNYVV-GFP (Fig. 4i-l). Visualisation of viral populations in systemically infected leaves again showed a clear separation of both populations. A reassortant made of BSBMV-RNA1 and BNYVV-mRFP was not infectious and therefore could not be tested. PVX (Genus *Potexvirus*) and TRV (Genus *Tobravirus*) were included in the experiments, representing two unrelated viruses. BSBMV-mRFP was either co-inoculated with TRV-GFPuv or with PVX-GFPuv. Confocal imaging revealed that virus populations in the combinations BSBMV-mRFP + TRV-GFPuv (Fig. 4m-o) and BSBMV-mRFP + PVX-GFPuv (Fig. 4q-s) infected the same areas in systemically infected leaf tissues. Close-ups of mesophyll cells showed a high number of cells displaying yellow fluorescence, indicating that both viruses replicate within the same cell (Fig. 4p, t).

As agroinoculation was used for cDNA clone inoculation, it was not possible to show when both viral populations start to separate if the infection is initiated from a single mixed infected cell. Therefore, co-infection exclusion was also studied using microprojectile co-bombardment of viral cDNA clones that allows initiating a mixed infection in a single cell. TRV-dsRED and TRV-GFPuv were chosen for this experiment as they displayed the highest infection rate and fasted fluorescent signal development after particle bombardment on detached leaves. When cDNA clones TRV-dsRED and TRV-GFPuv were co-bombarded, both viral populations replicated in distinct areas after 2 dpi (Fig. 5c). High resolution imaging of the border between both viral populations showed a clear spatial separation (Fig. 5d). Primary infection sites could be identified by the yellow appearance of single mesophyll cells (indicated by arrows) (Fig. 5e-h). Mixed infected cells were surrounded by spatially separated viral populations, indicating a rapid onset of exclusion after co-infection of single cells following bombardment. Combinations of two unrelated viruses were also co-bombarded as control. In case of BSBMV-mRFP and PVX-GFPuv, both viral populations were identified in the same area indicated by many co-infected cells displaying yellow fluorescence (Fig. 5k-l). A similar pattern was observed for TRV-dsRED co-infected with PVX-GFPuv (Fig. 5o-p).

Super-infection exclusion of differentially labelled viruses

Differentially labelled full-length clones were also applied to study the interaction between BNYVV and BSBMV in super-infection exclusion experiments. *N. benthamiana* plants were inoculated with BNYVV-GFP as the primary virus. After 21 dpi, leaves displaying systemic symptoms and fluorescence were mechanically super-inoculated with BNYVV-mRFP, BSBMV-mRFP, PVX-dsRED or TRV-dsRED as secondary viruses. Occurrence of virus expressed fluorescence was analysed in inoculated and upper non-inoculated leaves displaying systemic symptoms at 42 dpi (Table 1). When plants were super-infected with BNYVV-mRFP or BSBMV-mRFP as secondary virus, small fluorescence clusters of BNYVV-mRFP and BSBMV-mRFP were observed in inoculated leaves. However, only green fluorescence generated by the primary virus BNYVV-GFP was detected in newly emerged leaves, thus indicating that the secondary virus could not establish a systemic infection. In contrast, super-infection with the two unrelated viruses PVX-dsRED or

TRV-dsRED resulted in a mixed fluorescence in newly emerged leaves, demonstrating that the secondary virus could successfully establish an infection and moved systemically to non-inoculated upper leaves. The infectivity of the inoculum used for super-infection was additionally confirmed by parallel mechanical inoculation of healthy *N. benthamiana* plants.

DISCUSSION

In this study, fluorescently labelled viruses derived from full length-clones of two closely related Benyviruses, namely BNYVV and BSBMV, were developed. Labelling was achieved by a deletion of the RT domain and replacement with different fluorescent marker genes. Fluorescent labelling did not hamper the infectivity as both viruses moved systemically, induced characteristic disease symptoms. It can be concluded that the RT domain including the P75 minor coat protein is dispensable for systemic infection and symptom development. Furthermore, the presence of virus particles in TEM analysis demonstrated that particle formation is not prevented by the absence of the RT domain and the incorporation of the fluorescent protein. The diameter of particles from modified viruses was indistinguishable from the wild type. The suppression of the leaky stop codon occurs at an estimated rate of 10% when ribosomes encounter it [32]. This probably leads to a partial incorporation of the CP-fluorescent marker fusion protein that cannot be visualized by standard TEM.

The fluorescence of mRFP expressed by BNYVV and BSBMV was clear and homogeneously distributed throughout the cytoplasm of infected cells, whereas the fluorescence of GFP was unevenly distributed in small bright clusters indicating that the CP-GFP fusion interfered somehow with subcellular localisation. It was suspected that the clusters represent virus particles that localise to mitochondria as reported by Erhardt *et al*. [31], but this can be excluded because the mitochondria localisation signal, previously demonstrated to localize to the RT [34], was removed by the replacement with the marker. It was also reported by Erhardt *et al*. [31] that at later times during infection virus particles relocated to semi-ordered clusters in the cytoplasm. However, we assume that the CP fusion to GFP reduced the solubility. Although the fluorescence could be improved by a mutation in the coding sequencing, cluster formation was not completely prevented.

The results from co-infection experiments showed that populations of identical, but differentially labelled Benyviruses replicated predominantly in discrete areas. The same spatial separation was observed when BNYVV and BSBMV were co-inoculated. The presence of both viruses was restricted to a few cells, indicating that both viral species, in principle, can replicate within the same cell. A similar observation was reported with populations of identical but differentially labelled Potyviruses [12]. A pseudorecombinant viral population consisting of BNYVV-RNA1 + BSBMV-RNA2-mRFP inoculated together with BNYVV-GFP showed also a clear spatial separation, indicating that trans-replication does not prevent triggering the exclusion mechanism. In contrast, viral populations of BSBMV and two distantly related viruses (PVX and TRV) showed large leaf

areas with mixed fluorescence. This confirms previous observations that viral species of the same family remain spatially separated whereas viral species belonging to different families co-infect the same cells [12, 16]. An identical pattern was observed after particle bombardment of detached leaves confirming that spatial separation occurs in primary and systemically infected leaves [12]. Co-bombardment revealed that two viral populations co-infected only a few cells before they started to separate. This observation should be confirmed by protoplast inoculation to prove that both species can indeed replicate within the same cell. Furthermore, a local lesion host like *Chenopodium quinoa* could help to determine the point of separation of BNYVV and BSBMV as movement is restricted to a few cells.

The exclusion pattern of BNYVV and BSBMV in super-infection experiments was similar to the observations in co-infection experiments. Neither BNYVV-mRFP nor BSBMV-mRFP could establish a secondary infection in *N. benthamiana* plants previously infected with BNYVV, even though small fluorescence clusters of the secondary viruses could be observed in super-infected leaves. This indicates that virus replication was possible in primary infected cells but systemic movement was inhibited. It has to be emphasised here that the absence of fluorescence from the secondary virus can be also due to a mutation or deletion in the coding sequence of the fluorescent protein. However, we have never observed this in our experiments and all control plants showed fluorescence in systemic infected leaves. Different mechanisms have been proposed for the exclusion of two viral species after super-infection. It was shown very early that super-infection exclusion can be elicited by RNA silencing [35]. More recently, a viral protein from *Citrus tristeza virus* (p33) was identified that mediates super-infection exclusion at the whole-organism level but not on cellular level [20, 36]. Similarly, Tatineni & French [13] demonstrated that *Wheat streak mosaic virus* and *Triticum mosaic virus* encoded CP and NIa-Pro proteins trigger super-infection exclusion independently of each other. Moreover, recent findings on *Turnip crinkle virus* support the idea that superinfection-exclusion is a mechanism that prevents progeny viruses replicating their genomes in the cells of their “parents” and highly similar superinfecting viruses that are indistinguishable from progeny viruses are collaterally targeted [37-38]. In case of BNYVV and BSBMV, the percentage of identical amino acids is highest for the RdRp (92%) on RNA1 and ranges between 32% and 81% for the proteins on RNA2 [4]. This similarity is sufficient to form viable RNA1 and RNA2 reassortments between both viruses [39] indicating that both viruses share a highly similar replication and movement strategy. Further studies will be needed to identify the viral protein responsible for the exclusion.

The data presented here provide the first evidence that BNYVV and BSBMV remain spatially separated when colonising the same host plant. Considering that BNYVV is the nearest known relative of BSBMV, co-and super-infection exclusion of both viral species seems to be plausible in terms of virus evolution. The ability of viral variants to exclude each other in mixed infections eliminates the competition for the host’s resources. Furthermore, it has great implications for the

stability of viral sequences and the genetic structure of a virus population [20]. Newly emerging viral variants have a benefit by favouring uninfected cells rather than already infected host cells [11]. However, replication of two or more viral genomes in one cell increases the likelihood of recombination and reassortments. This is of particular relevance as it can increase the genetic diversity within a viral population, leading to new viral variants. Considering the results of the present study, the likelihood of recombination and pseudorecombinants between BNYVV and BSBMV in mixed infections seems to be relatively low due to the spatial separation on cellular level. Moreover, both viral species seem to have developed different colonisation strategies in their host sugar beet as BNYVV infections are mainly restricted to the root while BSBMV causes more systemic foliar symptoms [3]. The fluorescence labelled viruses derived from full length-clones developed in this study represents versatile tools to address this question in the natural host sugar beet.

METHODS

Construction of fluorescent labelled full-length clones

Both BSBMV and BNYVV RNA2 cDNA clones for agroinoculation [39] were modified to express mRFP [40] and GFP [41]. Labelling was achieved by replacing the readthrough part of the CP-RT, thereby retaining the leaky stop codon of the CP and the first two codons of the RT domain. Replacement of the RT by the different fluorescent proteins was followed by two stop codons (TGATAG) and the remaining 249 nucleotides of the CP-RT, containing the putative sg promotor for the 42 kDa TGB1 (Fig. 1b). This modification resulted in a large read-through protein made of the CP and the fluorescent protein. The DNA fragments of fluorescent marker genes coding sequences were cloned into linearised plasmids of BNYVV/BSBMV RNA2 clones by means of Gibson assembly [42]. The marker genes coding sequences were amplified with Phusion Flash High-Fidelity PCR Master Mix (ThermoScientific) according to the manufacturer's instructions. Specific primers (Supplementary Table 1) contained 5´- and 3´-extensions overlapping with RNA2 of BNYVV/BSBMV (25-28 nt). The BNYVV/BSBMV RNA2 clones were linearised by PCR amplification using primers BNYVV2-s/BNYVV2-as and BSBMV2-s/BSBMV2-as (Supplementary Table 1). All PCR products were gel-purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. After Gibson assembly, *in vitro* recombination products were transformed into chemical competent *Escherichia coli* cells (strain DH5α) as described by Inoue *et al*. [43]. Plasmids carrying cDNA fragments of the fluorescent proteins were identified by means of appropriate restriction enzyme digest and all mutations were verified by commercial capillary Sanger sequencing (Eurofins MWG Operon). The resulting clones were named BNYVV-RNA2-mRFP/-GFP and BSBMV-RNA2-mRFP/-GFP

To study the effect of the RT deletion on particle assembly, the RT coding sequence was deleted from the BSBMV RNA2 cDNA clone. The leaky stop codon TAG was mutated to TGA. The RT

sequence downstream of the stop codon was deleted except for the last 249 nucleotides of the RT. This was achieved by PCR amplification of BSBMV-RNA2 with the primers BSBMV-deltaRT-fw and BSBMV-deltaRT-rv (Supplementary Table 1). The resulting clone was named BSBMV-RNA2-deltaRT.

The infectivity of the above mentioned constructs was tested in *N. benthamiana* using agroinoculation. For this purpose, viral cDNA clones of RNA1 and RNA2 were transformed into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) strain C58C1. Bacterial cultures were prepared according to Voinnet *et al*. [44] with an optical density at 600 nm (OD_{600}) of 0.1. The first two pairs of true leaves were inoculated. Different cDNA components from multipartite viruses were mixed in a 1:1 ratio prior to inoculation. All plants were grown under greenhouse conditions with 24°C for 14h and 18°C for 10h. CLSM (see below) was applied to visualise fluorescent labelled full-length clones in systemically infected leaf tissue.

Co-infection and super-infection exclusion of BNYVV, BSBMV, PVX and TRV

Co-infection exclusion was studied with BNYVV/BSBMV and two unrelated viruses (PVX and TRV). PVX-GFPuv was constructed as described by Draghici & Varrelmann [45] using a PVX expression plasmid obtained from David Baulcombe (Sainsbury Laboratory, Norwich, United Kingdom). The construction of the PVX vector expressing dsRED has been described previously (PVX201-*optRed*) [46]. TRV vectors composed of pTRV1 and pTRV2 (pYL156) [47] were modified as described by Ghazala & Varrelmann [49] to express either dsRED (TRV-dsRED) or GFPuv (TRV-GFPuv). Two differentially labelled viruses were inoculated simultaneously but in separate leaves of 3-week old *N. benthamiana* plants using agroinoculation as described above. After symptom development, virus distribution was visualised in systemically infected leaf tissue by means of CLSM. Particle bombardment was also applied to study the spread of differentially labelled viruses starting from a single doubly infected mesophyll cell. Detached leaves from 4- to 5-week old *N. benthamiana* plants were subjected to microprojectile co-bombardment with a particle inflow gun [49] using 10 µl purified plasmid DNA corresponding to each viral RNA component. Following bombardment, detached leaves were placed in a petri dish with watered filter paper and incubated at room temperature in the dark. Virus distribution was visualised with CLSM after 2-5 days.

Super-infection exclusion experiments were performed in *N. benthamiana* plants using BNYVV-GFP as primary virus and BNYVV-mRFP, BSBMV-mRFP, TRV-dsRED as well as PVX-dsRED, respectively, as secondary virus. *N. benthamiana* plants were first infected with BNYVV-GFP using agroinoculation as described above. After three weeks, leaves displaying systemic symptoms were mechanically super-inoculated with the challenging virus. Prior to the secondary infection, the establishment of the primary infection was confirmed by means of CLSM. The inoculum for the secondary infection was produced in *N. benthamiana* using agroinoculation as described above.

Leaves displaying systemic symptoms were grinded in phosphate buffer (10 mM Na_2SO_3, pH 7.0) and rub-inoculated on *N. benthamiana* leaves infected with the protecting virus. After another three weeks, the establishment of the secondary infection was checked in inoculated and upper non-inoculated leaves using CLSM. Each variant comprised of five repetitions.

Confocal laser scanning microscopy

Systemically infected leaf tissue from *B*. *macrocarpa* and *N*. *benthamiana* was harvested and visualised with the TCS-SP5 confocal laser scanning microscope (Leica Microsystems). Excitation/emission wavelengths for the different fluorescent proteins were as follows: mRFP 561 nm/520-540 nm, dsRED 561 nm/520-540 nm, GFP 488 nm/515-523 nm and GFPuv 405 nm/490-520 nm. All confocal images were processed with the LAS-AF software version 2.6.3.8173 (Leica Microsystems).

Transmission electron microscopy

For negative staining of virus particles, sample material from *N*. *benthamiana* was bound to a glow discharged carbon foil covered grid. After staining with 1% uranyl acetate the samples were evaluated at room temperature with a CM 120 transmission electron microscope (FEI). Summed averaged images of the virions were calculated using the software RELION (MRC Laboratory of Molecular Biology). Overall, 7000 individual overlapping segments of the virions, respectively, were boxed using RELION. The images were sorted by MSA and summed to obtain a class average image of the virions. For immunogold labelling, sample material from *N*. *benthamiana* was absorbed to formvar-carbon coated Ni-grids. They were fixed with 4% paraformaldehyde, quenched with 20 mM glycin, and immunostained using the described sera, followed by addition of Protein A-gold (10 nm). The preparations were then washed repeatedly with TPBS and high-salt TPBS (0.5 M NaCl) and post-fixed with 2% glutaraldehyde. After counterstaining with 1% uranylacetate, samples were investigated using a CM120 Philips electron microscope using a TemCam F416 CMOS camera (TVIPS).

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Conflict of interest

The authors declare that there are no conflicts of interest

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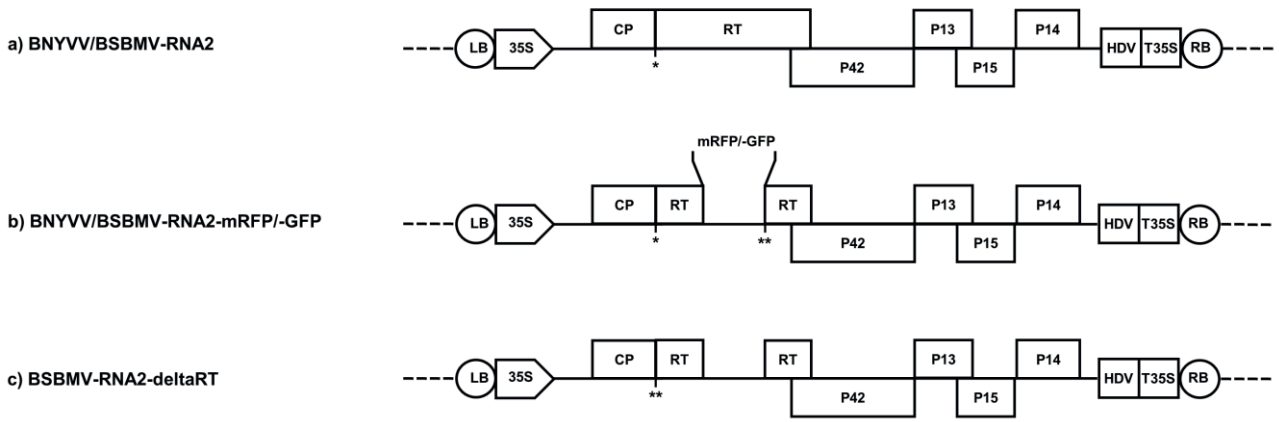

Fig. 1: Schematic representation of infectious full-length cDNA clones of BNYVV-/BSBMV-RNA2 (a) and modified variants carrying either a fluorescent marker gene (b) or a deletion in the RT-ORF (c). LB: Left border; 35S: *Cauliflower mosaic virus* (CaMV) 35S promoter; CP: coat protein for encapsidation; RT: read-through domain for transmission; P42, P13 and P15: triple gene block for movement; P14: viral silencing suppressor; mRFP: monomeric red fluorescent protein; GFP: soluble modified red-shifted green fluorescent protein;; HDV: *Hepatitis delta virus* ribozyme; T35S: CaMV Terminator 35S; RB: Right border; *: leaky stop codon; **: stop codon.

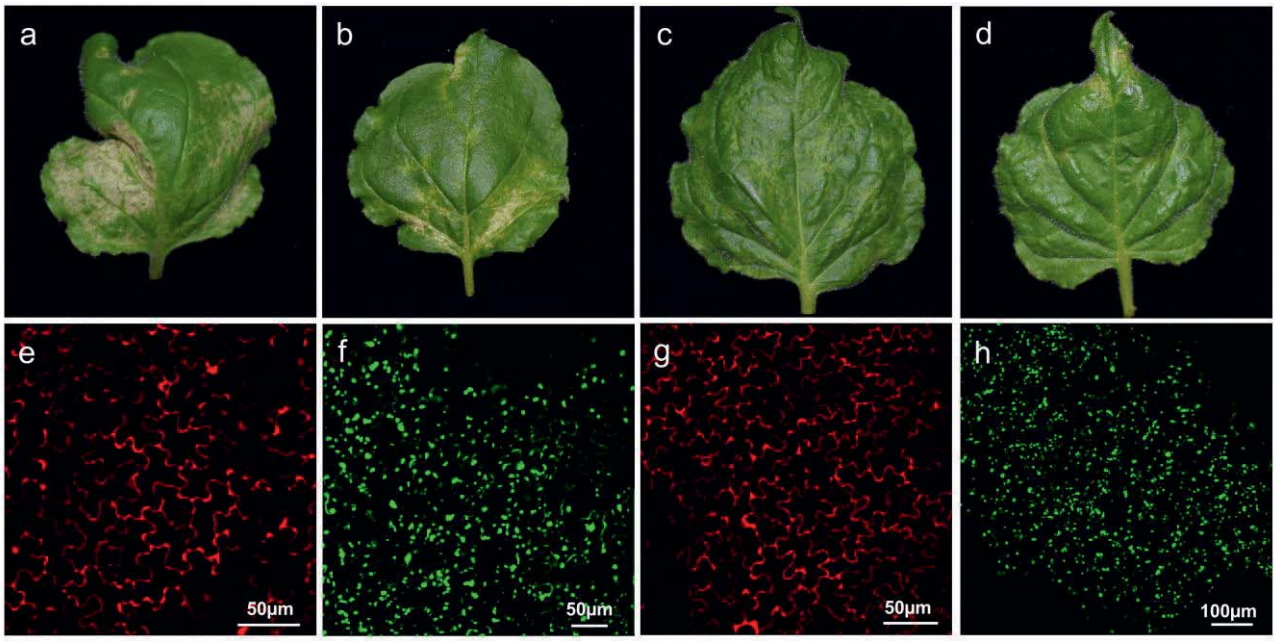

Fig. 2: *N. benthamiana* leaves displaying systemic symptoms following agroinoculation (16-24 dpi) with RNA1-2 cDNA clones of (a) BSBMV-mRFP, (b) BSBMV-GFP, (c) BNYVV-mRFP and (d) BNYVV-GFP. Confocal imaging of fluorescence in *N. benthamiana* leaf tissue is shown for (e) BSBMV-mRFP, (f) BSBMV-GFP, (g) BNYVV-mRFP and (h) BNYVV-GFP. Bars indicate the selected scale.

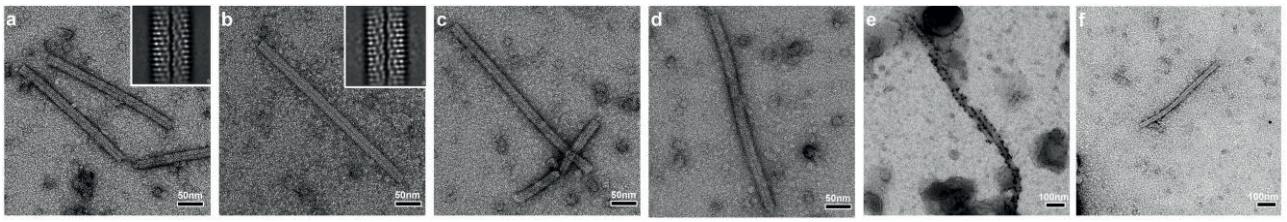

Fig. 3: Electron microscope images after negative staining of virus particles derived from the RNA1 and 2 cDNA clones of (a) BSBMV, (b) BSBMV-deltaRT, (c) BSBMV-GFP and BSBMV-mRFP (d). Virus particles of BSBMV-GFP (e) and BSBMV (f) were also treated with 10 nm colloidal gold-labelled GFP antibodies. All virus particles were isolated from leaf tissue displaying systemic symptoms. Bars indicate the selected scale.

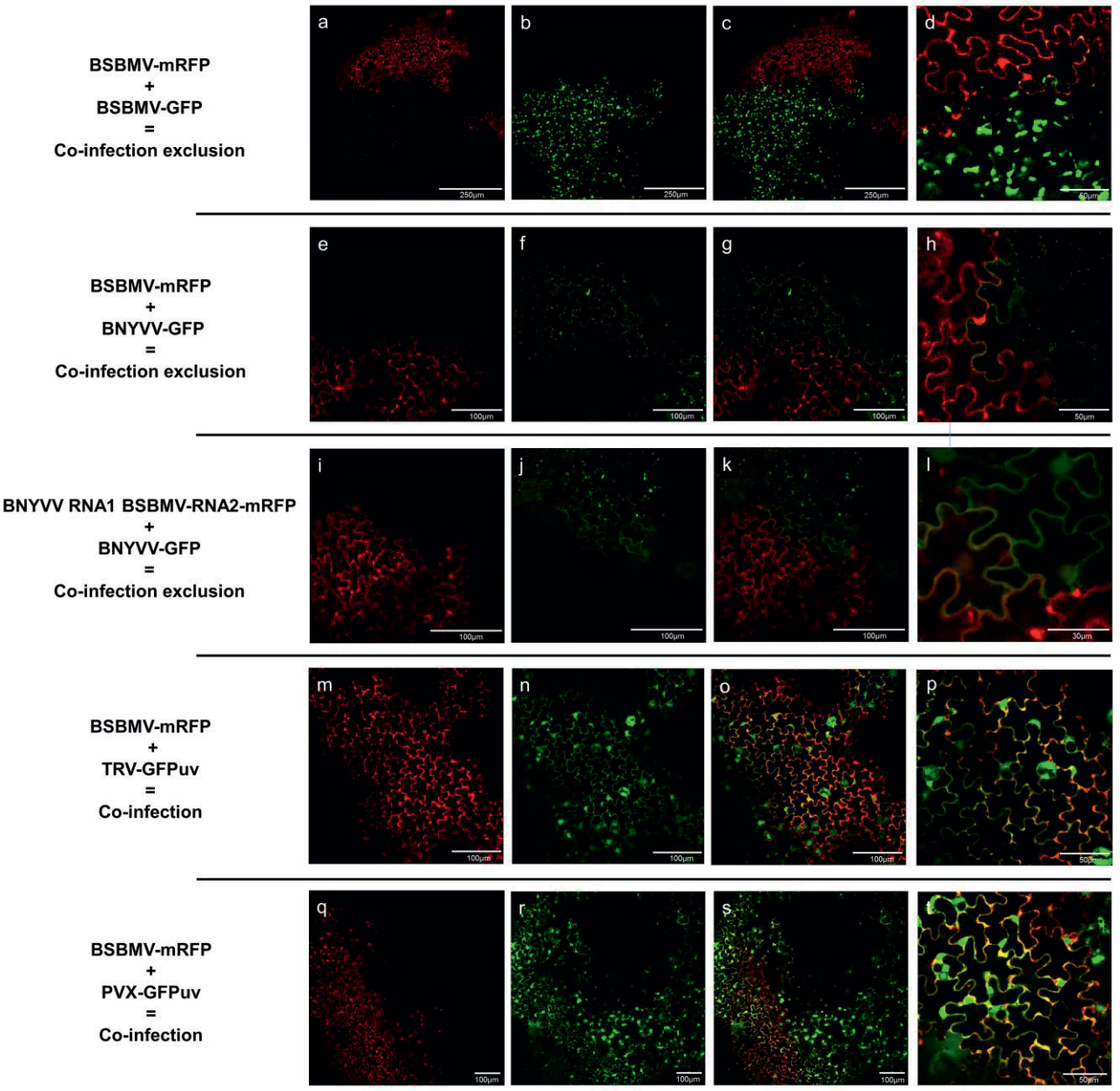

Fig. 4: Virus distribution in systemically infected *N. benthamiana* tissues following agroinoculation with cDNA clones of BSBMV-mRFP (a) + BSBMV-GFP (b); BSBMV-mRFP (e) + BNYVV-GFP (f); BNYVV RNA1 BSBMV-RNA2-mRFP (i) + BNYVV-GFP (j); BSBMV-mRFP (m) + TRV-GFPuv (n) and BSBMV-mRFP (q) + PVX-GFPuv (r). Confocal images (c), (g), (k), (o) and (s) are merged

images of RFP (a, e, i, m, q) and GFP (b, f, j, n, r) channels. Spatially separated populations are indicated by differently coloured fluorescence clusters (c, g, k), whereas mixed populations show large yellow clusters (o, s). Close-ups of co-infected mesophyll cells are shown in (d), (h), (l), (p) and (t). Co-infection in spatially separated populations is restricted to a few cells at the border between both populations (d, h, l), whereas mixed populations show many co-infected cells (p, t). Bars indicate the selected scale.

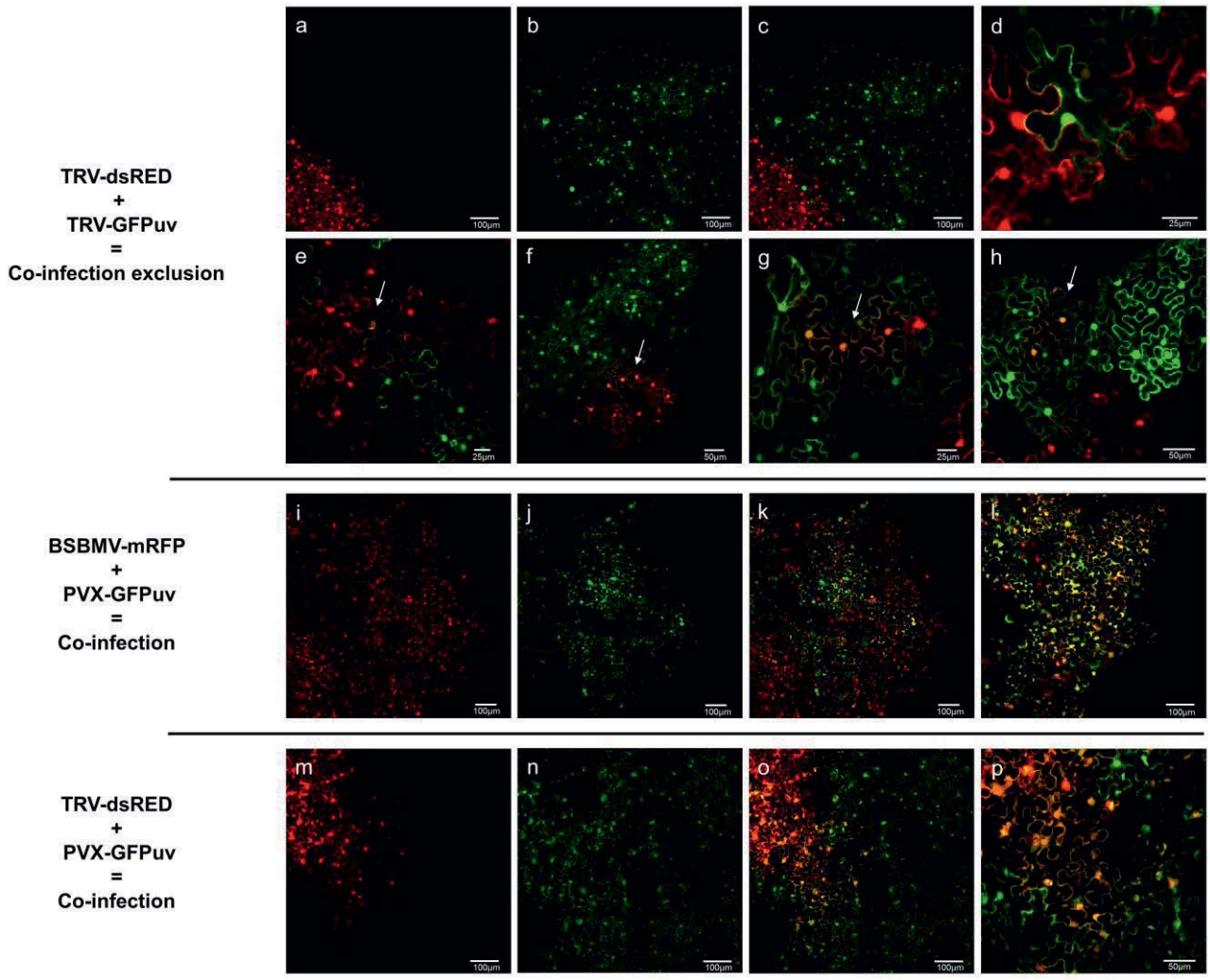

Fig. 5: Virus distribution in detached leaves of *N. benthamiana* after microprojectile co-bombardment with cDNA clones of TRV-dsRED (a) + TRV-GFPuv (c); BSBMV-mRFP (i) + PVX-GFPuv (j) and TRV-dsRED (m) + PVX-GFPuv (n). Confocal images (d-h), (k-l) and (o-p) are merged images of RFP (a, i, m) and GFP (b, j, n) channels. After co-bombardment, differentially labelled virus populations of TRV started cell-to-cell movement from a single infected cell that appears yellow (e-h). Both viral populations co-infected a few cells (indicated by arrows) and then started to separate, leading to spatially separated populations (c-d). In contrast, co-bombardment of BSBMV-mRFP with PVX-GFPuv and TRV-dsRED with PVX-GFPuv led to mixed populations, represented by large yellow clusters (k, o). Close-ups of co-infected cells are shown in (l) and (p). Bars indicate the selected scale.

Table 1: Evaluation of super-infection experiments with fluorescently labelled full-length clones BNYVV-GFP/-mRFP, BSBMV-mRFP, PVX-dsRED and TRV-dsRED in N. benthamiana. Plants were infected with a primary virus and after 21 dpi, leaves displaying systemic symptoms were mechanically super-inoculated with a secondary virus. Virus fluorescence was then evaluated in upper, non-inoculated leaves after 42 dpi with the primary virus. Each variant comprised five repetitions.

Primary virus	**Secondary virus**	**Viral fluorescence detected in non-inoculated leaves after mechanical inoculation with the secondary virus**
BNYVV-GFP	BNYVV-mRFP	BNYVV-GFP
BNYVV-GFP	BSBMV-mRFP	BNYVV-GFP
BNYVV-GFP	PVX-dsRED	BNYVV-GFP + PVX-dsRED[b]
BNYVV-GFP	TRV-dsRED	BNYVV-GFP + TRV-dsRED[b]
Healthy	BNYVV-mRFP	BNYVV-mRFP
Healthy	BSBMV-mRFP	BSBMV-mRFP
Healthy	PVX-dsRED	PVX-dsRED
Healthy	TRV-dsRED	TRV-dsRED
BNYVV	-	No fluorescences observed
BSBMV	-	No fluorescences observed
Mock[a]	-	No fluorescences observed
Healthy control	-	No fluorescences observed

-: Non inoculated.

[a]: Plants inoculated with phosphate buffer.

[b]: Mixed viral populations without spatial separation.

Supplementary Table 1: Sequences of oligonucleotides used for generation of viral cDNA constructs. Underlined bases at the 3'-end represent fluorescent marker gene sequences.

Plasmid	Primers	Sequence (5'- 3')
BSBMV-RNA2-mRFP	BSBMV2-mRFP-s	CGTTCCACGCACCAATTAGCAATTAATGGCCTCCTCCGAGGACGTCA
	BSBMV2-mRFP-as	AACACCACGTGTTCGTAACATACTATCAGGCGCCGGTGGAGTGGCGG
BSBMV-RNA2-GFP	BSBMV2-smRSGFP-s	CGTTCCACGCACCAATTAGCAATTAATGAGTAAAGGAGAAGAACT
	BSBMV2-smRSGFP-as	AACACCACGTGTTCGTAACATACTATCATTTGTATAGTTCATCCA
BSBMV-RNA2-deltaRT	BSBMV-deltaRT-fw	ATGGCGCGCCTCAATTGGTGCGTGGAACGGCAGGAGTAACACCCC
	BSBMV-deltaRT-rv	GATTAATTAA TAGTATGTTACGAACACCGTGGTGTTAGTAATA
Linearisation of BSBMV-RNA2	BSBMV2-s	TAGTATGTTACGAACACGTGGTGTTAGTAATA
	BSBMV2-as	TAATTGCTAATTGGTGCGTGGAACGGCAGGAG
BNYVV-RNA2-mRFP	BNYVV2-mRFP-s	GAACCAGTCCACCCGGACAATAGCAATTAATGGCCTCCTCCGAGGAC
	BNYVV2-mRFP-as	GAAGAACCAGCCCCACGTCTATCAGGCGCCGGTGGAGTGGCG
BNYVV-RNA2-GFP	BNYVV2-smRSGFP-s	GAACCAGTCCACCCGGACAATAGCAATTAATGAGTAAAGGAGAAGAAC
	BNYVV2-smRSGFP-as	GAAGAACCAGCCCCACGTCTACTATTTGTATAGTTCATCCATGCC
Linearisation of BNYVV-RNA2	BNYVV2_s	ACGTGGGGCTGGTTCTTC
	BNYVV2_as	TTGTCCGGGTGGACTGGTTC

5. Manuscript III

will be submitted to Journal of General Virology

Short Communication

First description of *Beet necrotic yellow vein virus* and *Beet soil-borne mosaic virus* as tools for virus-induced gene silencing

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Abstract

The RNA2 encoded read-through domain (RTD) of *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) is dispensable for virus encapsidation, systemic colonisation and symptom development in *Beta macrocarpa* (*B. macrocarpa*) and *Nicotiana benthamiana* (*N. benthamiana*). Therefore, we tested if the RTD in infectious full-length clones of BNYVV and BSBMV can be replaced by untranslatable cDNA fragments from *magnesium chelatase H subunit* (*chlH*) and *phytoene desaturase* (*pds*), repectively, to create a virus-induced gene silencing system (VIGS). Agroinoculation of *N. benthamiana* resulted in systemic infection and development of a photobleaching phenotype with green and white/yellow leaves, indicative for systemic virus movement and silencing of *chlH* or *pds*. Quantitative real-time PCR displayed significant reductions in plant derived *pds* (59-77%) and *chlH* (67-85%) transcripts in the photobleached leaves of both BSBMV and BNYVV VIGS-treated plants, respectively. VIGS-constructs with sense or antisense fragments displayed similar silencing efficiencies indicating that development of *chlH* or *pds*-VIGS is independent of insert orientation.

The creation of transgenic plants to study gene functions by downregulation of gene expression is often laborious and time-consuming, particularly for non-model plant species (Robertson, 2004). Virus-induced gene silencing (VIGS) constitutes an alternative approach by exploiting the mechanism of RNA interference (RNAi) to achieve downregulation of a target gene. RNAi is a genetically conserved mechanism involved in several biological processes like regulation of gene expression, maintaining genome integrity and adaptive responses to abiotic and biotic stresses as well as in antiviral defence (Brodersen and Voinnet, 2006; Li and Ding, 2006; Meister and Tuschl, 2004). During RNAi, dsRNA is cleaved into small interfering RNAs (siRNAs) of 21-25 nucleotides by the RNase-like enzyme DICER. One strand of the siRNA is subsequently incorporated into the RNA-induced silencing complex (RISC). RISC targets specific single-stranded mRNA transcripts complementary to the siRNA. This procedure leads to degradation or a reduction in the accumulation of the target mRNA (Unver and Budak, 2009). In the last twenty years, several VIGS-vectors based on DNA and RNA viruses have been constructed to silence target genes in diverse plants species including also many non-model plants (Hiriart et al., 2002; Ratcliff et al., 2001; Robertson, 2004). Nowadays, VIGS is widely used because of its easy handling and the short time necessary to establish a phenotype (Robertson, 2004).

Beet necrotic yellow vein virus (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) are members of the genus *Benyvirus* in the family *Benyviridae* and naturally infect plant species in the family of *Amaranthaceae* and *Chenopodiaceae* (Gilmer et al., 2017). Both viruses are transmitted by zoospores of the plasmodiophorid *Polymyxa betae* (*P. betae*) (Adams et al., 2001). The two viruses possess a similar genome organisation and particle morphology but display sufficient sequence variability to be assigned to different species (Lee et al., 2001; Ratti et al., 2009). Both BNYVV and BSBMV possess a multipartite RNA genome, which is composed of four plus-sense single stranded RNAs. RNA1 is associated with the replication of viral RNAs, it possess one single open reading frame (ORF) encoding a 237 kDa protein that includes motifs for a helicase (HEL), methyltransferase (MTR), RNA-dependent RNA polymerase (RdRp) and a papain-like protease (PRO) (Link et al., 2005; Peltier et al., 2008). RNA2 encodes six proteins. At the 5′-terminus the cistron for the coat protein (CP) is located, terminated by a leaky stop codon and followed by the read-through domain (RTD), the triple gene block cluster (TGB 1-3) and the cistron for the suppressor of gene silencing (Haeberle et al., 1994). Proteins of RNA2 have a function in virus encapsidation, vector transmission, cell-to-cell movement, replication and suppression of posttranscriptional gene silencing (PTGS) (Dunoyer et al., 2002; Richards and Tamada, 1992). Beside RNA1 and RNA2, BNYVV and BSBMV contain two additional smaller RNAs: RNA3 and RNA4. RNA3 is important for the development of rhizomania symptoms in roots of sugar beet, whereas RNA4 is involved in virus transmission by *P. betae* (Chiba et al., 2008; Jupin et al., 1992). In

principle, RNA1 and RNA2 are sufficient to initiate systemic movement and distribution throughout different tissues in *N. benthamiana* (Rahim et al., 2007).

Recently, we have shown that the RTD on RNA2 of the newly developed full-length cDNA clones of BNYVV and BSBMV is dispensable for systemic colonisation and symptom development in both *B. macrocarpa* and *N. benthamiana* (Laufer et al., 2018). Moreover, the RTD can be replaced by different open reading frames encoding fluorescent proteins which allowed the construction of fluorescently labelled recombinant viruses retaining the ability of systemic movement (Laufer et al., submitted). In this study, we addressed the question whether cDNA clones of BNYVV and BSBMV can also be used as tools for VIGS by partial replacement of the RTD encoding sequence with untranslatable cDNA fragments from *N. benthamiana magnesium chelatase H subunit* (*chlH*) and *phytoene desaturase* (*pds*). Furthermore, both VIGS-constructs should be optimised using coding sequences from *chlH* and *pds* in different orientations.

For this purpose, total RNA was extracted from *N*. *benthamiana* leaves using a NucleoSpin® RNA Plant Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. In a reverse transcription PCR (RT-PCR), cDNA was produced using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific, USA). To amplify a 549 bp *chlH* as sense and antisense cDNA fragment using Phusion Flash High-Fidelity PCR Mastermix (Thermo Fisher Scientific, USA), oligonucleotides were designed based on the *chlH* sequence of *N. tabacum* (GenBank accession number 2318136). To produce a 578 bp *pds* sense and antisense fragment, primers were designed according to the *N. benthamiana pds* sequence (GenBank accession number 93117609) (Table S1). All primers were extended with *Asc*I (5′-GGCGCGCC-3′) and *Pac*I (5′-TTAATTAA-3′) recognition sites. PCR products of *chlH* and *pds* were digested with *Asc*I and *Pac*I restriction enzymes, purified from agarose gels with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Dueren, Germany) and cloned into RNA2 cDNA clones of BNYVV and BSBMV, respectively (Laufer et al., 2018). For this purpose, plasmids were initially re-amplified, thereby deleting the RTD but leaving 249 bp upstream of the TGB1 ORF retaining the TGB1 subgenomic promotor and creating the single restriction enzyme recognition sites *Asc*I and *Pac*I followed by two stop codons (TGATAG) to facilitate the cloning of *chIH* and *pds* fragments downstream of the CP-ORF. Additionally, all VIGS-constructs comprise the mutated opal (TGA) stop codon of the coat protein and the first two codons (CAATTA) of the RTD. *In vitro* recombination products were transformed into chemical competent *Escherichia coli* cells (strain DH5α) (Inoue et al., 1990). Subsequently all constructs (Table S2) were electroporated into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) strain GV2260 via heat shock transformation (Mattanovich et al., 1989) and inoculated into 2-3 leaves of 3-4 week old *N*. *benthamiana* plants as described by Voinnet et al. (1998).

Both non-inoculated plants and plants inoculated with the vector containing the empty plasmid developed no systemic symptoms (Fig. 1A and B; Fig. 2A and B). All plants inoculated with BNYVV RNA1-2 (BN1BN2) without *chlH* or *pds* insert showed only typical systemic BNYVV symptoms in form of a chlorosis and necrotic yellow veins (Fig. 1C). Similarly, plants inoculated with BSBMV RNA1-2 (BS1BS2) displayed mosaic patterns and yellow blotches (Fig. 2C) without a *chlH* or *pds* silencing phenotype. In contrast, all plants inoculated with BNYVV/BSBMV containing *chlH* or *pds* fragments showed a typical silencing phenotype. First symptoms of photobleaching caused by BNYVV RNA1 + RNA2-PDS (BN1BN2-PDS) and BNYVV RNA1+ BNYVV RNA2-ChlH (BN1BN2-ChlH) both in sense (s) and antisense (as) orientations, respectively, were observed about 19 days post inoculation (dpi), and became more pronounced after 28 dpi (Fig. 1D, E, G and H). In the same manner, all plants infected with the BSBMV RNA1+ BSBMV RNA2-PDS (BS1BS2-PDS) and BSBMV RNA1+ BSBMV RNA2-ChlH (BS1BS2-ChlH) VIGS constructs in different orientations, respectively, developed photobleaching after 21 dpi (Fig. 2D, E, G and H). PDS and ChlH silencing phenotype appeared first with a white/yellow colour and faint green regions in new upper leaves above the inoculated leaves, without any photobleaching in stems and petioles. PDS and ChlH silencing was already described in previous reports using *Potato virus X* (PVX), *Tobacco rattle virus* (TRV) and *Tobacco mosaic virus* (TMV) with *pds* or *chlH* inserts (Kumagai et al., 1995; Lange et al., 2013; Thomas et al., 2001; Yuan et al., 2011). The observed white/yellow colour of PDS and ChlH silencing phenotype in the BNYVV or BSBMV RNA2 background is similar to PDS or ChlH silencing phenotype in *N. benthamiana* described in preceding studies (Kjemtrup et al., 1998; Thomas et al., 2001; Voinnet et al., 2000).

In the next step, the silencing effect on transcript levels of *chlH* and *pds* was analysed by means of quantitative real-time PCR (qRT-PCR). Therefore, leaf samples from five systemically infected plants displaying silencing symptoms were collected and subjected to RNA extraction using the NucleoSpin® RNA Plant Kit. The first strand cDNA was synthesised from 1 µg of total RNA using RevertAid RT Reverse Transcriptase. A primer pair for qRT-PCR was used to generate a 66 bp *pds* and a 95 bp *chlH* PCR-product targeting a region outside of the coding sequences inserted in the BNYVV/BSBMV based VIGS-vectors (Table S1). The 60S rRNA gene and the F-BOX gene served as endogenous controls for normalisation (Liu et al., 2012). The relative mRNA expression of *pds* and *chlH* to the non-inoculated control plants was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Fold change values were log10 transformed prior to statistical analysis with SAS Version 9.4 (SAS Institute Inc., Cary, USA). The qRT-PCR analysis revealed that expression of *pds* was reduced by 7% and *chlH* by 16% in BN1BN2 infected plants compared to the non-inoculated control plants (Fig. 3A and B) However, plants infected with VIGS constructs

from BNYVV displayed in both target genes a significant reduction of the transcript level. In case of *chlH*, no significant differences between sense (85%) and antisense (86%) constructs were observed (Fig. 3A). In contrast, the silencing effect of *pds* with a sense construct (77%) was significantly higher compared to the antisense construct (60%) (Fig. 3B). Plants infected with BS1BS2 displayed also a reduction in mRNA expression level of *chlH* (25%) whereas no reduction in the *pds* mRNA expression level was detected (Fig. 3C and D). VIGS constructs of BSBMV carrying a *chlH* fragment in sense (67%) or antisense (74%) orientation caused also a markedly reduction of the transcript level (Fig. 3C) but this effect was not significantly different from the empty full-length clone. In contrast, the transcript level of *pds* was significantly reduced by sense (59%) and antisense (49%) constructs as well (Fig. 3D).

Our data show that there is no apparent difference between sense and antisense constructs with one exception in case of BNYVV-PDS. This indicates that the development of ChlH or PDS-VIGS is independent of insert orientation. Similar results were also reported in *N. benthamiana* and *N. tabacum*, where the sense and antisense insertion in TMV, PVX or in a hybrid viral vector consisting of sequences from *Tomato mosaic virus* (ToMV) and TMV had the same PDS silencing effect (Gosselé et al., 2002; Hiriart et al., 2002; Kumagai et al., 1995; Ruiz et al., 1998). However, many approaches have been proposed to insert a target gene into a VIGS-vector. Among other, inserting a fragment of *pds* between the large coat protein and movement protein and in frame with the viral polyprotein of *Bean pod mottle virus* (BPMV) (Zhang et al., 2010). In the previous TRV-VIGS vector a target gene fragment was inserted into the multiple cloning sites (MCS) of the TRV vector in different orientations (Lee et al., 2017). Similarly, fusion protein expression was used for both BPMV and *Apple latent spherical virus* (ALSV) VIGS studies (Igarashi et al., 2009).

In this study, we inserted the target genes (*pds, chlH*) after the mutated opal stop codon of the CP of BNYVV and BSBMV. VIGS efficiency seems to be dependent on the virus-host interaction as well as on the replication cycle of the virus (Senthil-Kumar and Mysore, 2011). However, different target genes (*pds*, *chlH*) display different quantitative effects. ChlH constructs in BNYVV as well as in BSBMV delivered higher silencing levels than the PDS constructs. This might be explained by the fact that RNAi produces in many cases variable effects, which may occur when the siRNA molecules cannot bind to the target mRNA, because the target region is bound to proteins or is not accessible due to secondary structures (Tomari and Zamore, 2005). Recent studies demonstrated that the silencing efficacy and stability might be influenced by the sequence of the insert (Bruun-Rasmussen et al., 2007; Pignatta et al., 2007; Zhong et al., 2005). Additionally, several observations showed that the 3`-end derived siRNAs were better for PDS VIGS compared to siRNAs

derived from the 5`-end of the gene, which was found particularly more pronounced for the antisense orientation than the sense orientation (Igarashi et al., 2009; Zhang et al., 2010).

In summary, we have shown that full-length cDNA clones of BNYVV/BSBMV can be used as tools for VIGS in *N. benthamiana*. To the best of our knowledge, BNYVV and BSBMV are the first benyviruses modified for efficient VIGS. These VIGS-constructs should be now optimized to be applicable in their natural host plants *B. vulgaris.*

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Figures and Figure legends

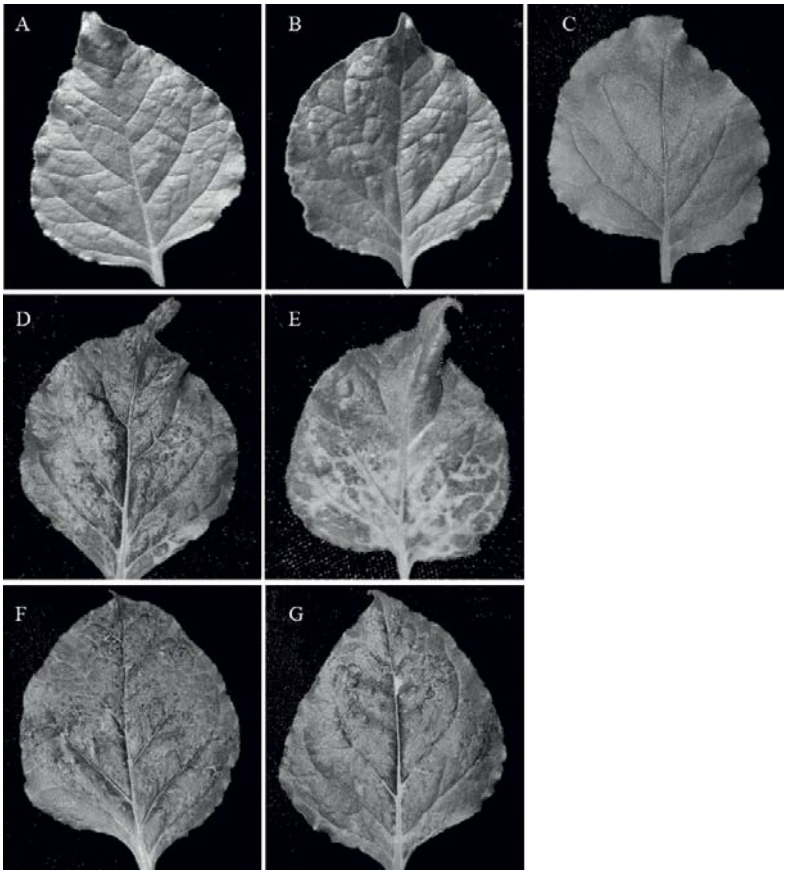


Figure 1. Silencing of endogenous phytoene desaturase (pds) and magnesium chelatase H subunit (chlH) genes in Nicotiana benthamiana after agroinfiltration with different VIGS-vectors from BNYVV (31dpi). A) Healthy N. benthamiana plants. B) Plants inoculated only with infiltration buffer containing only A. tumefaciens GV 2260 without vector. C) N. benthamiana plants with typical BNYVV symptoms after infection with BN1BN2. D) Phenotype caused by the BN1BN2-PDS-s silencing vector. E) Phenotype of the BN1BN2-PDS-as silencing vector. G) An intensive white/yellow photobleaching occurring at 31 dpi in upper non-inoculated leaves of plants infected with BN1BN2-ChlH-s and H) with BN1BN2-ChlH-as.

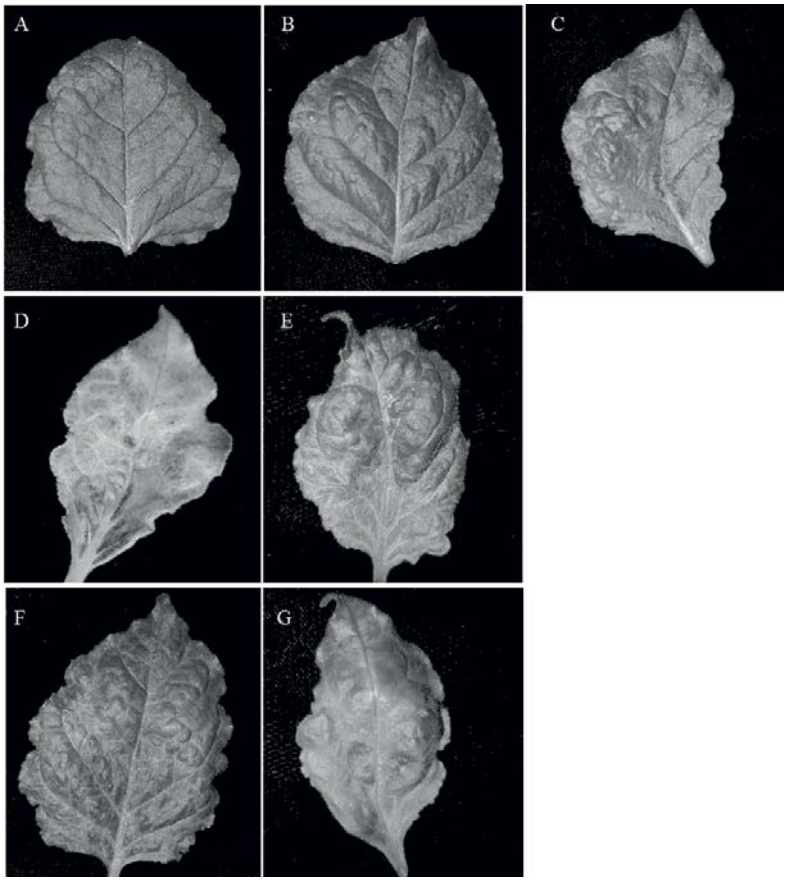


Figure 2. Silencing of endogenous *phytoene desaturase* (*pds*) and *magnesium chelatase H subunit* (*chlH*) genes in *N. benthamiana* after agroinfiltration with different VIGS-vectors from BSBMV (31dpi). A) Healthy *N.benthamiana* plant. B) Plants inoculated only with infiltration buffer containing only *A. tumefaciens* GV 2260 without vector. C) *N. benthamiana* displaying typical BSBMV symptoms after infection with BS1BS2. D) Phenotype caused by the BS1BS2-PDS-s silencing vector. E) Phenotype of the BS1BS2-PDS-as silencing vector. G) An intensive white/yellow photobleaching occurring at 31 dpi in upper non-inoculated leaves of plants infected with BS1BS2-ChlH-s and H) with BS1BS2-ChlH-as.

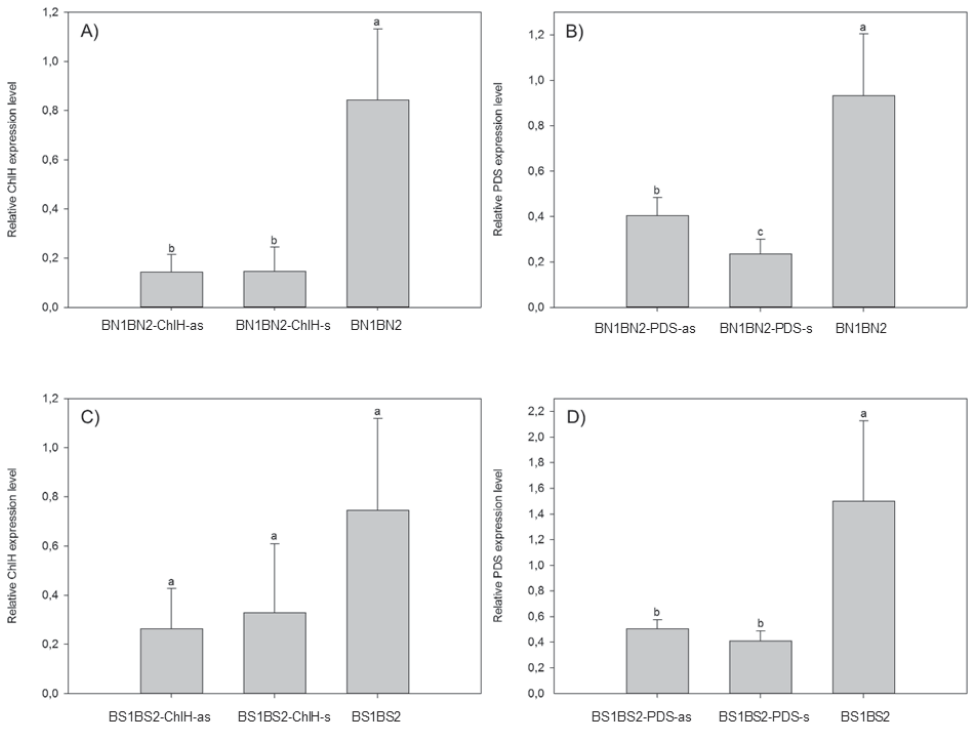
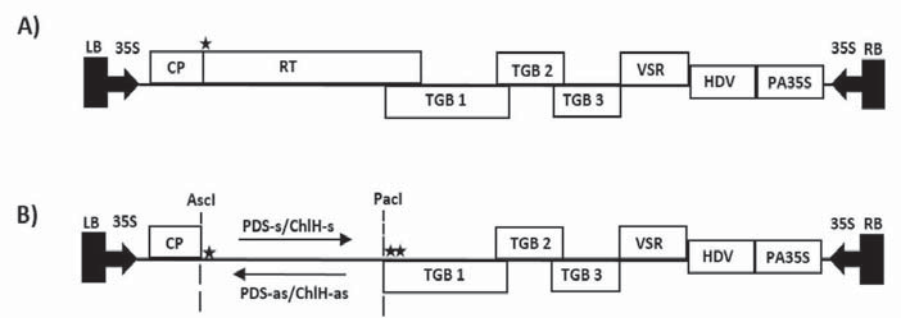


Figure 3. *Magnesium chelatase* (*chlH*) and *phytoene desaturase* (*pds*) expression levels for different silencing constructs of BNYVV (A and B) and BSBMV (C and D). Fold change values are mean values of five *N*. *benthamiana* plants calculated relative to the mock-inoculated plants. Error bars indicate standard deviation. Small letters represent different statistical groups based on a 0.05 confidence level.

Supporting Information



Supplementary Figure 1. Schematic representation of BNYVV/BSBMV-RNA2 infectious cDNA full-length clones and genomic modifications. A, Organisation of BNYVV/BSBMV plasmids used in VIGS analysis of PDS/ChlH. The BNYVV/BSBMV open reading frames are shown as CP (coat protein); RT (read-through domain); TGB1-3 (triple gene block ORF1-3) and VSR (viral silencing suppressor). LB and RB (left and right borders of the binary vector); p35S (Cauliflower mosaic virus 35S promoter); HDV (Hepatitis delta virus ribozyme); pA35S (Cauliflower mosaic virus 35S polyadenylation signal). B, BNYVV/BSBMV-RNA2 after insertion of phytoene desaturase (PDS) sense; (PDS) antisense; magnesium chelatase (ChlH) sense and (ChlH) antisense. ★ : refer to stop codon.

Table S1. Oligonucleotide primer used in this study. Restriction sites are indicated in the name and underlined within the sequence

No.	Primer	Sequence (5′-3′)	Purpose
1	S-ChlHNb-AscI-for	5′-TTGGCGCGCCGAATCTCCTTGACCGAGCAGTC-3′	pDIVA:BNYVV-RNA2-CP-NbPDS-s
2	S-ChlNb-PacI-rev	5′-CGTTTAATTAAATGCCTTCATACCACTTGGGGT-3′	pDIVA:BNYVV-RNA2-CP-NbPDS-s
3	AS-ChlNb-PacI-for	5′-GATTAATTAAGAATCTCCTTGACCGAGCAGTC-3′	pDIVA:BNYVV-RNA2-CP-NbPDS-as
4	AS-ChlNb-AscI-rev	5′-TTGGCGCGCCATGCCTTCATACCACTTGGGGT-3′	pDIVA:BNYVV-RNA2-CP-NbPDS-as
5	S-PDSNb-AscI-for	5′-TTGGCGCGCCTTGTTATTGCTG-3′	pDIVA :BNYVV-RNA2-CP-NbChlH-s
6	S-PDSNb-PacI-rev	5′-GATTAATTAAAGTTCAAAGCAATCAAAATGCA-3′	pDIVA :BNYVV-RNA2-CP-NbChlH-s
7	AS-PDSNb-PacI -for	5′-CGTTTAATTAAGTTCAAAGCAATCAAAATG-3′	pDIVA :BNYVV-RNA2-CP-NbChlH-as
8	AS-PDSNb-AscI-rev	5′-TTGGCGCGCCTTGTTATTGCTGGTGCAGG-3′	pDIVA :BNYVV-RNA2-CP-NbChlH-as
9	BN-RNA2-AscI-rev	5′-ATGGCGCGCCTTGTCCGGGTGGACTGGTTC-3′	pDIVA:BSBMV-RNA2-CP-PDS-s
10	BN-RNA2-PacI-for	5′-GATTAATTAATGATAGACGTGGGGCTGGTTCTTC-3′	pDIVA:BSBMV-RNA2-CP-PDS-s
11	BS-RNA2-AscI-rev	5′-ATGGCGCGCCTCAATTGGTGCGTGGAACGGCAGGAGTAACACCCC-3′	pDIVA:BSBMV-RNA2-CP-PDS-as
12	BS-RNA2-PacI-for	5′-GATTAATTAATAGTATGTTACGAACACGTGGTGTTAGTAATA-3′	pDIVA:BSBMV-RNA2-CP-PDS-as
13	PDSNb-for	5′-TTCTTTTGCCTGAAGACTGGAAA-3′	Quantitative real-time PCR
14	PDSNb-rev	5′-GAACTCCCACTAGCTTCTCCAACT-3′	Quantitative real-time PCR
15	ChlHNb-for	5′-AGCGTGACCTTGTGGTAGGAA-3′	Quantitative real-time PCR
16	ChlHNb-rev	5′-TGGAGGTTCACCAATGATGTGA-3′	Quantitative real-time PCR
17	60S-Nb-for	5′-AAGGATGCCGTGAAGAAGATGT-3′	Quantitative real-time PCR
18	60S-Nb-rev	5′-GCATCGTAGTCAGGAGTCAACC-3′	Quantitative real-time PCR
19	FBOX-Nb-for	5′-GGCACTCACAAACGTCTATTTC-3′	Quantitative real-time PCR
20	FBOX-Nb-rev	5′-ACCTGGGAGGCATCCTGCTTAT-3′	Quantitative real-time PCR
21	PDSNb-1for	5′-CGAGCTGAATGAGGATGGAAGTG-3′	Quantitative real-time PCR
22	PDSNb-1rev	5′-GCACCTTCCATTGAAGCCAAG-3′	Quantitative real-time PCR
23	ChlHNb-1for	5′-GCTGATGCAGTTCTCCACTTTGG-3′	Quantitative real-time PCR
24	ChlHNb-1rev	5′-GACGGAGCAGCTCCACATCTC-3′	Quantitative real-time PCR

Table S2. List of plasmids and VIGS constructs used in this study.

Construct	Insert	Virus	Abbreviation
pDIVA:BNYVV-RNA1	None	BNYVV	BN1
pDIVA:BSBMV-RNA1	None	BSBMV	BS1
pDIVA:BNYVV-RNA2	None	BNYVV	BN2
pDIVA:BSBMV-RNA2	None	BSBMV	BS2
pDIVA:BNYVV-RNA2-CP-NbPDS-s	PDS-s (578 bp)	BNYVV	BN2-PDS-s
pDIVA:BNYVV-RNA2-CP-NbPDS-as	PDS-as (578 bp)	BNYVV	BN2-PDS-as
pDIVA :BNYVV-RNA2-CP-NbChlH-s	ChlH-s (549 bp)	BNYVV	BN2-ChlH-s
pDIVA :BNYVV-RNA2-CP-NbChlH-as	ChlH-as (549 bp)	BNYVV	BN2-ChlH-as
pDIVA:BSBMV-RNA2-CP-PDS-s	PDS-s (578 bp)	BSBMV	BS2-PDS-s
pDIVA:BSBMV-RNA2-CP-PDS-as	PDS-as (578 bp)	BSBMV	BS2-PDS-as
pDIVA:BSBMV-RNA2-CP-PDS-SDP-IR	PDS-SDP (578 bp)	BSBMV	BS2-PDS-SDP-IR
pDIVA:BSBMV-RNA2-CP-ChlH-s	ChlH-s (549 bp)	BSBMV	BS2-ChlH-s
pDIVA:BSBMV-RNA2-CP-ChlH-as	ChlH-as (549 bp)	BSBMV	BS2-ChlH-as

6. General Discussion

6.1 Construction of infectious cDNA full-length clones of BSBMV and challenges of the inoculation technique

For a better understanding of the molecular biology of BSBMV and the molecular interactions between benyviruses and their hosts, the first aim of this thesis was the construction of infectious full-length cDNA clones of a Californian BSBMV isolate. To circumvent the difficulties of classical cloning, the approach of Gibson assembly was successfully applied. The generated BSBMV clones replicated, encapsidated and moved systemically in different host plants (*N. benthamiana, C. quinoa, B. macrocarpa* and *B. vulgaris*) by means of agroinoculation or mechanical inoculation, respectively. Based on the results described in manuscript I, it was clearly demonstrated that the constructed infectious full-length cDNA clones of BSBMV in terms of symptom and particle formation as well as transmission by *P. betae*, were comparable to the wild-type isolate.

Yamaya et al. (1988), Quillet et al. (1989) or Delbianco et al. (2013) used the classical cloning approach by applying DNA ligase and restriction endonucleases for construction of infectious full-length cDNA clones. This technique is often time consuming, laborious and error-prone because of its many sequential cloning and sub-cloning steps (Tuo et al., 2015). Using the classical cloning technique can also be a limitation for the construction of infectious clones of some viruses (Desbiez et al., 2012). For example Desbiez and co-workers failed in the construction of different potyvirus clones using the classical cloning approach. In the last few years, many different sequence- and ligation-independent methods for one step DNA assembly into a plasmid vector have been developed (Patron 2014; Tuo et al., 2015). In 2009, Gibson et al. described the approach of Gibson assembly as a simple and fast handling approach allowed the construction of infectious clones of BSBMV within a short time. Ziegler-Graff et al. (1988) and Quillet et al. (1989) produced the first *in vitro* transcripts of a benyvirus (BNYVV). Later, Ratti et al. (2009) and D'Alonzo et al. (2012) constructed the first *in vitro* transcripts of BSBMV RNA3 and RNA4, respectively. All working groups used a bacteriophage T7 *in vitro* transcription system instead of *in vivo* transcription. *In vitro* transcription is associated with a high workload, high production costs, low number of transcripts, capping of the 5'-end and unreliable mechanical inoculation. Furthermore, the RNA2 clones generated in this way were associated with stability or toxicity problems in *E. coli* (Quillet et al., 1989). Dawson et al. (1986) showed that just six extra nucleotides at the 5'-end reduced the infectivity of the transcripts of TMV. This observation was shared by Ziegler-Graff et al. (1988), who demonstrated that non-viral extensions on the 5'-end of BNYVV RNA3 transcripts had a negative effect on their biological activity; additionally a lower infectivity could arise due to an incomplete capping.

For introducing clones into plants, *Agrobacterium*-mediated transformation is the most common and effective method (Nagyová and Šubr, 2007). Leiser et al. (1992) were the first to use this technique successfully for the inoculation of a monopartite ssRNA virus (BWYV). Wang et al.

(2009) and Crivelli et al. (2011) showed that this technique is also suitable for inoculation of a bipartite ssRNA virus and a tripartite ssRNA virus, respectively. Finally, Delbianco et al. (2013) used agroinoculation for the first time, to inoculate a benyvirus (BNYVV B-type) on *N. benthamiana* and *B. macrocarpa*. In this study *N. benthamiana* and *B. macrocarpa* were successfully agroinoculated with BSBMV clones. At first, some complications arose during *B. macrocarpa* agroinoculation and the inoculated plants displayed no systemic symptoms. The cause of unsuccessful *B. macrocarpa* transformation was found in the environmental conditions. It has to be considered that light and temperature influence plant physiology. Plants belonging to the *Beta* species are naturally long-day plants. To avoid stem extension (bolting) and flowering, they should be kept under short-day conditions. *B. macrocarpa* is a wild Mediterranean form and typically an annual plant (Draycott 2008). Finally, different environmental conditions in the greenhouse were tested to find a light-temperature ratio suitable for *Agrobacterium*-mediated transformation and plant growth. No additional lighting, a shading of the plants for 8 hours during the day as well as a temperature of 24°C/14h 18°C/10h led to success after leaf infiltration of *B. macrocarpa*. Limitations of BSBMV agroinoculation occurred for *C. quinoa* leaf infiltration and agroinoculation of *B. vulgaris*. Therefore, different strategies have been investigated. Infiltation with a needleless syringe, drenching, as well as picking and stabbing the bacterial colonies with a toothpick into the leaf were tested for *C. quinoa*, but no symptoms occurred (data not shown). Finally mechanical rub-inoculation induced local lesions on *C. quinoa* plants. Already, Flores Solís et al. (2003) reported about difficulties in transformation of the close relative *Chenopodium rubrum.* They unsuccessfully tested different approaches like vacuum-infiltration of adult plants and co-cultivation of germinating seed with agrobacterium. Flores Solís and co-workers (2003) overcame the barriers by using SAAT. The authors emphasised that the efficiency is dependent on plant age and that just two days old seedlings are susceptible to infection. Results within this study and lacking information in the literature point out that further improvements in agroinoculation techniques for *C. quinoa* are needed to replace the time-consuming mechanical rub-inoculation.

For *B. vulgaris*, leaf infiltration with a needleless syringe, drenching, vacuum-inoculation, as well as vortex-inoculation with a solution of *Agrobacterium* cells carrying BSBMV cDNA clones did not lead to a systemic virus infection (data not shown). After leaf infiltration, *B. vulgaris* displayed local symptoms but no systemic colonisation was observed. Drenching, vortex-inoculation and vacuum-inoculation with a solution of *Agrobacterium* cells failed as well. Generally, *Agrobacterium*-mediated transformation is influenced by many different factors like plant species and genotype, bacterial strain and cell density, light and temperature and plant growth regulators and antibiotics (Gurel et al., 2008; Karami 2008; Leiser et al., 1992; Nagyová and Šubr, 2007). In addition, Koenig and Stein (1990) reported on their experience with mechanical vortex-inoculation that a certain plant age (6-9 days old) as well as sugar beet cultivar is of importance. For example, sugar beet seedlings older than 12 days never showed BNYVV symptoms and temperature

between 25-30°C led to a stronger symptom formation than temperatures around 22°C. In accordance with Koenig and Stein (1990), different BBCH stages of *B. vulgaris* seedlings, different sugar beet cultivars and different growing conditions were tested, but did not lead to any visible symptoms on newly emerged leaves after agroinoculation. Our experiments showed that the environmental conditions successfully applied for *B. macrocarpa* cultivation were not practicable for *B. vulgaris*. One hypothesis for this disparity between the plants could be the influence and effect of environmental conditions on *Agrobacterium*. Karami (2008) reviewed that light and temperature may have a stimulatory effect for T-DNA delivery, but this effect differs between plants. Hatlestad et al. (2012) successfully used a TRV based vector to agroinoculate seven-day-old beet seedlings by vacuum-infiltration. After the infiltration, they cultivated the infiltrated seedling under special conditions; 24h 100% humiditiy followed by 24h fluorescent light. Based on the environmental conditions described by Hatlestad et al. (2012), they should be examined for their applicability to BSBMV/BNYVV. If these experiments fail, it has to be considered whether the benyvirus is responsible for the failure. On the other hand, Klimek-Chodacka and Baranski (2014) applied different cell densities (OD_{600}: 0.05-0.5) and concluded that the OD is dependent on the duration of the inoculation treatment. Following this approach, different OD_{600} (0.05-0.5) were tested in this study, in combination with different length of inoculation treatments, but without any success. In addition, two different bacterial strains (GV2260 and GV3101) have been tested, however, this attempt also failed. Nonetheless, Klimek-Chodacka and Baranski (2014) concluded that the transformation efficiency is more dependant on the genotype of the plant than on the bacterial strain used. As reviewed by Karami (2008), the recalcitrance of some plants to *Agrobacterium*-mediated transformation can be explained by several factors like the suppression of the *vir* gene expression, an inefficient synthesis and transfer of the T-strand DNA into the plant or the presence of inhibitors in the host plant. This nonspecific defence response to agrobacterium was for example observed for some potato genotypes (Du et al., 2014), but not reported for sugar beet so far. Another factor affecting the transformation efficiency is the fact that sugar beet cells have a low ability to regenerate after agroinoculation (Gurel et al., 2008). Also the low efficiency of *A. tumefaciens* to transform root cells has to be considered (Grevelding et al., 1993). Lacroix and Citovsky (2011) assumed in their study that the addition of exogenous VirB5, an agrobacterium virulence protein, may enhance agrobacterium infectivity. An enhancement of 30-70% on transient T-DNA expression due to the addition of VirB5 was identified in sugar beet hypocotyl. In a few studies for sugar beet transformation, other scientists used *A. rhizogenes*, the causal agent of hairy root disease, instead of *A. tumefaciens*. Klimek-Chodacka and Baranski (2014) reported a good transformation rate of sugar beet explants by using *A. rhizogenes* and applying the SAAT technique. Also Cai et al. (1997) and Pavli et al. (2010) used *A. rhizogenes* for efficient sugar beet root transformation. Kifle et al. (1999) achieved a higher transformation rate of sugar beet roots by using *A. tumefaciens* and *A. rhizogenes* together. Furthermore, to alleviate undesired hairy roots

phenotypic effect, disarmed *A. rhizogenes* are required (Mankin et al. 2007). So far, the authors have not approved to share their disarmed *A. rhizogenes* and to construct a disarmed bacterial strain is difficult, time-consuming and labour intensive. To conclude, until now, there is a lack of an easy handling and efficient *Agrobacterium*-mediated inoculation technique that can transform sugar beet roots, leading to a colonisation of the whole plant with BSBMV and BNYVV respectively. Besides the advantages (fast and high expression level of a virus, systemic spread and low production cost) of *Agrobacterium*-mediated transformation, one drawback of this technique is that naturally, RNA viruses do not enter the cell nucleus. Consequently, a lower efficiency caused by a possible degradation of the transcript before it leaves the nucleus has to be considered (Gleba et al., 2007; Gleba et al., 2013). Moreover, the results confirm the statement of Mutterer et al. (1999) that every host plant-virus combination may need an optimisation of agroinoculation conditions.

To circumvent the problems of agroinoculation of *B. vulgaris*, it was tested whether *B. vulgaris* could be infected mechanically with BSBMV cDNA clones by vortex-inoculation using sap from infected *N. benthamiana* tissue. Koenig and Stein (1990) successfully infected *B. vulgaris* with BNYVV by means of mechanical vortex-inoculation with a sap of infected *T. expansa.* Likewise, Bornemann and Varrelmann (2011) successfully used the method of Koenig and Stein (1990), with some modification, to infect *B. vulgaris* with BNYVV. The approach described by Bornemann and Varrelmann (2011) of mechanical vortex-inoculation was optimised and resulted in systemic colonisation of *B. vulgaris.* Foliar symptoms, yellow blotches and bands, were similar to those observed in naturally BSBMV infected plants (Heidel et al., 1997; Peltier et al., 2008). A drawback is that the propagation step is time-consuming and labour intensive compared to an agroinoculation. Another disadvantage is the fact that BNYVV RNA1+2 segments alone are sufficient for systemic infection in *N. benthamiana* and that BNYVV isolates tend to lose their small RNAs during passages on *C. quinoa* (Koenig et al., 1986; Rahim et al. 2007). Thus, experiments including the small RNAs could result in lower infection rates or an infection could fail. These facts lead to the suggestion that a vortex-inoculation of *B. vulgaris* with a sap of infected *B. macrocarpa* tissue could result in higher infection rates.

To control the spread of viruses and to develop antiviral strategies, it is necessary to understand the molecular biology as well as the various functions of the expressed proteins (Urcuqui-Inchima et al., 2001). The ability to modify and inoculate the infectious clones easily helps to speed up investigations. Experiments could now be easily conducted in the absence of *P. betae.* Physical and biological properties of artificially induced mutants can be compared with the parent virus after inoculation of a host plant and can dissect the properties of viral genomes (Hull, 2009; Hull, 2013). The BSBMV and BNYVV clones represent a powerful tool for further investigations to understand their functional differences and interaction e.g. formation of reassortants (see 6.3) or interaction on

cellular level (see 6.4.2). Moreover, clones could be used for resistance tests to identify resistance factors within *B. vulgaris* and BSBMV/ BNYVV, respectively.

6.2 Interaction of BSBMV and BNYVV on the whole plant organism level

As summarised and described in detail under 1.2.4, information about the interaction of BSBMV and BNYVV is still limited (Heidel et al., 1997; Wisler et al., 2003). Contradictory statements can be found in the literature (Mahmood and Rush 1999; Piccinni and Rush 2000; Wisler et al., 2003). For this reason, an artificial co-infection experiment with BSBMV- and BNYVV-wildtype isolates was carried out in sugar beet. Seedlings were mechanically vortex-inoculated with a sap of infected *C. quinoa* tissue. Initial inocula were relative quantified by qRT-PCR (Germer et al., 2000) and adjusted to each other (BSBMV 0,5g/BNYVV 1.0g). The experiment was repeated two times under the same environmental conditions and plants harvested at 84dpi and 120dpi, respectively. Lateral roots were analysed by means of ELISA and both repetitions showed that the viral content of BNYVV significantly decreased by 87.95% and 93.54% in co-infected sugar beet compared to a single BNYVV infection (data not shown). With regard to beet weight, the BNYVV infected beets had a significantly lower weight than the healthy control and the co-infected beets (Fig. 4). Additionally, the evaluation of the lateral root weight showed a higher lateral root weight of BNYVV single infected sugar beets compared to the other variants (Fig. 4).

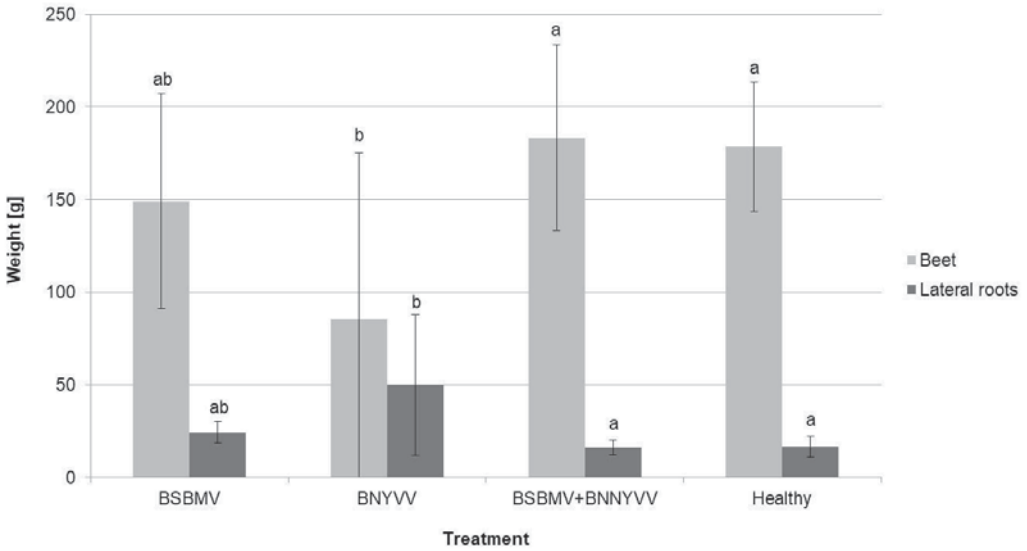


Fig. 4: Comparison of beet and lateral root weight of vortex-inoculated sugar beet seedlings (cultivar KWS03) after 120dpi. Inoculation was done with sap obtained from local lesions of *C. quinoa* infected wild-type BSBMV and BNYVV. Treatments are separated into single infections of BSBMV and BNYVV respectively, and a co-inoculation of BSBMV plus BNYVV. Healthy control sugar beet seedlings were treated with a sap of non-infected *C. quinoa* leave tissue. Analysis by ANOVA, post-hoc Tukey $p \leq 0.05$. Error bars indicate standard deviation.

Uchino and Kanzawa (1995) determined the yellowing intensity of infected BNYVV sugar beet leaves by using a SPAD (single-photon avalanche diode) meter. They verified that there is a

distinction of the relative chlorophyll content of sugar beet leaves infected with BNYVV and healthy plants. In our experiments, a visual effect was observed between BSBMV and BNYVV infected sugar beet leaves. In contrast to BSBMV infected leaves, which are dark green with a yellow mosaic pattern, leaves infected with BNYVV seem to be more yellow to bright green. Consequently, to quantify the relative chlorophyll content of sugar beet leaves a SPAD 502-meter (Minolta, Japan) was used. The numerical SPAD-values showed that the leaf chlorophyll content over a time period of 84dpi of co-infected sugar beets are more related to the single infected BSBMV beets as to the BNYVV infected beets, with one exception at 56dpi (Fig. 5). A documentation and comparison of leaf symptoms as well as the results described indicate that BSBMV suppresses BNYVV in co-infected beets and it seems likely that an antagonistic interaction between the two viruses occurs.

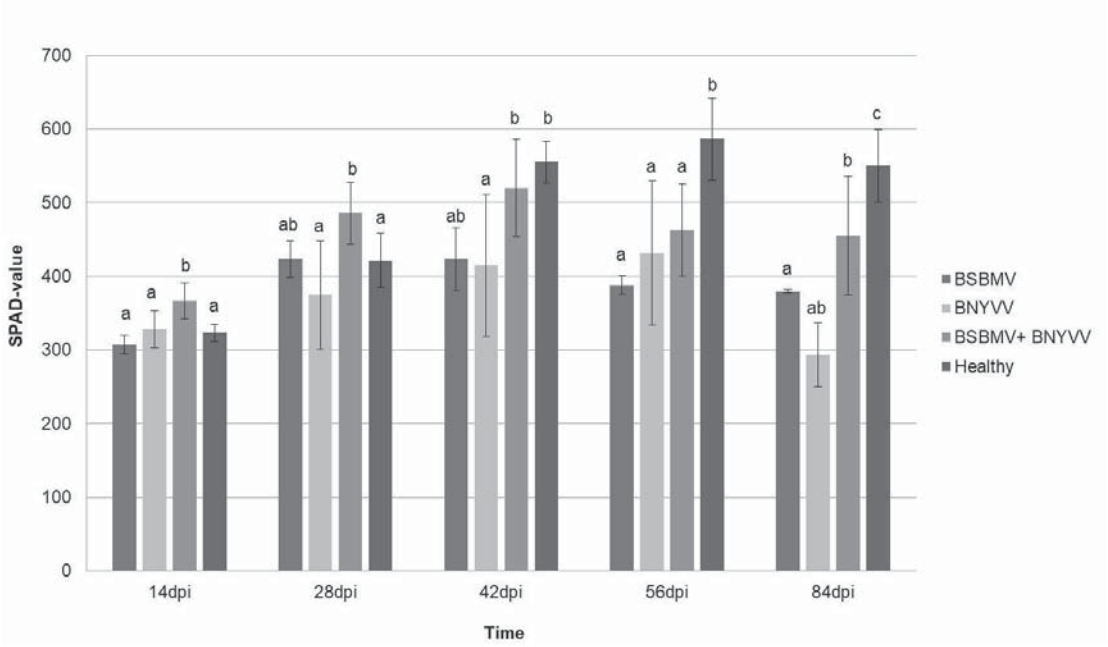


Fig. 5: SPAD-values of infected sugar beets measured over a time period of 84dpi. Seedlings were vortex-inoculated with a sap obtained from local lesions of *C. quinoa* infected wild-type BSBMV and BNYVV. Treatments are separated into single infections of BSBMV and BNYVV respectively, and co-inoculation of BSBMV plus BNYVV. Healthy control sugar beet seedling were treated with a sap of non-infected *C. quinoa* leave tissue. Ten repetitions per leaf and two leaves per plants were tested. Plants showing a positive ELISA were used to calculate an average SPAD-value. Analysis by ANOVA post-hoc Tukey, $p \leq 0.05$. Error bars indicate standard deviation.

According to a field experiment by Piccinni and Rush (2000), an infection with both viruses resulted in a higher root yield and a lower disease impact compared to a single BNYVV infection, but in a lower yield as a single BSBMV infection. Contrary, Wisler et al. (2003) concluded that BSBMV infection is lowered by BNYVV. The authors showed that ELISA values of BSBMV were significantly lower in mixed infections with BNYVV as in single BSBMV infections. They studied the interaction of the two species in sugar beets grown in naturally infested soils. However, a

mechanical inoculation of sugar beet seedlings led to a cross-protection between BSBMV and BNYVV; where primarily BSBMV infected beets showed a lower BNYVV titer, reduced infestation pressure, and were less diseased (Mahmood and Rush 1999). Similar results were achieved by Prillwitz and Schlösser (1993) who pre-infected sugar beets with *Beet soilborne pomovirus* (BSBV) and challenged with a BNYVV infection. This resulted in 50% less rhizomania-damaged beets. However, the predomination of a virus might be determined by multiple factors including environmental conditions, inoculum density or inoculation techniques (Rush, 2003).

The described experiments show that BSBMV suppresses BNYVV in co-infected sugar beets, but still both viruses were detectable within a plant. Therefore, it cannot be ruled out that a reassortant or a recombinant can be formed. At this point, the infectious full-length cDNA clones are helpful to study the genetic stability and to expand our knowledge about the exchange of RNA segments and recombinant production between the two benyviruses. Thus, experiments with artificially formed BSBMV/BNYVV reassortants could show whether these reassortants would be generally viable (see 6.3).

6.3 Artifical formation of reassortants of BSBMV and BNYVV

To study the interaction and possible consequences of a mixed infection, experiments with cDNA clones of BSBMV and BNYVV A-type reassortants in *N. benthamiana* and *B. macrocarpa* hosts were performed as described in manuscript I. Results show that RNA1+2 reassortants were viable and capable of viral long-distance movement in *N. benthamiana*. Reassortant experiments including the small RNA components of the species resulted in an exchangeability and long-distance movement in *B. macrocarpa*.

Systemic colonisation of RNA1+2 of BSBMV- and BNYVV-clones was observed in *N. benthamiana*, confirming previous observations (Chiba et al., 2013; Rahim et al., 2007). Interestingly, BSBMV RNA1+BNYVV RNA2 showed a lower efficiency in systemic movement (1/10 plants) compared to BNYVV RNA1+BSBMV RNA2 (10/10 plants) and the wild-types (BSBMV RNA1-2 and BNYVV RNA1-2; 10/10 plants). Moreover, a delayed symptom formation of the artificial reassortants compared to the wild-types was observed. These observations confirmed previous studies. For example, studies with two isolates of *Tomato aspermy virus* (TAV, *Cucumovirus,* +ssRNA), V-TAV and C-TAV, showed that artificial reassortants cannot infect *N. tabacum* as efficiently as C-TAV (Asaoka et al., 2010). Generally, cucumovirus reassortants are less effective as the wild-type virus and in most cases the interaction between the viral proteins and host factors are limited (Shi et al., 2003). Similar results were achieved by Hill et al. (1998) who performed reassortant experiments in *N. benthamiana* with the bipartite geminiviruses *Squash leaf curl virus* (SqLCV) and *Cabbage leaf curl virus* (CLCV). In comparison to the wild-types CLCV and SqLCV, a reduced infection and less severe symptoms of CLCV DNA A+SqLCV DNA B was reported after agroinoculation.

In principle, our results show that the housekeeping functions of RNA1 and RNA2 of both viruses are exchangeable. In regard of symptom expression and formation of the artificial BSBMV/BNYVV reassortants compared with the wild-types, it has to be considered that RNA2 is probably involved in symptom formation in *N. benthamiana*. However, which protein or UTR of RNA2 is affecting symptom formation is unclear. Investigation with artificially formed recombinants or deletion mutants to study the influence of the single ORFs and the UTRs could be helpful. Fitness penalty of the reassortants was observed due to a delayed symptom formation and the low infection rate of BSBMV RNA1+BNYVV RNA2. Reason for the low infection rate could be sequence differences within the UTRs, which might lead to an interference with the RdRp of BSBMV and BNYVV RNA2. The viral replication process is initiated by specific sequences confined to the extremities. An alignment of the 5'-UTR and 3'-UTR of BSBMV and BNYVV RNA2-clones, differences within the UTRs were detected (data not shown). By using the approach of site directed mutagenesis, the sequence differences within the 3'-UTR and 5'-UTR of BNYVV RNA2 could be introduced in the infectious clone of BSBMV RNA2. A comparison of agroinoculated *N. benthamiana* plants with either BSBMV RNA1+BNYVV RNA2 and BSBMV RNA1 supplemented with the modified BSBMV RNA2 3'-UTR/5'-UTR clones could provide evidence. Moreover, experiments by heterologous expression of the single ORFs of BNYVV in BSBMV background could give an idea about the interference between BSBMV RNA1 and the housekeeping functions of BNYVV RNA2. In addition, northern blot analysis was applied to estimate the accumulation of viral RNAs (RNA1 and RNA2) in *C. quinoa* and to confirm the effective replication of reassortants. Our results showed that in most cases there is no negative effect on RNA replication of artificial reassortants. Nevertheless, BSBMV RNA1+BNYVV RNA2 showed a lower accumulation of both genomic RNAs compared to the other reassortant and the wild-type controls. However, these results should be verified by qRT-PCR, which allows absolute quantification of single RNA components. Whether BNYVV RNA1+BSBMV RNA2 really possess a fitness penalty compared to the wild-types, the virus content was determined by ELISA. Results of the analysed *N. benthamiana* leaves showed a similar mean absorbance between reassortants and wild-types. Thus, the delayed symptom formation of the reassortants did not have a negative impact on the viral content.

Furthermore, we confirmed that BSBMV/BNYVV RNA3 is essential for long-distance movement in *B. macrocarpa* (Lauber et al., 1998; Ratti et al. 2009). The formation of reassortants resulted in systemic spread, indicating the close relationship and high sequence similarities especially of the presence of the core-region in both species. Additionally, BSBMV RNA4 can complement BNYVV RNA4 in BNYVV A-type background, confirming the results of D'Alonzo et al. (2012), who conducted the experiments with BNYVV B-type clones. Moreover, BSBMV RNA1-3 can transreplicate and transencapsidate BNYVV A-type RNA4. The observed high infection rate among reassortants in *B. macrocarpa* in this investigation has been previously reported for artificially formed reassortants of BNYVV B-type and BSBMV by D'Alonzo et al. (2012). However, infection

rate and symptom occurrence differed between the individual variants of reassortants and wild-types. Already Ratti et al. (2009) observed a delayed symptom formation of the reassortant (BNYVV B-type RNA1-2+BSBMV RNA3) compared to wild-type BNYVV B-type which is in consent with our findings (BNYVV A-type background). Reasons for a delayed symptom formation of BNYVV RNA1-2+BSBMV RNA3 could be due to interference with replication and encapsidation. In regard of the low efficiency of the reassortant BSBMV RNA1+BNYVV RNA2 in *N. benthamiana* (1/10 plants) the following result is remarkable. *B. macrocarpa* inoculated with BSBMV RNA1+2 and supplemented with BNYVV A-type RNA3 resulted in earlier and more severe symptoms than plants inoculated with BSBMV RNA1-4 and BSBMV RNA1-3 respectively. All-over, our findings are consistent with previous investigations by Ratti et al. (2009) and D'Alonzo et al. (2012) who demonstrated that BNYVV RdRp can replicate BSBMV RNA3/4 under artificial conditions. Sequence alignments of RNA3 UTRs from BSBMV and BNYVV showed high identities and conserved UTR structures (α, β and γ structures; Box I, Box II and Box III) comparable to BNYVV RNAs (Gilmer et al., 1993; Lauber et al., 1997; Lee et al., 2001; Ratti et al., 2009) favouring the hypothesis that both virus RdRps can replicate each other. Furthermore, they can utilise the protein functions of each other to fulfill the viral life cycle. However, due to the different severity of symptom formation of reassortants in *B. macrocarpa*, it can be concluded that BNYVV RNA3 in BSBMV background influences the severity. One possibility could be the different fitness of the full-length clones and the higher aggressiveness potential of BNYVV compared to BSBMV (Rush and Heidel, 1995; Heidel et al., 1997). Furthermore, BNYVV RNA3 encodes the pathogenicity factor P25, which is responsible for symptom formation (Koenig et al., 1991). In contrast, besides the involvement in long-distance movement, the function of BSBMV RNA3 P29 is unknown (Ratti et al., 2009). They speculated, derived from its 43% relatedness to P26 (BNYVV RNA5), that P29 is probably responsible for virus pathogenicity, but is less virulent as BNYVV. To study the role of BNYVV RNA5 in BSBMV background could be interesting, in order to see if RNA5 could complement BSBMV RNA3 or if RNA5 acts as an additional pathogenicity factor for BSBMV and consequently enhances symptom expression.

The fact that reassortants can be generated under artificial conditions does not mean that reassortants of the two viruses can be formed in nature at all. Moreover, the resulting properties of artificially formed reassortants cannot be so easily transferred to natural formed reassortants. In nature, reassortants can only survive if they are as efficient as their parental viruses and it is speculated that most reassortants have a weak competitiveness (Savory et al., 2014; Ohshima et al., 2016). However, in some cases, reassortment has led to viral strains with expanded aggressiveness and expanded host range (Briese et al., 2013). The risk for reassortment rises with the number of viral segments (Briese et al., 2013). For example, reassortment of CMV strains has resulted in a high genetic diversity and successful evolution, leading to worldwide distribution and to the broadest host range of any known plant virus (Roossinck, 2002; Nouri et al., 2014). Chen et

al. (2007) developed a PCR and restriction enzyme analysis-based method to identify reassortant of CMV strains in naturally infected tomato plants. Koenig et al. (2009) identified BNYVV reassortment in rootlets of field-grown sugar beets by sequence comparison. Nevertheless, the detection of natural reassortment remains challenging and difficult. An environmentally benign approach to control plant viruses is the use of resistant or tolerant varieties as for example resistant sugar beet varieties to control rhizomania (Peltier et al., 2008). An undesirable potential effect to overcome resistances is the reassortment or recombination between viral sequences. There are already presumptive indicators of BNYVV reassortment of A-, B- and P-type. During a sample collection in the area of Pithiviers between 2008 and 2012, BNYVV reassortants were found in 20% of sugar beet roots (Galein et al., 2013). The formation of BNYVV reassortants probably leads to resistance breaking varieties (Galein et al., 2013; Koenig et al., 2009). Likewise, Qiu and Moyer (1999) demonstrated that *Tomato spotted wilt tospovirus* (TSWV) overcomes host resistance by forming reassortants or as Tamarzizt et al. (2013) reported about resistance-breaking properties of CMV reassortants in Tunisian pepper crops. However, until now investigations about the formation of reassortments of BSBMV/BNYVV in sugar beet are lacking, although mixed infected plants with both viruses had been found in the USA. To identify reassortment and recombination it is necessary to determine the genome compontents and genome sequences of both viruses in mixed infected plants. Based on sequences analysis, it would be possible to identify recombination events between both viruses. In case of reassortant formation, it is difficult to provide experimental evidence because an isolate has to be found that contains a mixture of RNAs from BSBMV and BNYVV, but lacks the complete genome components of each single virus (e.g. BSBMV RNA1-3+BNYVV RNA4). This would indicate that the foreign RNA has complemented the function of the missing RNA. Under natural conditions, the genetic bottleneck has to be taken into account which minimises the extent of genetic variation within a virus population with the aim to maintain the genetic stability (Nouri et al., 2014).

In case of BSBMV and BNYVV, the risk of reassortment and recombination seems to be low because it is shown in this study that both viruses suppressed each other in sugar beet and reassortments displayed fitness penalty. However, it has to be taken into account that closely related viruses like BSBMV and BNYVV could be transmitted simultaneously by their vector *P. betae*, and that may have significant implications (Syller 2014). It may favour the recombination and reassortment of the two, which is caused by the assumption that BNYVV translation and movement exists within the vector (Lubicz et al., 2007). However, no information about simultaneous transmission is available. Although the probability of recombination and reassortment between BSBMV and BNYVV seems to be low, a positive selection pressure could favour the occurrence. This would be possible for example if BNYVV could overcome *Rz1* resistance with the RNA3 of BSBMV. However, this has to be explored in further experiments. To further investigate the likelihood of reassortment and recombination between BSBMV and BNYVV, it is crucial to

study their interaction on celluar level, as both viruses have to be able to colonise the same cell. Therefore, labelling of the infectious clones is required to further investigate the interaction and the likelihood of reassortment and recombination in more detail.

6.4 Fluorescent labelling and interaction studies

6.4.1 Strategies of fluorescent labelling of BSBMV and BNYVV cDNA full-length clones

It is of interest to study and to understand the interaction of both species on cellular level. For this purpose, a suitable cloning site to label the infectious cDNA clones with a fluorescent marker is essential. By applying fluorescent markers, the labelled viruses can be visualised by microscopy. At the beginning of this work, several strategies for labelling the cDNA clones have been tested (Fig. 6; Fig. 7). Different fluorescent markers were cloned via Gibson assembly at different positions of the viral genome of BSBMV. As described in manuscript II, labelling was finally achieved by a nearly complete replacement of the RT and leaving only 249 nucleotides of the RT domain upstream of the TGB to act as a sg promoter. This resulted in a fusion protein of the CP and the marker protein. Clones generated with this strategy successfully infected the host plants *N. benthamiana* and *B. macrocarpa* and showed a typical symptom expression. In addition, characteristic rod shaped virus particles of BSBMV were detected in systemically infected *N. benthamiana* leaves using electron microscopy. The following section describes several strategies that have been tested in this study. Although most strategies did not work, it could be useful for other researchers and is therefore described.

In previous studies, replicons based on BSBMV/BNYVV RNA3 (Erhardt et al., 2000; Ratti et al., 2009) and BNYVV RNA5 (Schmidlin et al., 2005) were used as viral expression vectors to express fluorescent markers in *C. quinoa* and *B. macrocarpa* respectively. However, replicon-derived BNYVV RNA3 does not move long distance in *N. benthamiana* and *Spinacia oleracea* (Gilmer, 2016). For both host plants, RNA1 and RNA2 are sufficient for a systemic infection. This observation is consistent with our findings for BSBMV. In this study, a replacement of P29 or P32 with a fluorescent marker did not result in systemically infected leaves. Only on local infected *N. benthamiana* leaves, fluorescence was detected with the epi-fluorescence microscopy (Epi-FM). RNA1 of BSBMV as well as BNYVV harbours one ORF responsible for replication of viral RNAs. A putative papain-like protease domain is located between the helicase and polymerase domain (Hehn et al., 1997). This domain can autocatalytically cleave the 237kDa protein into two products of 150kDa and 66kDa (Hehn et al., 1997). Thus, this domain would be conceivable as a possible marker site. However, the authors only speculate about two possible cleavage sites on BNYVV RNA1 and did not determine an exact position. Efforts to insert dsRED between the 150kDa and 66kDa product by flanking the fluorescent protein with the possible cleavage site (amino acid sequence: NMAGGK) failed (Fig. 6; personal communication Prof. Dr. E. Maiss).

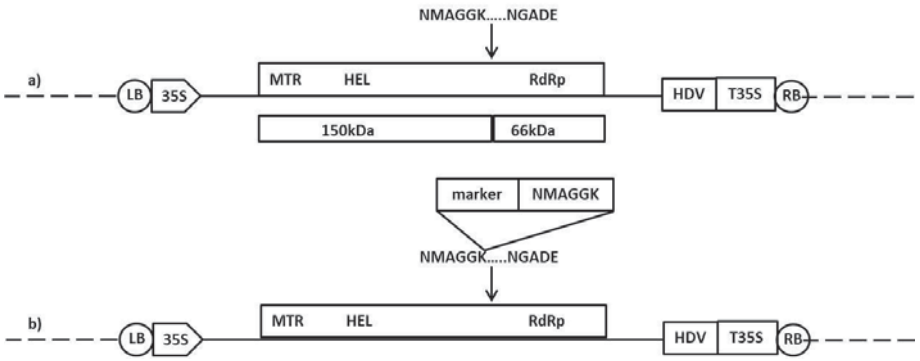


Fig. 6: Schematic representation of full-length infectious cDNA clones of the (a) BNYVV RNA1 based vectors and the modified variant (b). The approximate location and the two amino acid sequences of the predicted cleavage sites are indicated by an arrow. The resulting cleavage products 150kDa and 66kDa are shown below the BNYVV RNA1 polyprotein. LB: Left border; 35S: Cauliflower mosaic virus (CaMV) 35S promoter; MET: Methyltransferase domain; HEL: Helicase domain; RdRp: RNA-dependent RNA polymerase; HDV: Hepatitis delta virus ribozyme; T35S: CaMV Terminator 35S; RB: Right border; marker: Fluorescent protein dsRED. (a) Hehn et al., 1997, modified.

In regard to RNA2, different strategies for labelling were tested. One idea was to use the 16 amino acid long 2A region (NFDLLKLAGDVESNPG) of the *Foot-and-mouth disease virus* (FMDV), which has a cleavage activity. This 2A region is responsible for the procession of the polyprotein of FMDV (Ryan et al., 1991). This ability makes it attractive to use for foreign gene expression via full-length clones, but so far, the use has been limited. For the first time, Cruz et al. (1996) used it to modify a PVX based cDNA clone. Due to the fusion of 2A with GFP and CP, they were able to generate a PVX clone expressing GFP and the CP, resulting in systemically infected plants. In this work, all constructs based on the 2A mechanism failed and no fluorescence was observed on *N. benthamiana* (data not shown). Three different positions on BSBMV RNA2 for insertion of the 16 amino acids of the 2A peptide were investigated. Positions were chosen in a way that avoided the loss of functionality. A replacement of the RT domain by keeping only the last 249 nucleotides which serves as sg promotor for the TGB was unsuccessful (Fig. 7b). An insertion of the 2A peptide with the fluorescent marker downstream and upstream of the P14 respectively, as well as an insertion between P15 and P14 flanked by the sg promoter of P14 (Fig. 7c-e) failed. For all variants tested, no fluorescence in upper non inoculated leaves was detected. The most commonly used strategy to express a foreign sequence, is the replacement of a gene, which is not mandatory for the viral gen expression. Mostly, these “non-essential” gene sequences of the virus belong to the coat protein (Scholthof et al., 1996). French et al. (1986) replaced the CP of BMV with the reporter gene for chloramphenicol acetyltransferase. CP of BSMV and PVX were replaced by foreign sequences and both viruses provided foreign gene expression. In both cases, long distance movement was negatively influenced (Scholthof et al., 1996). Scholthof et al. (1993) used tomato bushy stunt virus to replace parts of the CP and to insert a foreign sequence. Still, a complete replacement of the CP can also lead to problems, as demonstrated by Takamatsu et al. (1987).

The replacement of TMV CP resulted in high levels of the foreign protein in the inoculated leaf; however, the modified virus could not spread systemically. To explore localisation of potato leafroll virus (family: *Luteoviridae*), the virus was labelled with GFP by truncating the RT, which is involved in virus movement and mandatory for aphid transmission, and fused to CP (Haupt et al., 2005). Boissinot et al. (2017) successfully tagged turnip yellows virus (TuYV; family: *Luteoviridae*) by replacing the C-terminal part of the RT and agroinoculated three different host plants. Erhardt et al. (2001) successfully integrated the GFP gene into the RNA2 of BNYVV by replacing a part of the RT domain of the P75. Obtained clones were infective in the local lesion host *C. quinoa*, but infectivity was not studied in other host plants. Based on these studies, a partial replacement of RT, leaving the last 249 nucleotides of the RT domain to act as sg promotor of P42, plus adding this sg promotor in front of the fluorescent gene was tested (Fig. 7f). No fluorescence was detected in *N. benthmania* (data not shown). A construct without the addition of the sg promoter of P42 in front of the fluorescent protein resulting in a fusion protein of the CP and the fluorescent protein displayed fluorescence and a typical symptom expression in the host plants *N. benthamiana* and *B. macrocarpa* (Fig. 7g). In addition, characteristic rod shaped virus particles of the modified BSBMV clone were detected in systemically infected *N. benthamiana* leaves using electron microscopy. Remarkably, no significant differences in particle diameter between the labelled clones and the unmodified BSBMV clones were observed. Moreover, a modified clone with a complete deletion of the RT was not distinguishable from wild-type BSBMV particles. The results obtained here emphasise that the RT domain, including the P75 minor coat protein, is dispensable for particle formation, systemic infection and symptom development. These observations are in contrast to Schmitt et al. (1992), who postulated that the N-terminal half of the RT domain is essentially for virus particle assembly. Moreover, Haeberlé et al. (1994) and Erhardt et al. (2001) assumed that the resulting P75 minor coat protein is incorporated only at the extremities of rod-shaped viral particles. In contrast, in our study a decoration with gold particles to localise the GFP marker protein showed that the read-through protein made of CP and GFP is incorporated over the entire surface of virus particles. However, Haeberlé et al. (1994) detected P75 with an RT-specific antiserum and that due to the modification, the RT domain (54kDa) is replaced by GFP with a size of 27kDa. Whether the smaller size and the deletion of specific sequences within the RT is responsible for the localisation over the entire particle surface needs to be further investigated. Probably the modified clones are not vector transmittable anymore, because the KTER motif which is located in the RT region and is associated with efficient transmission by *P. betae* was replaced (Lee et al., 2001; Tamada et al., 1996). All-over, it can be concluded that a suitable cloning site within BSBMV/BNYVV RNA2 has been identified and therefore experiments to study the interaction between both viruses in co- and super-infection experiments on cellular level could be performed.

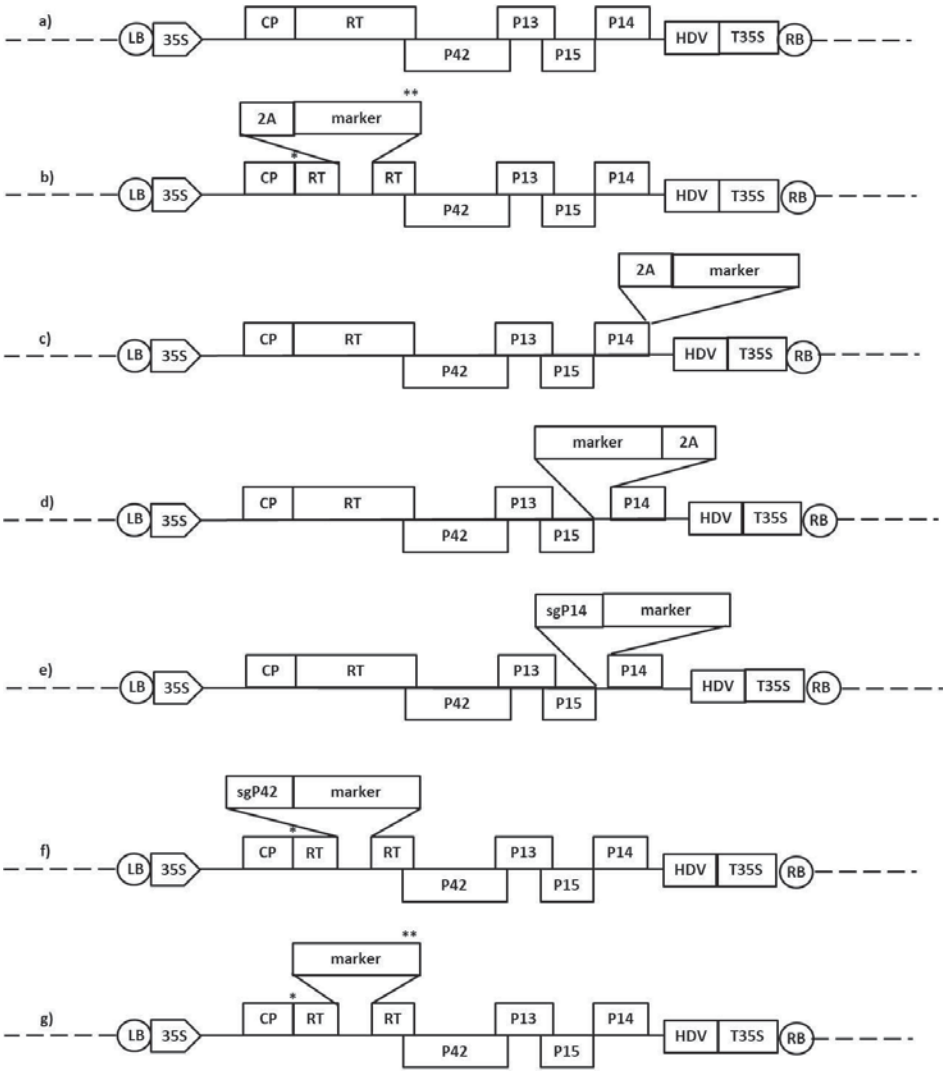


Fig. 7: Schematic representation of full-length infectious cDNA clones of the (a) BSBMV RNA2 based vectors and modified variants (b-g).
LB: Left border; 35S: Cauliflower mosaic virus (CaMV) 35S promoter; CP: coat protein for encapsidation; RT: read-through domain for transmission; P42, P13 and P15: triple gene block for movement; P14: viral silencing suppressor; HDV: Hepatitis delta virus ribozyme; T35S: CaMV Terminator 35S; RB: Right border; *: leaky stop codon; **: stop codon; 2A: 16 amino acid long 2A region of the foot-and-mouth disease virus; marker: different fluorescent proteins.

6.4.2 Challenges of fluorescent labelling

As described in manuscript II, BSBMV/BNYVV RNA2 were successfully labelled with different green fluorescent proteins (GFPuv, GFPuv^{A206K}, smRS-GFP) and mRFP. After transformation into *A. tumefaciens*, host plants like *N. benthamiana* and *B. macrocarpa* were inoculated via leaf agroinoculation. Plants inoculated with BSBMV/BNYVV clones harbouring a fluorescent protein were viable and did not interfere with symptom expression and systemic movement. Leaf tissue displaying symptoms of systemic infection was analysed with CLSM. A clear homogenous fluorescence of mRFP was detected in mesophyll cells of *N. benthamiana* and *B. macrocarpa.* Striking was the uneven distribution of smRS-GFP and GFPuv in small bright clusters for both viruses. A similar cluster formation has been reported in *N. benthamina* plants agroinoculated with

TuYV labelled with enhanced green fluorescent protein (EGFP) (Boissinot et al. 2017). It was speculated that these clusters are membrane proliferations and probably sites of viral replication or encapsidation. Based on the mitochondrial targeting sequence on P75 (Erhardt et al., 2001; Valentin et al., 2005), an assumption was that the clusters represent virus particles which accumulate at the mitochondria, the site of virion assembly. Therefore, it was tested whether the green fluorescence protein co-localises with cell organelle markers for mitochondria. Simultaneous agroinoculation of *N. benthamiana* with smRS-GFP labelled clones and a red mitochondrial marker (Nelson et al., 2007) was conducted. The co-localisation experiment did not give information about the cluster formation because virus and marker fluorescence did not co-localise after overlay imaging. In contrast it might be possible that virus particle assembly, which is associated with the N-terminal half of the 54kDa RT domain (Tamada and Kusume, 1991; Schmitt et al., 1992) could be influenced by the incorporation of GFP because cluster formation was not observed when mRFP was incorporated. This could also explain the delayed symptom formation of the labelled GFP clones compared to the unlabelled variants. Von Stetten et al. (2012) reported that GFP dimerises at high concentrations. Consequently, artefacts in microscopy experiments could arise, which might lead to a cluster formation. By mutating amino acid position 206 from alanine to lysine (A206K), the authors achieved a minimal improvement. Therefore, clones with an alanine to lysine mutation at amino acid position 206 were generated. CLSM images of BSBMV-GFPuv^{A206K} and BNYVV-GFPuv^{A206K} displayed a better distribution of the fluorescence but several small bright clusters remained. This might be explained with a poor solubility of the green fluorescent proteins. It was assumed that in co- and super-infection experiments, the cluster formation had no effect on the spatial separation of viral populations.

6.4.3 Interaction studies of BSBMV and BNYVV on cellular level

Co- and super-infection experiments (manuscript II) with labelled BSBMV and BNYVV clones were conducted in *N. benthamiana* and examined by means of CLSM. In co-infection experiments, differentially labelled isolates of the same species as well as the two different virus species remained spatially separated, which can be interpreted as co-infection exclusion. Furthermore, spatial separation was also observed with RNA1+2 reassortant indicating that a specific genome component combination was not required for this effect. In contrast, co-infection of BSBMV with an unrelated virus like TRV or PVX, resulted in many mixed infected cells. In super-infection experiments, BSBMV and BNYVV acted similar compared to co-infection experiments and showed super-infection exclusion. Only the unrelated viruses PVX and TRV were able to establish an infection in super-infected plants and spread systemically.

By the application of different methods, the observation of spatial separation has been confirmed by several scientists (Dietrich and Maiss, 2003; Hall et al., 2001; Hull and Plaskitt, 1970; Takeshita et al., 2004). Decades ago, it was already shown by electron microscopy that two strains of AMV,

which form distinct aggregation bodies, keep separate after co- and super-infection (Hull and Plaskitt, 1970). By using molecular markers, Hall et al. (2001) showed co-infection exclusion and super-infection exclusion for different strains of *Wheat streak mosaic virus* (WSMV). Takeshita et al. (2004) analysed the spatial separation of CMV subgroups by tissue-blotting and *in situ* hybridisation. Nowadays, fluorescent markers have become the most important tool to study the spatial separation of different plant viruses like *Potyvirus* and *Potexviurs* (Dietrich and Maiss, 2003), *Cheraviurs* (Takahashi et al., 2007), *Tobamovirus* (González-Jara et al., 2009; Julve et al., 2013), *Potyvirus* (Gutiérrez et al., 2015), *Tritimovirus* and *Poacevirus* (Tatineni and French 2016). Consequently, BSBMV/BNYVV experiments were conducted by using fluorescent markers. After co-infection of differentially labelled virus populations from BSBMV and BNYVV, contact at the border of different fluorescent cell clusters was observed but it was restricted to a few cells. This observation indicates that even though a co-infection of the same cell is not very common, replication within the same cell of both viruses could still be supported. In contrast to this, inoculation experiments with a benyvirus and an unrelated virus (PVX or TRV) lead frequently to double infected cells. This is in accordance with Dietrich and Maiss (2003) and Takahashi et al. (2007). Dietrich and Maiss (2003) co-inoculated *N. benthamiana* with differentially labelled potyvirus populations, what resulted in a replication in discrete areas and only a few cells were double infected. Moreover, they co-inoculated a potyvirus with an unrelated virus (PVX), which resulted in a co-infection of the same cells. Takahashi et al. (2007) have made the same observation in co-infected *N. benthamaina* plants using labelled ALSV (genus: *Cheravirus*) and BYMV (genus: *Potyvirus*). However, the mechanistic basis and viral determinants behind this phenomenon of spatial separation of related viruses following co-infection is not known. It can be speculated, that the border cells represent the cells where both related viruses initiated infection simultaneously, but one virus prevents the other to spread to other cells where one of the viruses has already been established. This indicates mechanisms, which are discussed in terms of super-infection.

In super-infection exclusion experiments, the related viruses BSBMV and BNYVV interact in an antagonistic manner. Only small fluorescence clusters of the challenging virus were observed in super-infected leaves. However, super-infection with an unrelated virus resulted in a synergistic spread of the virus. Similar to co-infection, exclusion after super-infection seems to be strongly driven by the degree of relationship. For example, Folimonova et al. (2010) demonstrated super-infection exclusion of CTV only between isolates of the same strain and not between isolates of different strains. As early as 1929, McKinney observed that tobacco was protected by TMV against a secondary infection of another strain of TMV. Over the years different theories have been proposed but explanations behind the mechanism are missing. In 1969 Gibbs speculated that maybe a passive competition for the same host factors, which are necessary for the viral life cycle, exists between the related viruses. Based on TMV studies, González-Jara et al. (2009) speculated

that the primary virus has a selective advantage and consequently prevents super-infection of another virus. García-Cano et al. (2006) speculated that probably a certain threshold titre of the virus or of proteins or a time lapse is needed to interfere or downregulate the challenging virus. Alternatively, exclusion could be conceivable due to the fact that the protective virus occupies most of the virus-specific replication sites (Hull and Plaskitt, 1970). This is in accordance with Dodds et al. (1985), who suggested that somehow the replication of the challenging virus is suppressed and a lack of accumulation may exist. Baulcombe (1996) reviewed that spatial separation could occur due to the competition for plasmodesmatal binding sites between the movement proteins. This hypothesis was refuted by Julve et al. (2013) who showed that TMV movement protein is not responsible for super-infection exclusion. Another hypothesis is that the CP of the protecting virus which has already been established in the cell influences and prevents the assembly of the challenging virus (de Zoeten and Fulton, 1975; Powell Abel et al., 1986). It seems that this mechanism is determined on the CP production level in infected cells (Baulcombe 1996). However, both CPs may interact with each other and the inhibition is greater when CP sequences are more similar as shown for TMV and *Sunn-hemp mosaic virus* by Beachy (1999). Recently, Tatineni and French (2016) demonstrated that WSMV and *Triticum mosaic virus* encoded CP and Nia-Protease proteins trigger super-infection exclusion independently of each other. Moreover, a partly expression of CP is enough to induce super-infection exclusion (Tatineni and French, 2016). Folimonova (2012) assumed that CTV requires the production of a specific viral protein namely P33, which has the function of extending the virus host range. A lack of P33 prevents super-infection exclusion by the same or closely related viruses. Later, it was shown that P33 mediates super-infection exclusion at the whole organism level but is not required for exclusion at the cellular level (Bergua et al., 2014), indicating that two different mechanisms exist. More recently, Atallah et al. (2016) elicited that P33 alone is not sufficient for virus exclusion. Their experiments indicated that a substitution of the proteases L1 and L2 of CTV affected the exclusion ability. Whether the proteases function independently or together with P33 remains unknown. Likewise, Zhang et al. (2017) assumed for turnip crinkle virus (TCV) that super-infection exclusion is a protein-based mechanism. They showed that the exclusion is triggered by P28 of the protecting virus and blocks the replication the challenging virus**.** It is suggested by the authors that the mechanisms of co- and super-infection exclusion are identical. Moreover, they suppose that super-infection prevents replication of progeny viruses in the cells of their “parents” with the aim to maintain viral genome integrity and to reduce errors made by the RdRP (Zhang et al., 2018). In contrast, Ratcliff et al. (1999) showed that super-infection exclusion can be elicited by RNA silencing. Small RNAs processed from the former dsRNA guide the degradation of RNA of the challenging virus, which contain homologous sequences (Ratcliff et al., 1999). Tatineni and French (2016) suggested that RNA silencing is not involved in super-infection exclusion because 98% sequence homology at RNA level between WSMV CP and Nia-Protease to a defective mutant

failed to trigger super-infection exclusion in their experiments. A further mechanism of super-infection exclusion but so far only reported in animal viruses, is associated with virus entry into cells, which is supported by specific cell surface receptors (Lee et al., 2005).

So far, different mechanisms have been postulated for several viruses. Remarkably, the exclusion mechanism is strongly depending on the host and the infecting virus. Whether the co- and super-infection exclusion of benyvriuses is influenced by the encoded proteins, their RNA sequences or host-pathogen interaction has to be determined. Thus, further studies to elucidate the mechanism are required. The labelled BSBMV- and BNYVV-clones, are valuable tools to explore the mechanism behind. Viral effectors involved in the exclusion mechanism can be studied by loss-of-function experiments by deletions of viral ORFs or genomic exchanges between the two viruses. Moreover, individual virus-encoded proteins can be expressed by a heterologous virus to study their effect on the exclusion mechanism. Based on Culver (1996), who showed that PVX based vectors expressing TMV CP are able to decrease the TMV accumulation when plants were challenged with TMV, function of BSBMV/BNYVV proteins could be examined in the same way. Therefore, a primary infected plant with a heterologous virus can be challenged with a labelled clone to examine super-infection exclusion. Despite the unknown mechanism, it can be hypothesised that the risk of reassortment and recombination between BSBMV and BNYVV in mixed infections is low due to the observed spatial separation on the cellular level. So far, the experiments were conducted in *N. benthamiana* and have to be performed in *B. vulgaris* to verify the observations.

6.5 BSBMV and BNYVV as expression vector

Lastly, the generated clones (BSBMV and BNYVV) were applied as vectors for VIGS (manuscript III). Two different target genes in sense and antisense orientation were used for the BSBMV and BNYVV VIGS approach and inserted by partial replacement of RT as described above. The target gene *phytoene desaturase* (*pds*) is represented by a 578 bp fragment from a *B. macrocarpa pds* fragment (Acc. No. XM_010686886.1) displaying only two nucleotide changes compared to the *B. vulgaris pds* sequence. The second target gene was a 549 bp fragment of *magnesium chelatase subunit H* (*chlH,* Acc. No. XM_010674548.1) which is involved in chlorophyll biosynthesis. Downregulation of the encoding gene *chlH* leads to yellow patches on leaves in which chlorophyll biosynthesis is interrupted but carotenoid biosynthesis is not affected (Hiriat et al., 2002; Igarashi et al., 2009). Constructs containing *pds* cause a white phenotype (Kumagai et al., 1995). Agroinoculation of *N. benthamiana* resulted in systemic infection and silencing of *pds* and *chlH*, respectively. The observed white/yellow colour of PDS and ChlH silencing phenotype in the BNYVV or BSBMV RNA2 background was similar to PDS or ChlH silencing phenotype in *N. benthamiana* described in preceding studies (Hiriart et al., 2002; Pignatta et al., 2007; Ratcliff et al., 2001; Ruiz et al., 1998). Contrary to the report of Smith et al. (2000), no apparent differences

between sense and antisense constructs were observed. BSBMV VIGS constructs in which the sense and antisense PDS sequences are separated by an intron from *Solanum tuberosum* (ST-LS1, Libiakova et al., 2001) were not considered for further experiments because only one *N. benthamina* plant of ten displayed a mild photobleaching phenotype in newly emerged leaves. This indicates that maybe an insertion of a >1000 bp fragment negatively interferes with BSBMV infectivity and spread. Moreover, it was shown by Burch-Smith et al. (2004) that silencing efficiency is reduced with increasing sequence length. Large inserted sequences could be partially or completely lost (Dickmeis et al., 2014). To measure the silencing efficiency of *pds* and *chlH* quantitatively, plants infected with sense and antisense constructs were analysed by means of qRT-PCR. In case of PDS, sense and antisense constructs displayed a similar silencing efficiency for both viruses. In contrast, BSBMV equipped with ChlH sense and antisense showed no significant differences to the wild-type infected plant. Since plants infected with BSBMV ChlH constructs displayed the silencing phenotype, an experiment with more repetitions would help to confirm a significant silencing efficiency. Furthermore, the environmental conditions could be optimised to improve the effect of silencing. The experiments were always conducted under the same environmental conditions of 24°C/14h 18°C/10h, but the fact that both viruses have different temperature requirements (Rush, 2003), suggests that experiments should be conducted at varying temperatures. It also has to be considered that the silencing efficiency is influenced by the sequence of the insert as well as the selected part of a target gene (Pignatta et al., 2007).

For both target genes, BNYVV infected *N. benthamiana* plants displayed a more severe silencing effect (visually and quantitatively) compared to BSBMV. In a similar way, Ratcliff et al. (2001) conducted experiments with viruses equipped with PDS and observed a more extensively photobleaching phenotype in *N. benthamiana* infected with TRV as with PVX. Similar to BNYVV, TRV naturally does not induce visual symptoms compared to PVX and BSBMV. Caused by their symptom expression, it seems like BNYVV (this thesis) and TRV (Ratcliff et al., 2001) are more effective as VIGS vectors than BSBMV and PVX. This could be due to the fact that the virus-host combinations and interaction influence the extent of silencing and severity of viral symptoms (Robertson, 2004). Moreover, the replication cycle of the virus and environmental conditions, especially temperature, affect the effectiveness of VIGS (Burch-Smith et al., 2004; Senthil-Kumar and Mysore, 2011).

So far, both benyviruses are a useful and efficient tool for VIGS in *N. benthamiana*. Therefore, BSBMV and BNYVV are the first sugar beet infecting RNA viruses used as tools for VIGS and could probably be suitable as VIGS-systems in sugar beet. However, further investigations are needed to confirm their application in *B. vulgaris.* It can be speculated, due to the limited ability of BNYVV (in contrast to BSBMV) to induce a systemic infection, that BSBMV would be more suitable as VIGS vector in *B. vulgaris*. Moreover, it would also be interesting to test reassortants between

BSBMV and BNYVV for their silencing efficiency in sugar beet. This might help to reduce the severe symptoms associated with an infection of BNYVV or BSBMV, respectively.

7. Summary

The close relatives *Beet soil-borne mosaic virus* (BSBMV) and *Beet necrotic yellow vein virus* (BNYVV) represent plant viruses of the genus *Benyvirus* (family: *Benyviridae*). Both species induce diseases on sugar beet (*Beta vulgaris*) with different symptom severity and tissue colonisation. BSBMV produces leaf mosaic symptoms; BNYVV is the causal agent of rhizomania. In contrast to BNYVV, which is mainly restricted to the root system, BSBMV can colonise sugar beet systemically. While BNYVV in sugar beet is controlled by plant resistance, so far no natural BSBMV resistance is known. However, the BNYVV resistances are limited, since the BNYVV resistance gene *Rz1*, does not target BNYVV A-type isolates, carrying a mutation on RNA3, anymore. Naturally, both viruses can occur in mixed infections in sugar beet. Information on possible antagonistic interactions is still very limited. So far, there is no information about the tissue colonisation and exclusion mechanism by both species available. Moreover, a formation of reassortants and recombinants are unknown, but it can be expected that this would influence the virus evolution.

Thus, the aims of this thesis were to generate infectious cDNA clones of BSBMV and BNYVV for agroinoculation of different host plants. Also, to perform reassortants experiments with BSBMV and BNYVV A-type to assess the viability and consequences of reassortants. Moreover, it was the aim to find a suitable cloning site for fluorescence labelling, without losing the functionality of the recombinant virus, to study the molecular interaction of BSBMV and BNYVV on cellular level. Lastly, another aim was to develop a virus-induced gene-silencing (VIGS) system based on BSBMV and BNYVV.

A Californian isolate of BSBMV was extracted from *C. quinoa* virus-induced local lesion and the single components were amplified and assembled as one fragment (RNA3 and RNA4), two fragments (RNA2) or as three fragments (RNA1). Vectors suitable for agroinoculation of all genome components of BSBMV were constructed by using the approach of Gibson assembly.

The functionality of the recombinant virus was demonstrated by displaying a comparable symptom expression to the wild-type in different host plants, displaying characteristic virus particles and replication of all components as well as transmission by *P. betae*. *N. benthamiana* symptoms inoculated with BSBMV RNA1+RNA2 or BSBMV RNA1-RNA4, respectively, were undistinguishable. This leads to the assumption that systemic movement and symptom induction are not affected by the smaller genomic RNA species.

The generated BSBMV sequences were compared with the published sequences of the characterised BSBMV isolates MRM06 (originating from Texas, USA; D'Alonzo et al., unpublished; D´Alonzo et al., 2012; Ratti et al., 2009) and EA (originating from Colorado, USA; Lee et al., 2001). The sequence comparisons revealed in a closer sequence similarity at nucleotide level over all RNA components of the Californian BSBMV isolate to isolate MRM06 than EA. To study a possible interaction of both viral species during mixed infection, an artificial formation of reassortants of

BSBMV and BNYVV were conducted. Plant infection was performed by means of agroinoculation. Results demonstrated that *in vitro* reassortants (BSBMV RNA1+BNYVV RNA2 and *vice versa*) were viable and capable to systemically infect *N. benthamiana*. Agroinoculation of *B. macrocarpa* with BSBMV and BNYVV RNA1-2, respectively, did not lead to systemic infection. However, when RNA3 was supplemented to the inocula systemic movement occurred for both viruses. Moreover, small genomic RNAs were exchangeable and resulted in systemic spread.

To understand the colonisation strategy of the two benyviruses, a labelling of individual genome components was required. Therefore, several strategies have been tested to find a suitable cloning site. Finally, a nearly complete replacement of the read-through domain on RNA2 resulted in a successful labelling of both viruses. Contrary to Schmitt et al. (1992), Haeberlé et al. (1994) and Erhardt et al. (2001), electron microscopy revealed a partial overcoat of virus particles with the fluorescent marker protein, demonstrating that the read-through domain is dispensable for particle formation. The monomeric red fluorescent protein (mRFP) and different green fluorescent proteins (GFP) were used as fluorescent markers.

N. benthamiana and *B. macrocarpa* were agroinoculated and local as well as systemically infected leaves were analysed by means of epi-fluorescence microscopy and confocal laser scanning microscopy. The labelled clones were infectious, moved systemically and produced wild-type like symptoms. Striking was the uneven distribution of smRS-GFP and GFPuv in small bright clusters for both viruses. This problem was partly solved due to a mutation from alanine to lysine at amino acid position 206 (A206K). Co-infection experiments with labelled BSBMV and BNYVV showed that both viruses remained spatially separated. In contrast, a mixture of BSBMV with an unrelated virus either *Potato virus X* (PVX) or *Tobacco rattle virus* (TRV) resulted in no co-infection exclusion. Moreover, super-infection exclusion experiments confirmed the results generated by the co-infection experiments. This demonstrated a possible antagonism between BNYVV and BSBMV.

In addition, a virus-induced gene-silencing (VIGS) system based on BSBMV and BNYVV was developed. Therefore, RNA2 (position as described above) of both viruses were equipped with fragments of the *magnesium chelatase subunit H* (*chlH;* 549 bp) and *phytoene desaturase (pds;* 578 bp) genes in sense and in antisense orientation. Silencing phenotypes in *N. benthamiana* induced by both target genes were comparable to those described in the literature. The silencing effect on transcript levels of ChlH and PDS was analysed by means of qRT-PCR. The qRT-PCR analysis showed a significant reduction of ChlH and PDS mRNA level in infected *N. benthamiana* compared to the non-inoculated control plants, except for BSMBV equipped with ChlH. The data show that BSBMV and BNYVV are suitable candidates for VIGS in *N. benthamiana*. The next step would be to apply the VIGS system to *B. vulgaris* to study the exact role and function of different genes e.g. resistance genes in sugar beet, genes influencing host-virus interaction or functions associated with the virus.

8. Literature

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