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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).

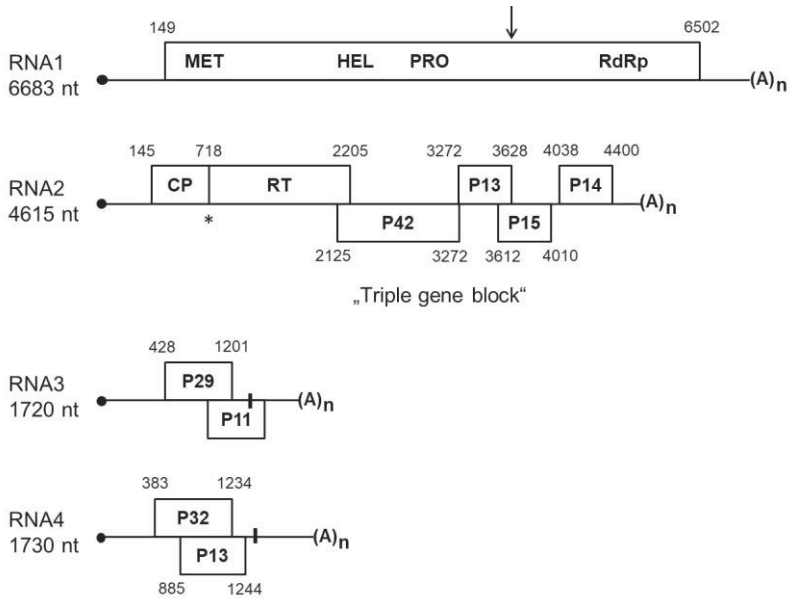

Fig. 1: Genome organisation of *Beet soil-borne mosaic virus* (BSBMV). BSBMV consists of four RNA segments, each segment possess a cap structure (filled circles) at the 5´end and a poly A-tail (A) at the 3´end. Rectangles display the open reading frames (ORF) in the genome. Inside each rectangle the 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.

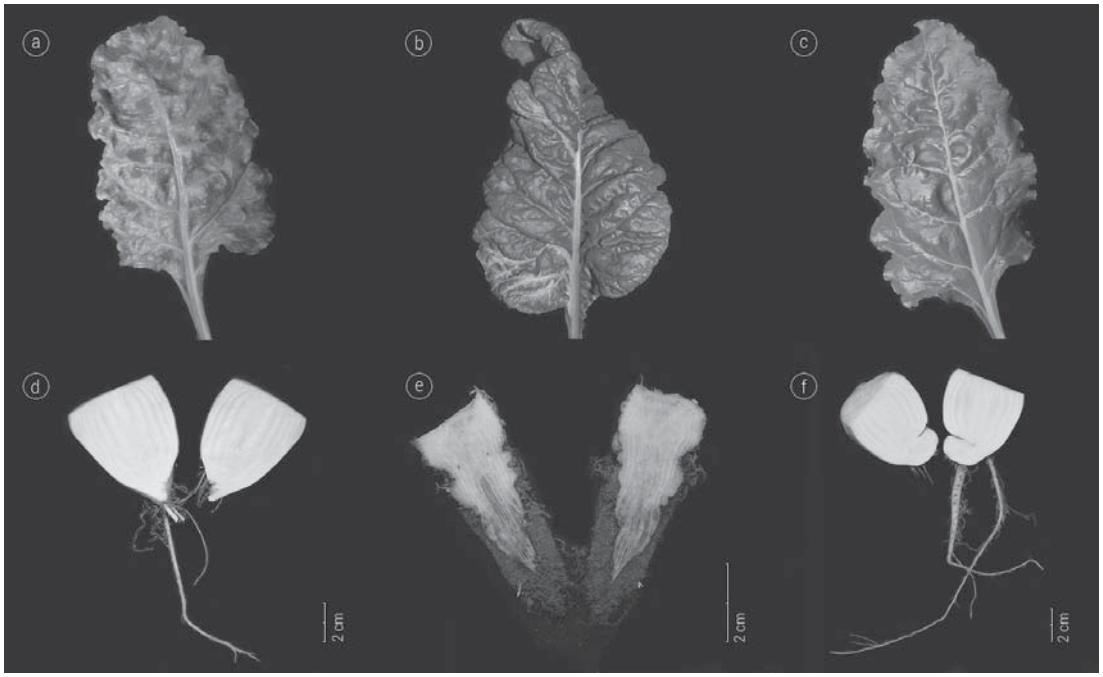

Fig. 2: Symptom expression on *Beta vulgaris* leaves (a-c, at 48 dpi) and taproots (d-f, at 84 dpi) produced after mechanical root vortex-inoculation with plant sap from *Chenopodium quinoa* local lesions infected with (a;d) wild-type BSBMV and (b;e) wild-type BNYVV compared to (c;d) healthy control.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