Construction of a Virus-Induced Gene Silencing System based on *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV)

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ABSTRACT

Rhizomania is currently one of the most important plant diseases in all sugar beet growing-regions that causes great yield and sugar losses. Despite breeding progress, a higher yield is required under rhizomania conditions. Several biotechnology approaches have been employed to further understand and enhance disease resistance. Among these, virus-induced gene silencing (VIGS) has lately attracted special interest e.g. for identification of plant gene functions.

The aim of this study was to verify if two benyviruses, namely *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) are in general suitable tools for VIGS and to investigate the molecular interaction between both viruses. Both viruses belong to the genus *Benyvirus* in the family *Benyviridae*. They have a similar morphology and genome organisation, are transmitted by the same vector and share the same host range. In this study, infectious full-length cDNA clones of BNYVV (A-type) and BSBMV for *Rhizobium radiobacter*-mediated infection have been successfully constructed by the Gibson Assembly *in vitro* recombination technique. In addition, clones with the monomeric red fluorescent protein (mRFP) or green fluorescent protein (GFP) based on RNA2 of BNYVV or BSBMV were first prepared by replacing a part of the read-through domain open reading frame. These clones were *R. radiobacter* inoculated together with a full-length clone of RNA1 into *Nicotiana benthamiana* and *Beta macrocarpa*. With this experimental approach the labeled viruses were detectable in locally and systemically infected leaves by fluorescence microscopy. On the basis of these vectors, RNA2 of both viruses was equipped with appropriate restriction sites. A 578 bp fragment of *phytoene desaturase* (*pds*) gene from *N. benthamiana* and a 549 bp fragment of *magnesium chelatase* (*chlH*) gene from *Nicotiana tabacum* were amplified for the silencing experiments and were integrated by restriction enzyme digest and ligation in sense and antisense orientation into the modified RNA2 of BNYVV and BSBMV. The clones were inoculated in *N. benthamiana* and silencing phenotypes of PDS and ChlH were recorded at 26 days past inoculation. Subsequently, significant reductions in both *pds* sense (59-77%) and antisense (49-60%), as well as *chIH* sense (67-85%) and antisense (74-86%) mRNA levels were measured by quantitative real-time PCR in the photobleached leaves of both BSBMV/BNYVV VIGS-treated plants, respectively.

As a further step in optimization of the VIGS vectors, the biological characteristics of both viruses were studied, using reassortants of BNYVV/BSBMV. In this part of the thesis we demonstrated that both (RNA1+2) *in vitro* reassortants were viable and capable of viral systemic movement in *N. benthamiana*. However, the plants infiltrated with both reassortants displayed a difference in symptom severity. In addition, RNA3 or 4 of BNYVV or BSBMV were exchangeable and able to move systemically in *B. macrocarpa* plants. Furthermore, co-infection and super-infection experiments based on labeled BNYVV and BSBMV as well as the two unrelated viruses *Tobacco rattle virus* (TRV) and *Potato virus X* (PVX) were conducted in *N. benthamiana*. From previous results of this study, the conclusion can be drawn that BSBMV and BNYVV tend to be spatially separated during plants infection whereas distant related viruses show clear co-infected cells in both co- and super-infection experiments.

Keywords: *Beet necrotic yellow vein virus*, *Beet soil-borne mosaic virus*, Virus-induced gene silencing

ZUSAMMENFASSUNG

Rizomania ist derzeit eine der wichtigsten Pflanzenkrankheiten in allen Zuckerrübenanbaugebieten, die zu hohen Ertrags und Zuckerverlusten führt. Auch die Fortschritte in der Züchtung können den negativen Effekten der Krankheit in Bezug auf Ertrag und Zuckergehalt nicht entgegenwirken. Mehrere biotechnologische Ansätze wurden eingesetzt, um die Krankheitsresistenz besser zu verstehen und zu optimieren. Vor allem hat das Virus-induzierte Gen Silencing (VIGS), z.B. zur Identifizierung von Pflanzengenfunktionen in den letzten Jahren zunehmend an Aufmerksamkeit gewonnen. Ziel dieses Projektes war die Überprüfung zweier Benyviren, *Beet necrotic yellow vein virus* (BNYVV) und *Beet soil-borne mosaic virus* (BSBMV) als generelle Werkzeuge für VIGS, sowie die molekulare Interaktion zwischen beiden. Beide Viren gehören zur Gattung *Benyvirus* in der Familie der *Benyviridae*. Sie haben eine ähnliche Morphologie und Genomorganisation, werden von demselben Vektor übertragen und teilen sich den gleichen Wirtsbereich. In dieser Studie wurden infektiöse Volllängen-cDNA-Klone von BNYVV (A-Typ) und BSBMV für *Rhizobium radiobacter*-vermittelte Infektionen erfolgreich durch *Gibson Assembly in vitro* Rekombinationstechnik konstruiert. Zunächst wurden mehrere Klone mit dem *monomeric red fluorescent protein* (mRFP) oder dem *Green Fluorescent Protein* (GFP) basierend auf RNA2 des BNYVV beziehungsweise BSBMV hergestellt, indem ein Teil des read-through Domain offenen Leseframes ersetzt wurde. Diese Klone wurden dann zusammen mit einem Volllängen-Klon von RNA1 mittels *R. radiobacter* in *Nicotiana benthamiana* und *Beta macrocarpa* inokuliert. Mit diesem Versuchsansatz ist es gelungen, die markierten Viren mittels Fluoreszenzmikroskopie in lokal und systemisch infizierten Blättern nachzuweisen. Auf Basis dieses Konstrukts wurde RNA2 erfolgreich mit Restriktionsschnittstellen ausgestattet. Ein 578 bp-Fragment des *phytoenedesaturase* (*pds*) Gens aus *N. benthamiana* und ein 549 bp-Fragment des *magnesium chelatase* (*chlH*) Gens aus *Nicotiana tabacum* wurden für die *silencing*-Experimente amplifiziert und durch Restriktionsenzymverdau und Ligation in sense und antisense-Orientierung in die modifizierte RNA2 von BNYVV und BSBMV integriert. Diese Klone wurden daraufhin in *N. benthamiana* inokuliert und nach 26 Tage nach Inokulation die *silencing*-Phänotypen des PDS und ChlH bonitiert. Anschließend wurden signifikante Reduktionen des *pds* in sense (59-77%), beziehungsweise in antisense (49-60%) und des *chIH* in sense (67-85%), beziehungsweise in antisense (74-86%)

Transkriptionslevel mittels *Real-Time quantitative* PCR in den gebleichten Blättern der beiden BSBMV/BNYVV VIGS-behandelten Pflanzen gemessen. Als weiteren Schritt in der Optimierung der VIGS-Vektoren wurden die biologischen Eigenschaften beider Viren genauer studiert, in dem wir eine Reihe von Reassortanten des BNYVV/BSBMV getestet haben. In diesem Teil der Arbeit wurde gezeigt, dass beide (RNA1 + 2) *in vitro* Reassortanten vermehrungsfähig und zur systemischen Ausbreitung in *N. benthamiana* in der Lage waren. Allerdings zeigten die Pflanzen, die mit Reassortanten infiltriert wurden, einen Unterschied in der Symptomausprägung. Darüber hinaus waren RNA3 oder 4 von BNYVV oder BSBMV austauschbar und konnten sich systemisch in *B. macrocarpa* Pflanzen ausbreiten. Des Weiteren wurden Co-Infektion und Super-Infektion Experimente auf Basis von markiertem BNYVV und BSBMV sowie zwei nicht verwandten Viren, *Tobacco rattle virus* (TRV) und *Potato virus X* (PVX) in *N. benthamiana* durchgeführt. Aus den vorherigen Ergebnissen dieser Dissertation lässt sich die Schlussfolgerung ziehen, dass BSBMV und BNYVV während der Pflanzeninfektion räumlich getrennt bleiben, während nicht verwandte Viren bei Co- und Super-Infektion Experimenten klare co-infizierte Zellen aufweisen.

Schlagworte: *Beet necrotic yellow vein virus*, *Beet soil-borne mosaic virus*, Virus-induziertes Gen Silencing

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ABBREVIATIONS

%	percent
A.	*Arabidopsis*
AGO	Argonaute
A. tumefaciens	*Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*)
APG	Angiosperm Phylogeny Group
as	Antisense
ATP	Adenosintriphosphat
b	Base
B.	*Beta*
bp	basepair(s)
Blast	Basic Local Alignment Search Tool
°C	degree Celsius
cDNA	complementary DNA
ChlH	magnesium-Chelatase
CP	coat protein
Ct	threshold cycle
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dsDNA/RNA	double-stranded DNA/RNA
DSMZ	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	*et alii*
FD	Fast Digest
Fig.	figure
g	gramm
h	hour
HIGS	Host-induced gene silencing
ICTV	International Committee on Taxonomy of Viruses
kb	kilobasepair(s)
kD	kilodalton
LB	lysogeny broth
M	molar
MCS	multiple cloning site
min	minute(s)
miRNA	microRNA

ml	milliliter
mm	millimeter
mM	millimolar
MP	movement protein
mRNA	messenger RNA
N. benthamiana	*Nicotiana benthamiana*
NCBI	National Center for Biotechnology Information
NGS	next-generation sequencing
nm	nanometer
N protein	nucleoprotein
ORF	open reading frame
PCR	polymerase chain reaction
PDS	Phytoendesaturase
pH	potential hydrogen
PTGS	Post-transcriptional gene silencing
qRT-PCR	quantitative real-time polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RFLP	restriction fragment length polymorphism
R. radiobacter	*Rhizobium radiobacter*
RISC	RNA-induced silencing complexes
RNA	ribonucleic acid
RNase	ribonuclease
RNAi	RNA interference
RT-PCR	reverse transcription PCR
s	second(s)
s	sense
siRNA	small interfering RNA
ss	single stranded
sp.	species
spp.	species pluralis
TIGS	Transient-induced gene silencing
TGB	Triple Gene Block Cluster
Ta-siRNA	Trans-acting siRNA
tRNA	transfer RNA
U	unit(s)
UV	ultraviolet
v	volume
VIGS	Virus-induced gene silencing
VSRs	viral suppressors of RNA silencing
w	weight
µM	micromolar
µl	microliter
λ	Lambda

Viruses

ACMV	*African cassava mosaic virus*
ALSV	*Apple latent spherical virus*
BtMV	*Beet mosaic virus*
BNYVV	*Beet necrotic yellow vein virus*
BPMV	*Bean pod mottle virus*
BSBMV	*Beet soil-borne mosaic virus*
BWYV	*Beet western yellows virus*
BdMV	*Burdock mottle virus*
CaLCuV	*Cabbage leaf curl virus*
CaMV	*Cauliflower mosaic virus*
ClYVV	*Clover yellow vein virus*
CMV	*Cucumber mosaic virus*
CTV	*Citrus tristeza virus*
FHV	*Flock house virus*
PHYVV	*Pepper Huasteco yellow vein virus*
PPV	*Plum pox virus*
PVY	*Potato virus Y*
PVX	*Potato virus X*
RSNV	*Rice stripe necrosis virus*
RTBV	*Rice tungro bacilliform virus*
RYMV	*Rice yellow mottle virus*
SPMMV	*Sweet potato mild mottle virus*
STMV	*Satellite tobacco mosaic virus*
TBSV	*Tomato bushy stunt virus*
TCV	*Turnip crinkle virus*
TGMV	*Tomato golden mosaic virus*
TMV	*Tobacco mosaic virus*

ToMV	*Tomato mosaic virus*
TriMV	*Triticum mosaic virus*
TRSV	*Tobacco ringspot virus*
TRV	*Tobacco rattle virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mottling virus*
TYLCCNV	*Tomato yellow leaf curl China virus*
WSMV	*Wheat streak mosaic virus*

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1. Chapter 1: General Introduction

1.1. Sugar beet (*Beta vulgaris* subsp. *vulgaris*)

Sugar beet (*Beta vulgaris* subsp. *vulgaris*) is one of the most important agricultural crop and it is mainly cultivated for sugar production. Initially, at the end of the eighteenth century, sugar beet was developed in Europe from various *Beta* species, which proved to be the best alternative source to the tropical sugar cane for sugar production (Monteiro et al., 2013; Panella and Lewellen, 2007; Watson and Dallwitz, 1999). The cultivar *B. vulgaris* subsp. *vulgaris* was formerly assigned to the family *Chenopodiaceae* and the genus *Beta* L. This family includes about 1400 species, which is divided into 102 genera (Ajayi et al., 2017; Letschert et al., 1994). In the recent taxonomy of the APG II System (2003), *B. vulgaris* belongs to the family *Amaranthaceae* (Monteiro et al., 2013). *B. vulgaris* is a biennial root crop, but can reach flowering in the first year under certain conditions. In the first growing year (vegetative phase) the plant develops approximately 20 glabrous leaves, the beet body and an expanding storage root. In the second year (reproductive phase), after a period of vernalisation, sucrose is utilized for flower and seed production (Dohm et al., 2014; Panella and Lewellen, 2007).

In commercial production, the sugar beet root is harvested after the first growing season, as the time, when the sugar concentration in the root is the highest. The sugar beet has a cultivation period of about 6 months in the temperate climatic zone of the northern hemisphere. The sugar beet is planted in spring and harvested in the autumn of the same year. The ability of sugar beet to store sugar (sucrose) at high concentration within its root makes it commercially and physiologically very interesting for sugar production. Although the sugar concentration based on the fresh weight of the root has increased from about 4% in the last century to maximum of 20% today, there are still diverse factors that affect the sugar content of the root (Draycott, 2008; Kühnel et al., 2011). In recent years, improvement in chemical properties of the root and sugar concentration by breeding has created interest in growing sugar beet in many new areas worldwide. Additionally, tolerance and/or resistance to diseases and pests have positive impacts on sugar production (Joersbo, 2007). The main objectives of breeding are, as before, an increase in the sugar content in the root as well as an increase in the beet yield. However, there is a negative correlation between these properties, the cause of which is still unknown. For this reason, types of varieties have been developed which meet different perspectives: the

Z-type (high sugar concentration but low root yield), and the E-type (low sugar concentration but high root yield), as well as the intermediate normal genotype (N-type) (Loel et al., 2014).

Sugar beet is cultivated as raw material for sugar production (sucrose) and industrial production. The root of the sugar beet (taproot) consists in average of 14% of sugar (sucrose), 3.7 % of molasses, 5.5% of pulp and 76.8% of water, however, these data vary depending on the cultivar and growing conditions (Campbell, 2002; FAO, 2009). The viscous by-product molasses can be used for yeast and alcohol production or as a raw material for the production of citric acid. Beet vinasse is produced as a sugar free by-product after molasses has been fermented (Haaksma and Vecchiettini, 1988). The rest residue of sugar refining, sugar beet pulp can be used directly as feed for sheep and cattles or can be used as substrate in ethanol production (Sutton and Peterson, 2001). Currently, sugar beets are considered as energy crops, as they can be used to produce bioethanol and biogas (FAO, 2009; Schnepf, 2006). Aditionally, sugar beet serves as an important source for the chemical industry and for degradable materials (plastic, packaging) as well as in the cosmetics sector (Duke et al., 1984).

The sugar beet can be attacked by many pathogens like viruses, bacteria, fungi such as *Aphanomyces cochlioides*, *Cercospora beticola*, *Rhizoctonia solani*, *Ramularia beticola*, *Erysiphe betae* and *Peronospora farinose*, nematodes as *Meloidogyne* spp. and *Heterodera schacchtii.* Bacterial pathogens cause only a few significant diseases in sugar beet cultivation. Under German cultivation conditions *Pseudomonas syringae* pv. *aptata* is known as a causative agent for the bacterial leaf spot disease on the sugar beet (Lennefors et al., 2006; Stevens et al., 2006). The other bacterial diseases are harmless and therefore they are not economically significant. On the other hand, most diseases on sugar beet are caused by viruses such as *Beet mosaic virus* (BtMV), *Beet mild yellowing virus* (BMYV), *Beet yellows virus* (BYV), *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV). BNYVV is one of particular economic relevance worldwide that causes rhizomania disease on sugar beet (Tamada, 2007; Wintermantel et al., 2009). BNYVV and BSBMV are transmitted to the root of the sugar beet by the obligate root-infecting parasite Plasmodiophoromycete *Polymyxa betae* Keskin (Keskin, 1964; Tamada and Kondo, 2013). Rhizomania was first reported by Canova in Northern Italy in 1952 (Canova, 1959). Since that the virus was distributed to all other beet growing regions of Europe

and the Middle East (Kutluk Yilmaz et al., 2007; Lennefors et al., 2005), as well as USA, China and Japan (Gao et al., 1983; Schirmer et al., 2005). In contrast, BSBMV exists so far only in the United States (Koenig et al., 2008; Nielsen et al., 2001). The root yield reduction that caused by this disease can be as high as 90%, whereby the sugar content can decrease dramatically from 18% to under 10% (Joersbo, 2007; Stevens et al., 2006). It although caused undesirable enrichment of constituents (amino nitrogen, potassium and sodium) for the sugar production. This has made the disease very important in sugar beet cultivation (Heijbroek, 1989; Johansson, 1985; Rush and Heidel, 1995). The symptoms of Rhizomania can be observed in the whole sugar beet plant and vary greatly. The above-ground symptoms are less specific and occur only at the end of the vegetation period, in the form of leaf yellowing, wilting, chlorosis and necrotic yellow veins (Fig. 1C). Whereas the symptoms on the root are characterized by excessive lateral root proliferation and yellow-brown colouring of vascular bundles (Peltier et al., 2008). This abnormal proliferation of dark necrotic roots is the reason for the common name of the disease (rhizomania- root madness) (Canova, 1966; Rush and Heidel, 1995). Additionally, the infected beet body is small and woody compared to the healthy beet body (Fig. 1A and B) (Heidel et al., 1997; Peltier et al., 2008). However, BNYVV can cause only latent infections without visible symptoms under cool spring conditions (Lindsten, 1986; Pavli et al., 2011). Despite the plant protection products and pesticides were utilized to against various pathogens and their vectors, sugar beet cultivation and production still suffer from massive losses of up to 30% of the total yield. The sugar beet viruses cause worldwide income losses of 6-7% itself (Oerke and Dehne, 2004). Currently, the only practical means to control yield losses due to rhizomania infection is the use of genetically partially resistant cultivars. To date, such resistance is mainly based on dominant inherited genes (*Rz1*, *Rz2* and *Rz3*) that when exist reduce virus replication and movement from infected hair-roots to the main root (Biancardi et al., 2002; Pavli et al., 2011). The first partially rhizomania-resistant variety (Rizor) was introduced in 1985 in Italy and in the early 1990s grown in the infested fields of many European countries (Asher, 1993; De Biaggi, 1987). Later, following the primary field observations in 1983, the rhizomania-resistant cultivar (Holly source) has been introduced in 1987 in the USA (Lewellen, 1988). Further, it has been verified that “Holly” type resistance was simply inherited and based on the dominant *Rz1* gene (Lewellen and Biancardi, 1990; Pelsy and Merdinoglu, 1996). It has been suggested that both “Rizor” and “Holly” type resistances presumably share the same resistance gene (*Rz1*) (Barzen et al., 1997). Despite several resistance genes (*Rz1*-*Rz5*)

that lately have been identified, the majority of modern commercial sugar beet cultivars were developed based on the *Rz1* gene (Grimmer et al., 2007; Pavli et al., 2011). BNYVV can continue to replicate in the hair-roots of these resistant cultivars without penetrating into the main root (Scholten and Lange, 2000; Tamada et al., 1999). However, it has been assumed that the inoculum potential has no influence on the occurrence of resistance overcoming isolates (Pferdmenges and Varrelmann, 2009). Lately, resistance breaking (RB) isolates of BNYVV have been found in some sugar beet-growing areas in Europe (Acosta-Leal et al., 2008; Bornemann et al., 2015; Liu and Lewellen, 2007). To overcome these difficulties and hurdles in sugar beet production, new methods and strategies such as VIGS can be explored and investigated.

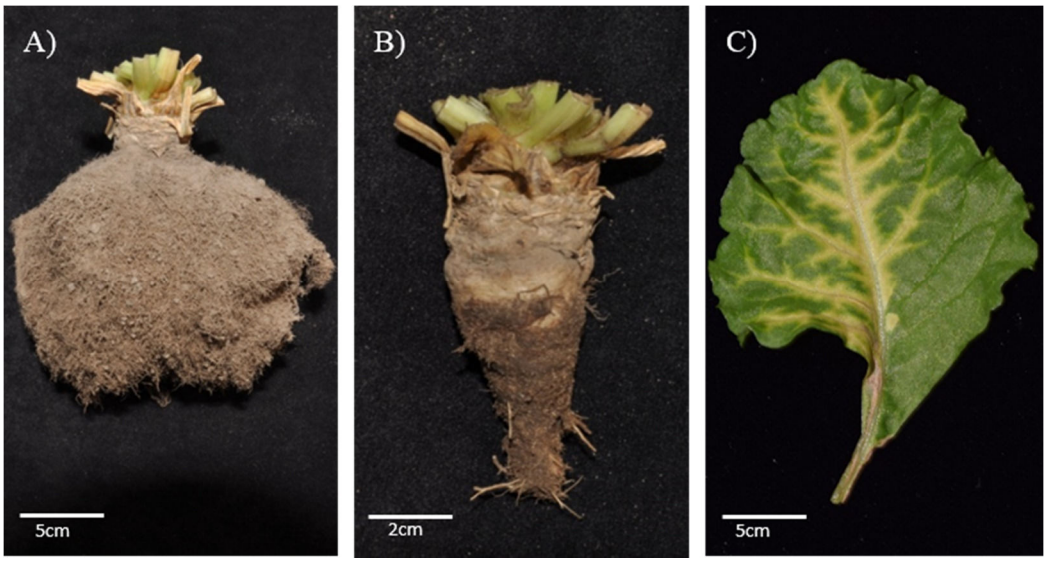

Fig. 1 Rhizomania symptoms on sugar beet. **A)** Beet body with many hairy secondary roots, which form a so-called root beard. **B)** Healthy sugar beet root. **C)** Leaf symptoms: yellowing along the leaf veins. (Photos: from M. Laufer and S. Liebe, IfZ, Göttingen).

1.2. Characteristics of benyviruses genomes

1.2.1. *Beet necrotic yellow vein virus* (BNYVV)

According to the International Committee on Taxonomy of Viruses (ICTV), *Beet necrotic yellow vein virus* (BNYVV), *Beet soil-borne mosaic virus* (BSBMV),

Rice stripe necrosis virus (RSNV) and *Burdock mottle virus* (BdMV) were assigned to the genus *Benyvirus* of the family *Benyviridae* (ICTV, 2017).

BNYVV is a multipartite RNA virus and depending on the isolate, it consists of 4 or 5 rod-shaped particles that include 4-5 genomic ss (+) strand RNAs (Gilmer et al., 2017; Jupin et al., 1991; Kiguchi et al., 1996; Koenig et al., 1997; Peltier et al., 2008) (Fig. 2). All RNA segments are encapsidated by the same viral coat protein (CP). Each RNA species has a poly-A sequence at the 3' end and a cap structure at the 5' end (Peltier et al., 2008; Putz et al., 1983). The various RNA particles are about 85-390 nm in length and 20-30 nm in diameter (Richards and Tamada, 1992). Under artificial conditions, RNA 1 and RNA 2 are sufficient to initiate systemic movement and distribution throughout different tissues in *Nicotiana benthamiana* (Chiba et al., 2013; Rahim et al., 2007). However, RNA3 particle is still required for long distance movement in other host plants, such as *Beta macrocarpa* (Lauber et al., 1998; Ratti et al., 2009). For the viral infection process under natural conditions, all RNA particles (1-4) are necessary.

RNA1 with 6,746 nucleotides in length is the largest RNA particle in the BNYVV genome. RNA1 is associated with RNA replication, comprises a single open reading frame (ORF), encoding a 237 kDa polypeptide (Bouzoubaa et al., 1987; Lennefors et al., 2005). The polypeptide own motifs for a helicase (HEL), methyltransferase (MTR), RNA-dependent RNA polymerase (RdRP) and papain-like protease (PRO) (Bouzoubaa et al., 1987; Quadt and Jaspars, 1989; Richards and Tamada, 1992). This protein is autocatalytically cleaved after translation by a papain-like protease (PRO), located between helicase (HEL) and RNA polymerase, into two smaller proteins of 150-kDa and 66-kDa (Hehn et al., 1997).

RNA2 has 4,588 nucleotides and encodes six proteins. At the 5′-terminus the cistron for the coat protein (CP) is located, followed by the read-through (RT) domain, the triple gene block cluster (TGB 1-3) and at the 3′-terminus with the cistron for the suppressor of gene silencing (p14) (Bouzoubaa et al., 1986; Richards et al., 1985; Ziegler et al., 1985). RNA2 is responsible for many biological and genetic viral functions such as encapsidation, cell-to-cell movment, vector transmission by *P. betae* and suppression of the posttranscriptional gene silencing mechanism (PTGS) (Dunoyer et al., 2002; Gilmer et al., 1992; Haeberle et al., 1994; Tamada et al., 1999). The p21, known as a coat protein (CP; 21-kDa) form together with read-through (RT; 54-kDa) the p75. The translation of p75 (75-kDa) can occur only when the leaky UAG termination codon, that located

between the 3' end of the 21-kDa ORF and the 5' end of the 54-kDa ORF undergo suppression (Haeberle et al., 1994; Niesbach-Klösgen et al., 1990; Richards et al., 1985; Ziegler et al., 1985). The C-terminal part of p75 contains the peptide motif (KTER) which appears to be particularly necessary for fungal vector transmission (Adams et al., 2001; Tamada et al., 1996). Remarkably, Tamada and Kusume (1991) and Schmitt et al. (1992) have shown that any type of mutations in the C-terminus of p75 can adversely affect the function of vector transmission.

The central region of RNA2 represents the triple gene block (TGB), which consists of TGB1 (p42), TGB2 (p13), and TGB3 (p15) (Gilmer et al., 1992; Lennefors et al., 2005; Verchot-Lubicz et al., 2010). These three overlapping proteins interact with each other in a highly specific manner to allow efficient movement of the virus between cells (Lauber et al., 1998). Bleykasten et al (1996) described that p42 can bind either single- and double-stranded DNA or RNA by a ATP / GTP binding domain (p-loop). This property probably allows the binding of p42 to genomic RNA of BNYVV. Furthermore, it was assumed that p13 and p15 facilitate the entry of p42 into punctate bodies that connect to the edge of plasmodesmata to enable BNYVV cell-to-cell movement (Bleykasten et al., 1996; Erhardt et al., 2000; Erhardt et al., 2005; Lauber et al., 1998; Niesbach-Klösgen et al., 1990). The last open reading frame on RNA2 translates into the cysteine-rich protein p14 (14-kDa), which expressed from a subgenomic RNA regulates the accumulation of RNA2 (Gilmer et al., 1992; Hehn et al., 1995). Additionally, p14 plays an important role *in planta* as a suppressor of post-translational gene silencing (PTGS) (Chiba et al., 2013; Dunoyer et al., 2002).

Beside RNA1 and RNA2, all BNYVV isolates contain two additional smaller RNAs: RNA3 and RNA4. RNA3 comprises of 1,775 nucleotides and encodes three proteins: the p25, p6.8 and p4.6 (Haeberle and Stussi-Garaud, 1995; Jupin et al., 1992; Tamada et al., 1999). The pathogenicity factor p25 is responsible for the symptom expression on roots in the natural host plant like *B. vulgaris* as well as for the formation of local lesions in leaves of inoculated experimental hosts like *C. quinoa* (Jupin et al., 1992; Jupin et al., 1991; Koenig et al., 1991; Tamada et al., 1999; Thiel and Varrelmann, 2009). In sugar beet, p25 mainly affects the intensity of typical BNYVV symptoms (leaf yellowing along the veins and formation of secondary roots). The defective type of p25, in which about half of the C-terminus was deleted, in comparison with wild-type p25, causes often milder symptoms following mechanical inoculation of leaves in experimental plants such as *C.*

quinoa (Commandeur et al., 1991; Jupin et al., 1991; Koenig et al., 1991). Haeberlé and Stussi-Garaud (1995) were able to show that the p25 possess a nucleo-cytoplasmic localisation signal KRIRFR (NLS) and a nuclear export signal VYMVCLVNTV (NES) that offer p25 the ability to enter both cytoplasm and nuclear compartment of the infected cells (Vetter et al., 2004). It has been suggested that p25 may act as the avirulence target that is recognised in the mechanically inoculated leaves of some BNYVV-resistant sugar beet genotypes (Chiba et al., 2011; Chiba et al., 2008). This was supported by the accordance of the occurrence of *B. vulgrais* resistance-breaking strains of BNYVV, overcoming a single *Rz1* gene in several sugar beet-growing areas, with p25 harboring specific mutations in the tetrad 67-70 (Bornemann and Varrelmann, 2011; Koenig et al., 2009). Furthermore, due to the interaction of p25 with proteins of sugar beet, there is evidence of a possible influence on plant defense and viral pathogenicity, respectively (Thiel and Varrelmann, 2009). It was also possible in further investigations to show that the p25 has the 26S proteasome of the sugar beet as the "target" involved in the hypersensitive response (HR) (Thiel et al., 2012).

In addition, RNA3 encodes two other small proteins: p6.8 and p4.6 (Jupin et al., 1991). p6.8 overlaps the 3'-terminus of p25 and if it is deleted, strong necrotic symptoms are caused in leaves of inoculated plants (Balmori et al., 1993; Bouzoubaa et al., 1985; Jupin et al., 1992). According to the studies of Jupin et al. (1991, 1992) the role of protein p4.6 that is expressed from the subgenomic RNA3 is so far unknown. Deletion of the p4.6 region in the same study had no effect on the symptoms expression in *Tetragonia expansa* (Balmori et al., 1993; Jupin et al., 1992). The noncoding RNA3 (ncRNA3), produced by 5'-3' exoribonuclease activity that is blocked by a structural motif involving the conserved coremin sequence present in the 'Core region' plays an important role in the systemic infection of the virus in *Beta* species. The "coremin" sequence of 20 nucleotides is present on RNA3 and RNA4 of BSBMV as well as on RNA3 and RNA5 of BNYVV (Gilmer et al., 2017; Lauber et al., 1998; Peltier et al., 2012).

Three major groups of BNYVV have been characterized, none of which is serologically distinct. The separation of the different BNYVV A-, B-, and P-pathotypes is based on molecular differences, identified by e.g. restriction fragment length polymorphism (RFLP), sequence comparisons, as well as single-strand conformation polymorphism (SSCP) of different BNYVV-RNA segments (Chiba et al., 2011; Koenig et al., 1995; Kruse et al., 1994). B-Pathotype is the most common in Germany, France and Japan,

while the A- pathotype is more prevalent worldwide. In the region of amino acid tetrad aa 67-70 of the BNYVV-RNA3 pathogenicity factor P25, B-pathotype has a low variability compared to the A-pathotype (Drake and Holland, 1999; García-Arenal et al., 2003; Peltier et al., 2008; Sohi and Maleki, 2004). The A- and B-pathotypes differ from the P-pathotype by possessing only 4 RNA segments in contrast to the P-pathotype, which additionally has a 5th RNA (Koenig et al., 1997; Koenig and Lennefors, 2000). The P-pathotype is mostly distributed in Japan and China and causes significantly more severe symptoms in the test plants compared to the A- and B-types (Miyanishi et al., 1999; Tamada et al., 1996). Schirmer et al. (2005) assumed a further seperation into P- and J-type and demonstrated that due to sequence variability of RNA 5 the J-pathotype can not be assigned to the P-pathotype.

The 1,470 nucleotides of RNA4 comprise two ORFs coding for a 31-kDa protein (p31) and for a 6.5-kDa protein (p6.5) (Bouzoubaa et al., 1985). P31 plays an important role for efficient transmission of the BNYVV by *P. betae* (D'Alonzo et al., 2012; Tamada and Abe, 1989), whereas the function of p6.5 is still unknown (Jupin et al., 1992; Jupin et al., 1991). P31 is also involved in both the suppression of RNA silencing in roots and enhancement of BNYVV symptom expression in *N. benthamiana* (Rahim et al., 2007).

The rarely occurring RNA5 is not found in all isolates, it is mainly found in some Asian isolates in Japan (Miyanishi et al., 1999; Tamada and Abe, 1989). In Europe, the RNA5 is found only in some isolates in limited regions of England and France (Heijbroek et al., 1999; Koenig and Lennefors, 2000; Ward et al., 2007). The RNA5 has 1,350 nucleotides and encodes a 228-amino-acid protein (p26) (Kiguchi et al., 1996). P26 of RNA5 displays a sequence homology with the pathogenicity factor p25 of RNA3 and this may support that p26 act as additional pathogenicity factor (Koenig et al., 1997). The p26 is involved in the long-distance movement of the virus in the vascular bundles, as well as in the enhancement of the rhizomania symptoms in sugar beet (Kiguchi et al., 1996; Koenig et al., 1997; Link et al., 2005; Miyanishi et al., 1999). Since the two proteins p25 and p26 can interact in a synergistic manner, this could be the reason why all BNYVV isolates with the additional RNA5 cause more severe symptoms than isolates that only possess RNA1-4 (Heijbroek et al., 1999; Koenig et al., 1997; Link et al., 2005; Tamada and Abe, 1989).

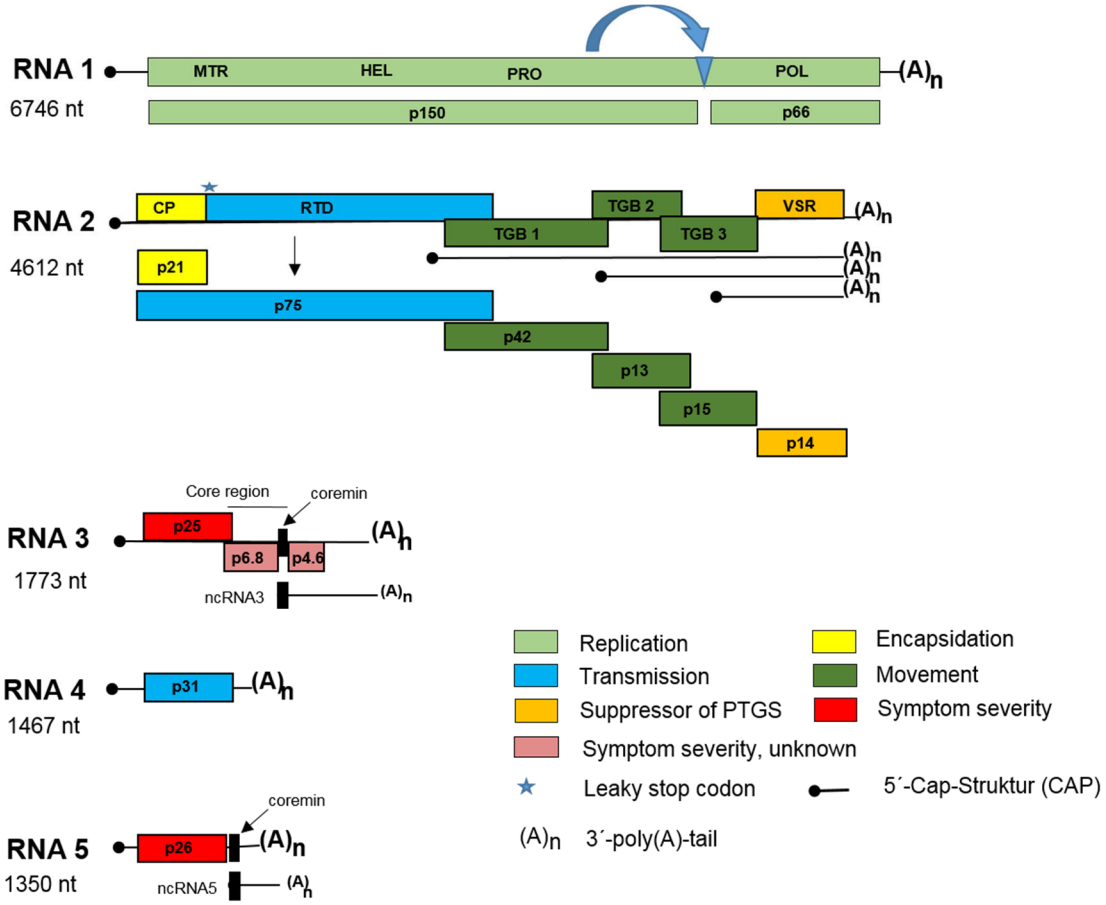

Fig. 2 Genome organization and expression strategy of the *Beet necrotic yellow vein virus* (BNYVV). All BNYVV RNAs 1-5 have a cap structure at the 5' terminus and a poly-A sequence at the 3' terminus. The open reading frame (ORF) are shown in different colors depending on the function. RNA1 encodes all important motifs for replication (green). Helicase (HEL), methyltransferase (MTR), RNA-dependent RNA polymerase (RdRP) and papain-like protease (PRO). CP "Coat protein" for encapsidation (yellow); "Read-through domain" (RTD) for the vector transmission (light-blue); "Triple gene block" (TGB) for cell-to-cell movment (dark green) and p14 "suppressor of gene silencing" (orange). RNA3 encodes p25, p6.8 and p4.6, which are responsible for symptom expression (red). P31 of RNA4 is used for vector transfer (light-blue). P26 acts as p25 during the symptom expression (red). Noncoding RNAs (ncRNA3 and ncRNA5) are responsible for long distance movement in *Beta* species. This figure is modified after (Gilmer et al., 2017; Varrelmann, 2007).

1.2.2. *Beet soil-borne mosaic virus* (BSBMV)

Beet soil-borne mosaic virus has been characterized and classified to the genus *Benyvirus* (Lee et al., 2001). BSBMV was first reported in 1988 in Texas, USA (Liu and Duffus, 1988). In contrast to the worldwide distributed BNYVV, the distribution of BSBMV is still limited to certain areas in the USA (Lee et al., 2001; Wisler G.C., 1994).

The BSBMV consists of four ss (+) strand RNAs and shows a similar genome organisation and morphological structure like BNYVV. Despite this similarity, both viruses exhibit a sufficient molecular difference to be classified as two different species (Lee et al., 2001; Rush, 2003). BSBMV differs primarily serologically from BNYVV and causes systemic leaf symptoms which have a different appearance from that of BNYVV (Heidel et al., 1997; Peltier et al., 2012; Rush and Heidel, 1995). BSBMV mainly induces systemic mosaic symptoms, mottling, yellow vein-banding on leaves or slightly disordered growth (Peltier et al., 2008; Rush and Heidel, 1995). The BSBMV symptoms in the open field can be observed more often on the sugar beet leaves than those caused by BNYVV. The effect of BSBMV on the sugar beet yield and the quality is very variable and depends on the soil moisture, as well as the temperature (Workneh et al., 2003). Generally, BSBMV causes significantly less yield reduction in sugar beets in comparision to BNYVV, which is due to the main infection of BNYVV of the roots, whereas BSBMV is prominent on leaves (Heidel et al., 1997; Workneh et al., 2003).

1.3. RNA interference (RNAi): Discovery and biological perspectives

1.3.1. Origins of RNA interference

RNA-mediated gene silencing, also commonly known as RNA interference (RNAi), is a complex molecular biology, evolutionarily conserved mechanism that can affect transcription by DNA methylation, influence mRNA stability, or inhibit translation (Guo et al., 2016; Matzke and Birchler, 2005; Schramke and Allshire, 2004; Tijsterman et al., 2002). This mechanism plays a key role in regulation of gene expression, maintaining genome integrity and adaptive responses to abiotic and biotic stresses as well as a natural defense mechanism against pathogens and foreign genetic elements such as viruses and transposons in the cells of eukaryotic organisms (Baulcombe, 2004; Tijsterman et al.,

2002; Voinnet, 2001; Wilson and Doudna, 2013). RNAi has been discovered and examined very early in plants (Napoli et al., 1990). Additionally, RNA silencing has been extensively studied in many different eukaryotic organisms, e.g. in *Caenorhabditis elegans*, in fungi (*Neurospora crassa*), insects, animals, and human (Elbashir et al., 2001; Ipsaro and Joshua-Tor, 2015; Kanakala and Ghanim, 2016; Misquitta and Paterson, 1999; Napoli et al., 1990; Romano and Macino, 1992). As early as 1928, this mechanism was described when some tobacco plants were infected with the *Tobacco ringspot virus* (TRSV), the new non-inoculated upper leaves could recover and show a kind of resistance to a secondary infection with the same virus (Baulcombe, 2004). Matzke et al. (1989) reported that after a rhizobium-mediated double transformation a T-DNA insert was inactivated by the insertion of a second T-DNA fragment. The authors hypothesized that the promoters in both T-DNA vectors possess a sequence similarity that could lead to methylation of the promoter sequence (Matzke et al., 1989).

The tale of RNA silencing began in 1990 when the research group C. Napoli and R. Jorgensen discovered the mechanism by chance in plants (Napoli et al., 1990). They attempted to intensify the pigmentation in *Petunia hybrida* (petunia) by upregulation the activity of the gene for *chalcone synthase* (*chs*) or *dihydroflavonol-4-reductase* (*dfr*) (Napoli et al., 1990; Van der Krol et al., 1990). In contrast to expectations, the transgenic petunias showed different patterns of flowering coloring varying between dark purple and mixtures of purple, white, and pure white (Napoli et al., 1990; Sen and Blau, 2006; Van der Krol et al., 1990). Similarly, the overexpression of a *Polygalacturonase* gene in the tomato during fruit maturation resulted in a strong reduction in expression of endogenes exhibiting sequence similarity with the *Polygalacturonase* gene (Smith et al., 1990). For the discovery of the mechanism of RNA interference in *C.elegans* in 1998, the two American scientists, Craig C. Mello and Andrew Z. Fire, received the Nobel Prize in physiology / medicine in 2006 (Fire et al., 1998).

This phenomenon was also described in different terms. In plants, it refers to post-transcriptional gene silencing (PTGS) (Agrawal et al., 2003; Cogoni and Macino, 2000). In fungi, especially in *Neurospora crassa* was called "quelling" (Cogoni and Macino, 1997). In animals and various insect species, e.g. *Trypanosoma*, *Drosophila* it is called RNA interference (RNAi) (Cogoni and Macino, 2000).

1.3.2. The RNA interference biogenesis machinery

Since the first discovery of RNA silencing in plants in the early 1990s, remarkable progress in understanding the molecular mechanisms of RNA silencing have been made (Agrawal et al., 2003; Guo et al., 2016; Ipsaro and Joshua-Tor, 2015; Wilson and Doudna, 2013). The description in this thesis refers mainly to posttranscriptional gene silencing (PTGS) in plants by mRNA degradation. This process is based on a natural process in which the cells of the infested organisms recognize the conserved molecular "markers" of the pathogens. Small double-stranded RNAs (dsRNA) are known pathogen markers classified as small interfering RNA (siRNA) or microRNA (miRNA) (Meister and Tuschl, 2004).

DsRNA can be produced by several processes in a plant cell, e.g. as an intermediate in the replication of many viruses in the infected cells, transgenic plants with a transgene in different orientation and translation of plant genes that may deliver mRNA with internal complementarity (Angell and Baulcombe, 1997; Axtell, 2013; Walkey, 2012). However, the RNAi can also be artificially triggered. For this purpose, specific constructs, e.g. the hairpin RNA (hrRNA) constructs or antisense constructs are introduced into a single cell or into the whole organism (Douchkov et al., 2005; Senthil-Kumar et al., 2010). To create a hairpin construct, the sequence of the target gene must occur twice between a promoter and a terminator. The sequences can be separated by an intron and installed in a reverse direction in the construct. The two complementary RNA strands join together after transcription to form a double-stranded RNA (dsRNA) (Smith et al., 2000). Therefore, the hrRNA do not require the RDRs to produce dsRNA arm (Guo et al., 2016). At the 3' end, the intron forms a loop (O-shaped piece) that is removed by splicing, which can contribute to improve RNA silencing efficiency in plants (Wang et al., 1997; Waterhouse, 2000). To introduce these constructs in different organisms numerous methods and strategies have been described. In plants, besides the transformation of protoplasts and the particle bombardment, viral vectors are most frequently used to introduce the dsRNA constructs into plants by *Rhizobium radiobacter* (Kanno et al., 2000; Klahre et al., 2002; Wesley et al., 2001). On the other hand, *C. elegans* can be fed with dsRNA, injected with dsRNA or inserted for 1-3 hours into a dsRNA solution (Ambros, 2003). In *Drosophila* other methods can be employed, e.g. introducing cultured *Drosophila* cells directly into dsRNA solution or production of transgenic fruit flies that are capable of transcribing the dsRNA (Kanakala and Ghanim, 2016; Schwarz et al., 2002). After the dsRNA has been

entered into the cytoplasm of the cell, it will be specifically cleaved by the cell. DsRNAs are recognized by Dicer-like enzymes in the cell nucleus or cytoplasm, bound and cut into small ~20-24 nucleotide long dsRNA fragments with 2-nt 3' overhangs at the 3' ends (Axtell, 2013; Guo et al., 2016; Papp et al., 2003).

The Dicer protein is a RNase III-like enzyme that possesses several domains (two dsRNA-BD, RNaseIII, RNA helicase / ATPase and PAZ domains) (Bernstein et al., 2001; Bologna and Voinnet, 2014; Ketting et al., 2001). Generally, Dicer is associated with Argonaute proteins in the cells. Also different Dicer enzymes are involved in the different RNA silencing pathways in different organisms. While *C. elegans* and humans can produce only a single Dicer enzyme, *Drosophila* has two Dicer (Dcr1 and Dcr2) and *Arabidopsis thaliana* has four core-localized Dicer-like proteins (DCL1-DCL4) (Henderson et al., 2006; Hiraguri et al., 2005; Papp et al., 2003). DsRNA is generally used as an RNAi initiator that can detect target mRNA specifically using siRNA. However, these two small RNAs, si- and miRNA, are similar in function but differ in their pathway and origin (Borges and Martienssen, 2015; Meister and Tuschl, 2004). In plants, dsRNA is bound by DCL4 and cut into double-stranded RNA fragments (siRNA) in 21-23 nucleotide lengths that have two base pairs of overhangs at the 3' end (Fig. 3A) (Guo et al., 2016; He et al., 2005). For the protection against degradation and the stabilization of the small RNAs, they are modified in various ways. It was shown that the 2'-OH group at the 3'-terminal of miRNAs is methylated by the methyltransferase HUA ENHANCER 1 (HEN1), which can protect it from polyuridylation and degradation (Allen et al., 2005; Zhai et al., 2013). For the same purpose, the 5'-terminal of siRNA in plants is phosphorylated by a kinase (Akbergenov et al., 2006). The production of siRNA in plants is based on the first line on DCL4, whereas the miRNAs are produced as products of DCL1 activity from primary miRNA transcripts that have partial dsRNA with hairpin loops (Fig. 3A and D). The miRNAs are approximately 21 base pairs long and cause the degradation of mRNA or the inhibition of translation depending on matching percentage (Fig. 3D). Thus, various cellular and biological processes in plants can be controlled, e.g. the flower development or the regulation of growth in plants (Palatnik et al., 2007; Schwab et al., 2006; Wang et al., 2008). Precursor transcripts from these small RNAs are generally converted by Dicer proteins in the nucleus to the 20-24 nt long dsRNAs (Castel and Martienssen, 2013; Fukunaga and Doudna, 2009). For the activities at the chromatin level, these stabilized small sRNA duplexes (21-23 nt) can remain in the cell nucleus or they can be exported to the cytoplasm via the exportin-5 homologous

protein HASTY (HST) (Bologna and Voinnet, 2014; Poulsen et al., 2013). In plants, the sRNA duplexes from the cell nucleus can also be transported into the cytoplasm via HST-independent mechanisms (Eamens et al., 2009; Park et al., 2005). A protein complex, that is known as RNA-induced silencing complex (RISC) with Argonaute protein (AGO), will bind these siRNA duplexes in an ATP-dependent reaction and separate them into two single-stranded RNA molecules (Bernstein et al., 2001; Fang and Qi, 2016; Wilson and Doudna, 2013). One of these two single-stranded RNAs serves as a guide and binds to RISC. This contributes that the RISC complex binds to specific complementary regions of the target mRNA and then degrades it. In this case, the structure and sequence of the siRNA play an important role in order to be selected as a leader strand "guide" by the RISC complex. If the guide strand remains in the RISC complex, it is further used to digest the target mRNA by splicer activity of the Argonaute enzyme. If the other strand binds to the RISC complex, it has no effect on the gene expression or on the inserted construct (Fang and Qi, 2016; Matranga et al., 2005; Tomari and Zamore, 2005).

In addition to siRNA and miRNA, the trans-acting siRNA (ta-siRNA) also belongs to the small RNA (sRNA). Ta-siRNAs are derived from non-coding transcripts from loci known as TAS genes, which suppresses gene expression by PTGS in plants (Fig. 3C). There are currently four TAS gene families (TAS1-4) in *A.thaliana* that are closely linked to miRNA for the synthesis of ta-siRNA and the targeted degradation of mRNA (Endo et al., 2013; Peragine et al., 2004; Vazquez et al., 2004). The primary ta-siRNA transcript is bound in the cytoplasm by a miRNA and cleaved by AGO1. The cleaved ta-siRNA is converted to a dsRNA by an RNA-dependent RNA polymerase 6 (RDR6) (Chen et al., 2010). The putative RNA binding protein SGS3 (Suppressor of Gene Silencing 3) probably plays a role in the stabilization of the cleavage fragments of the ta-siRNA (Cuperus et al., 2010; Peragine et al., 2004). This dsRNA is finally processed by the dsRNA-binding protein 4 (DRB4) and DCL4 into 21-nt siRNAs (Guo et al., 2016; Montgomery et al., 2008). In the same manner as for miRNA, the ta-siRNAs specifically suppress a sequence and lead to mRNA degradation. The regulatory factor AGO1 is involved in most ta-siRNA TAS1,2 and 4-mediated regulation (Baumberger and Baulcombe, 2005; Fang and Qi, 2016), Whereas in ta-siRNA TAS3- directed regulation probably acts AGO7 as a regulating factor (Adenot et al., 2006; Allen et al., 2005; Axtell, 2013).

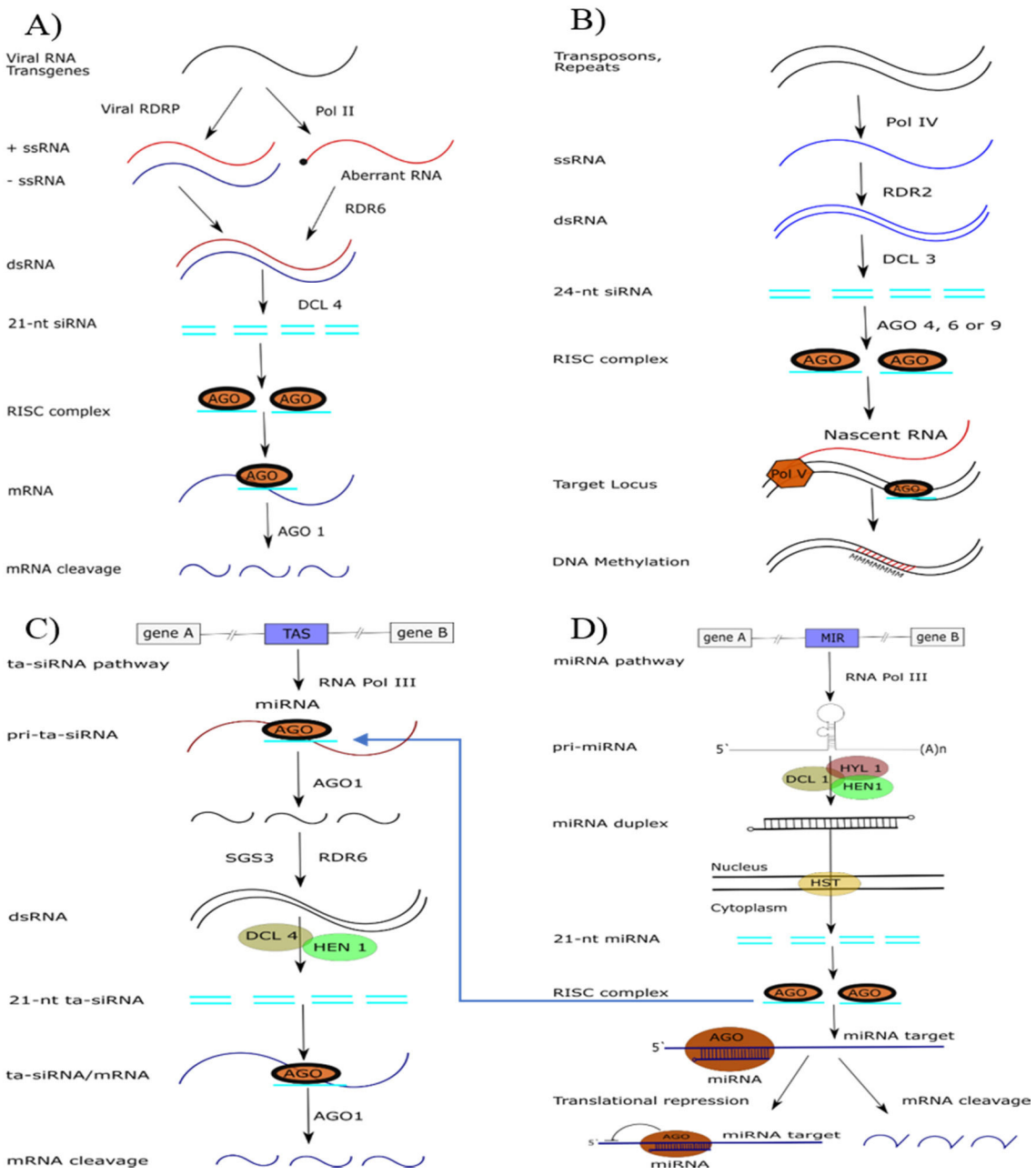

Fig. 3 Schematic representation of RNA silencing in plants. A) Exogenous siRNA-mediated gene silencing. Exogenous siRNAs are 21-nt long and are produced from directly introduced dsRNA, infected virus (VIGS) or transgenes. RNA pol II, RDR6, AGO1 and DCL4 are involved as host proteins in this silencing pathway. **B)** Silencing targeted to repetitive mobile genetic sequences and transposable elements (TEs) is caused by the RNA-dependent DNA methylation after binding to the siRNA duplex. In this pathway, DCL3, Pol IV, Pol V, RDR2, and AGO proteins (AGO4, 6 and 9) are involved. **C)** Trans-acting (ta-siRNA) mediated gene silencing. An additional class of small RNAs is the 21-nt long ta-siRNAs made from the endogenous TAS gene following the action of DCL4. RNA pol II, RDR6 and AGOs (AGO1 and probably 7) are involved in this ta-siRNA silencing as host proteins. **D)** MicroRNA (miRNA) mediates gene silencing. MiRNAs also belong the small RNAs that are about 21-nt long. MiRNAs are produced by the endogenous MIR gene after the action of DCL1 and RNA pol II. In this silencing pathway, AGO1 is involved as a host protein. This figure is modified after (Elvira-Matelot and Martínez, 2017; Ghildiyal and Zamore, 2009; Liu and Chen, 2016).

After many different experiments it was found that a locally triggered RNA silencing can spatially spread in various organisms (fungi, plants, *C.elegans*) (Voinnet and Baulcombe, 1997). However, it has to differentiate in plants between local silencing, cell-to-cell movment and systemic silencing resulting in a movement through the whole plant.

The possible signal molecules for local and systemic silencing are in particular dsRNA and siRNA (Hamilton and Baulcombe, 1999; Waterhouse et al., 2001). Dunoyer et al. (2005) described that the small siRNA (21 nucleotides) as components of the local silencing (10-15 cells) in *A. thaliana*, which can spread with the help of a protein via the plasmodesmata from one cell to the other (Dunoyer et al., 2005; Kobayashi and Zambryski, 2007). On the other hand, Kehr and Buhtz (2008) demonstrated that the larger siRNAs (24-26 nucleotides) act as a signal for systemic silencing, which can spread through the phloem over long distances (Hamilton et al., 2002; Kehr and Buhtz, 2008; Pant et al., 2008).

Transient-induced gene silencing (TIGS) and virus-induced gene silencing (VIGS) are among the most widely used methods to introduce dsRNA into an organism and thereby trigger RNAi. With the help of TIGS one is able to test a wide number of gene functions (thousands of genes) in a short time. The TIGS system is known as reverse genetics, but is also often associated with forward genetics (Nelson and Bushnell, 1997; Schweizer et al., 1999). This method is based on the biolistic gene transfer, this means that particles (tungsten or gold) are shot at the targeted tissue under high pressure. These microparticles may contain, in addition to an antisense or RNAi construct, a provided vector with reporter genes (e.g. *mrfp* or *gus*). The introduced genetic material (DNA) with the microparticles is firstly expressed in the cell when a particle encounters the cell nucleus of a cell (Nielsen et al., 1999; Panstruga, 2004). Thus, it is possible to investigate and evaluate the effect of an antisense or RNAi construct on the level of the individual cell. This method is employed, especially in the investigation of host-pathogen interaction (e.g. cereals and powdery mildew), since the cereal mildew interaction is restricted only to the level of individual cells of the epidermis in an early phase (Douchkov et al., 2005; Nowara et al., 2010; Zimmermann et al., 2006). Although the TIGS method can be used to test a wide range of genes in a short time, this method is only suitable for single cells. In contrast, the VIGS method offers the possibility to modify larger parts of the plants. Additionally, this natural phenomenon can be utilized to control agronomically important plant diseases, based on the observation that in vitro feeding of dsRNA can trigger PTGS

of target genes in several plant pathogens and pests, such as fungi, nematodes and insects (Nunes and Dean, 2012; Zhang et al., 2016). Expression of such pathogen-derived dsRNAs in the relevant host plant conferred protection from infection or infestation by pests. This method, termed host-induced gene silencing (HIGS), has arose as an encouraging alternative in plant protection because it is highly selective for the target organism and has only marginal side effects in comparison with chemical treatments (Baulcombe, 2015; Koch and Kogel, 2014).

RNA-silencing as modern technology brings many advantages. One of the advantages of RNA silencing is a huge variability and flexibility. In principle, any gene can be silenced in the plants. With these characteristics, it is possible to silence an entire multigene family and homologous genes in polyploids, which are of tremendously importance for the organisms with a complex genome, e.g. hexaploid wheat (*Triticum aestivum*) (Lawrence and Pikaard, 2003; Miki et al., 2005; Travella et al., 2006). Previous studies found effective interference with ~ 520 nucleotides long dsRNAs that were 88% identical to the target sequence (41 nucleotides maximum uninterrupted identity) (Parrish et al., 2000; Rual et al., 2007). This makes it possible to silence several genes at the same time with comparatively low costs and without great technical effort that makes the difficult and complicated production of stable mutants unnecessary (Matthew, 2004). In addition, RNA-silencing leads mostly to partial downregulation of the mRNA expression level, thus producing a range of silencing phenotypes that may differ in severity. RNAi offers the possibility to study essential genes, whose complete inactivation (mutation) would produce lethal effects for the studied organism (Senthil-Kumar and Mysore, 2011a).

Despite all the advantages of RNA silencing, it also has some disadvantages, like any other method. Off-target silencing is one of the greatest disadvantages of RNA silencing. The term means that not only the targeted gene but also other genes in the investigated organism could be silenced by RNAi. This can occur when partial sequence similarity permits siRNA to cleavage mRNA for genes that are a non target. The reason for this is the limited sequence specificity of siRNA. Earlier study prognosticated that about 50–70% of mRNA transcripts in *A. thaliana* produce off-target effects when used as silencing trigger for PTGS (Xu et al., 2006). Basically, it has been demonstrated that the off-target silencing correlated with the length and amount of the initial dsRNA (Qiu et al., 2005; Warthmann et al., 2008). Off-target effects lead to complications in identifying the precise functional role of target genes. This problem can be solved with the aid of

software, e.g. "SiRNA-scan" by checking the sequence in advance for its potential to cause an off-target effect (Xu et al., 2006). However, the problem remains for organisms with unknown or only partially known sequences. Furthermore, it is necessary to avoid selecting the required sequences for RNAi constructs from a highly conserved gene region if only a single gene is intended as silencing target.

Another problem of RNAi is often the occurrence of variable silencing effects. Inefficient RNAi constructs may give a false understanding of the importance of the gene studied for the observed phenotype. Reasons for this phenomenon can primarily be the target-sequence of the silencing-construct by localizing many secondary structures or proteins to the target site of the target-mRNA and thereby blocking the binding of the siRNA (Tomari and Zamore, 2005). Furthermore, the dsRNA might be introduced inversely into the RISC complex (Tomari et al., 2004).

Finally, the effect of RNAi on a target mRNA or as a secondary effect on non-target mRNA can be visually observed and evaluated. In addition, a number of the molecular biological methods are also contemplated to confirm silencing effects by measuring transcript levels by e.g. RT-PCR and qRT-PCR analyzes or arrays and ELISA experiments.

1.4. Overview of virus-induced gene silencing (VIGS)

1.4.1 Development of the VIGS technology

In addition to bacteria and fungi, viruses are the most frequently occurring pathogens in plants. However, the plant viruses are intracellular pathogens and possess a genome that can encapsidate and replicate only within the host cells. Plant viruses have been utilized for virus-induced gene silencing (VIGS) to produce rapid gene silencing phenotypes in several plant species. The term virus-induced gene silencing (VIGS) was first used by A. van Kammen in 1997, which described the phenomenon of plant recovery after virus infection (Gupta et al., 2014; Van Kammen, 1997). In the literature, the plant defense mechanism is referred to as VIGS only when the viruses naturally or artificially infected a plant to trigger RNAi. However, the term has been applied almost exclusively to the technique in which recombinant viruses have been used for "knock-down" experiments in the expression of endogenous genes (Baulcombe, 1999; Ruiz et al., 1998).

VIGS is an alternative and rapid method that can be used for the silencing of plant gene expression without generation of transgenic plants (Burgyán and Havelda, 2011; Ramegowda et al., 2014). It is a modern, molecular biological technique that exploits the advantages of RNAi-mediated antiviral defense mechanisms. VIGS has often been used in plants for the analysis of gene functions and adapted for high-throughput methods in functional genetics (Ramanna et al., 2013; Senthil-Kumar and Mysore, 2011c). To use VIGS, a short sequence of a plant gene is specifically cloned into a viral vector. The virus vector is transferred into *R. radiobacter* and then introduced into the host cells by rhizoinoculation in order to infect a young plant. After virus replication and local infection, the virus with the inserted sequence will spread through the whole plant. In a few weeks after inoculation, the natural defense mechanism of the plant can lead to the suppression of viral replication and also to a specific degradation of the mRNA of the endogenous plant gene (Benedito et al., 2004; Hileman et al., 2005). The viral siRNAs that are required for VIGS can come from many different sources. One of the most important source for siRNA is dsRNA, which is formed during the viral replication in the plant cell. The second source are the hairpin or sense (s) and antisense (as) constructs that have been incorporated into the viral background and introduced into the plant cell (Härtl et al., 2017; Voinnet, 2005). In addition, host-RNA-dependent RNA polymerases (RDR1, RDR2 and RDR6) are involved in some siRNA biosynthesis pathways, which is similar to the host endogenous siRNA pathway (Dunoyer et al., 2010; Wang et al., 2010).

In principle, VIGS could be used for the analysis of gene functions in all plants, but most of the work so far was done on *N. benthamiana*. Over the past ten years, efficient VIGS has been successfully used and established for many dicotyledons and monocotyledons plants using various DNA- or RNA-virus-based vectors (Kant and Dasgupta, 2017; Mei et al., 2016; Scofield and Nelson, 2009; Wang et al., 2016).

One of the first vectors based on *Tobacco mosaic virus* (TMV) was used to inhibit the biosynthesis pathway of carotenoids in *N. benthamiana* (Kumagai et al., 1995). Here, transcripts from a recombinant virus carrying a part of the sequence from the *phytoene desaturase* (*pds*) gene were prepared *in vitro* and inoculated into *N. benthamiana* for a successful silencing of the PDS. A further VIGS vector is based on *Potato virus X* (PVX) which was developed by Ruiz et al., (1998). Although this vector is more stable than the TMV-based vector, PVX has a more narrowly limited host spectrum as TMV (Ratcliff et al., 2001; Voinnet and Baulcombe, 1997). By developing

a VIGS vector based on *Tobacco rattle virus* (TRV) the host spectrum could be extended. Liu and colleagues (2002) demonstrated that the use of a recombinant TRV-based vector could produce an efficient gene silencing in tomato plants. Using this system, various genes like *pds*, *ctr1* and *ctr2* (constitutive triple response 1/2 genes) have been suppressed in tomato plants (Brigneti et al., 2004; Fantini and Giuliano, 2016; Zheng et al., 2017).

In monocotyledonous plants, a VIGS vector was developed for silencing of the *pds* gene expression in barley and wheat (Liu et al., 2016). This vector based on the *Barley stripe mosaic virus* (BSMV) is one of the few vectors for VIGS in monocots (Burch-Smith et al., 2004). A VIGS vector based on the *Tomato golden mosaic virus* (TGMV) was used as the first DNA virus for successful silencing of a meristematic gene in *N. benthamiana* (Kjemtrup et al., 1998; Peele et al., 2001). This TGMV-vector also has a restricted host spectrum. A further vector based on a DNA virus was constructed in the background of *Cabbage leaf curl virus* (CaLCuV) and used for silencing of endogenous genes in *A. thaliana* (Turnage et al., 2002). VIGS vectors are also described in the literature from *Pepper Huasteco yellow vein virus* (PHYVV), *African cassava mosaic virus* (ACMV), the satellite (DNAβ) associated with *Tomato yellow leaf curl China virus* (TYLCCNV) and the satellite *Tobacco mosaic virus* (STMV) (Beyene et al., 2017; Carrillo-Tripp et al., 2006; Stanley et al., 2005). All these vectors have been developed for silencing gene expression in different plants which are often used in the investigation of host-pathogen interactions (Tiwari et al., 2017; Whitham et al., 2016b). Recently, several viruses have been modified to be used as VIGS vectors in various plant species, e.g. *Apple latent spherical virus* (ALSV) in various *Prunus* species (Kawai et al., 2016), *Rice tungro bacilliform virus* (RTBV) in rice (Kant and Dasgupta, 2017), *Bean pod mottle virus* (BPMV) in soybean (Whitham et al., 2016a), TRV in Spinacia oleracea L. (Lee et al., 2017) and *Cucumber mosaic virus* (CMV) in maize (Wang et al., 2016).

1.4.2. Viral suppressors of RNA silencing (VSR)

In the course of evolution, viruses have developed many properties and proteins in response to the PTGS, with this function of which it is possible to suppress the PTGS in different host plants (Ikegami et al., 2016; Luna et al., 2017). These proteins, referred to as viral suppressors of RNA silencing (VSR), act on the PTGS in various ways. Not only the development of the PTGS but also the local or systemic movement of the silencing

signal in the host plant are influenced by VSR (Jiang et al., 2012; Moissiard and Voinnet, 2004). The VSRs target the most important elements of the RNAi to suppress them directly. For the suppression of RNAi, the VSRs can interact with and inhibit both the RISC complex and the enzymes of DNA methylation. In this process, the multifunctional viral proteins play the crucial role, e.g. the p14 protein of BNYVV and BSBMV, the p19 of *Tomato bushy stunt virus* (TBSV), the HC-Pro of *Potato virus Y* (PVY) and the p1 of *Rice Yellow mottle virus* (RYMV), which influence and suppress the accumulation of siRNA in plant cells (Csorba et al., 2015; Flobinus et al., 2016; Gillet et al., 2013). Recent studies postulated that the p19 protein binds the siRNA and can create a new conformation that prevents the incorporation of the siRNA in RISC and thereby disabling the plant defense system (Kontra et al., 2016).

Other proteins, such as the p38 of the *Turnip crinkle virus* (TCV), attack the defense system by binding the dsRNA and make it inaccessible to bind the Dicer or the AGO1 of the RISC and thus inhibit their cutting activity (Azevedo et al., 2010; Iki et al., 2017). The 2b protein of the CMV and the AC2 protein of ACMV are able to suppress the function of AGO proteins, which can prevent the endonucleolytic cleavage in viral RNAs (Dong et al., 2016; Du et al., 2014).

Another example of proteins that attack the AGO protein and inhibit their ability to cut is the p1 protein from the *Sweet potato mild mottle virus* (SPMMV). P1 interacts with AGO1 through glycine-tryptophan (GW) motifs and inhibits the mi/siRNA-programmed RISC activity (Machado et al., 2017). It has been shown that the conserved motif (GW / WG) located at the N-terminal half of p1 serves as a hook to bind to AGO proteins and is required for several RISC functions that play a crucial role in silencing system (Giner et al., 2010; Szabó et al., 2012).

The activity of the p14 as silencing suppressor of BNYVV and BSBMV is associated with the reduction of the accumulation of primary and secondary siRNAs (Chiba et al., 2013; Flobinus et al., 2016). The p14, the VSR of BNYVV and BSBMV is more efficient in the roots than in the leaves and also more efficient in the vascular tissue than in the mesophyll (Andika et al., 2012; Andika et al., 2016; Chiba et al., 2013). However, it is not yet known whether the reason for these efficiency differences in silencing suppression in different tissues is the p14 protein itself, or whether it is due to one of its so far unknown partners (Andika et al., 2005; Zhang et al., 2005).

Li and colleagues (2002) have shown that some of the animal viruses also have silencing suppressors, e.g. the protein B2 of the *Flock house virus* (FHV). The special feature of the B2 protein is that it can act as a silencing suppressor both in plant cells and in animal cells (Drosophila S2 cells) (Lu et al., 2005; Rao and Seo, 2017). The natural phenomenon has been artificially exploited for a long time in which the function of these effectors for PTGS suppression is used to improve the transient expression of transgenes (Csorba et al., 2015; Voinnet et al., 2003).

1.4.3. Reporter genes for VIGS

A reproducible silencing effect in VIGS experiments requires an optimal protocol for virus inoculation, stable infection rates and suitable plant growth conditions in the greenhouse (Burch-Smith et al., 2004; Senthil-Kumar and Mysore, 2011c). Studies have shown that various environmental factors, such as humidity, temperature and photoperiod influence the effectiveness and efficiency of VIGS in different plants like barley, tomato and *A. thaliana* (Bruun-Rasmussen et al., 2007; Fu et al., 2005; Padmanabhan and Dinesh-Kumar, 2009). In addition, inoculation methods, insert orientation, size and region of the insert play an important role in VIGS efficiency (Lacomme and Hrubikova, 2003). For the optimization of variables within an experiment, it is necessary to find a reporter gene with a reproducible, clear silencing phenotype. Various genes have been tested, such as the H subunit of the *magnesium chelatase* (*chlH*), *phytoene desaturase* (*pds*), *actin*, *chalcone synthase* and the β7 subunit of the 20S proteasome (20S-β7), which cause in case of a down-regulation a visual silencing phenotype. For these reasons, they have been used as reporter genes for the development and optimization of VIGS vectors in a number of different plant species (Tab.1).

Pds and *chlH* genes were most commonly used to test and to evaluate the VIGS efficacy (Hiriart et al., 2002; Holzberg et al., 2002; Kumagai et al., 1995; Ratcliff et al., 2001). Magnesium chelatase is an enzyme consisting of three components (ChlD, ChlI, and ChlH), that is associated with the chlorophyll biosynthesis pathway and required for inserting Mg2+ into protoporphyrin IX (Castelfranco and Jones, 1975; Walker and Weinstein, 1991). Phytoene desaturase (PDS) is the first enzyme in carotenoid biosynthesis that catalyzes the dehydrogenation (desaturation) from the colorless phytoene to the colored ζ-carotene in plants (Bartley and Scolnik, 1995; Garcia-Asua et

al., 1998; Grünewald et al., 2000). Carotenoids are color pigments that protect the chlorophyll in plants from photobleaching. Plants with ζ-carotene deficiency develop symptoms known as photobleaching, if they are exposed to light, cause of degradation of chlorophyll (Bartley and Scolnik, 1995; Norris et al., 1995). The silencing phenotype of *pds* as a reporter gene for VIGS varies only slightly depending on the test plants and the target tissues. In tomato, downregulation of *pds* renders plant leaves yellow and lead to lycopene-depleted fruits, whears in monocotyledonous plants symptoms are mostly restricted to narrow stripes parallel to leaf veins (Ding et al., 2006; Orzaez et al., 2009; Ruiz et al., 1998; Scofield et al., 2005).

According to Ding et al. (2002), the PDS silencing effects disappear usually by the fourth systemically infected leaf, depending on the host plant. In a previous study it has been demonstrated that even before the photobleaching appears, a significant 65% reduction of the relative *pds* transcript level was seen. On the other hand, even after silencing disappeared after 4 weeks a 48% decrease of the *pds* mRNA was observed (Bruun-Rasmussen et al., 2007). These studies underline the importance of molecular biology techniques to confirm and to verify the silencing phenotypes at the molecular level. Nowadays, numerous of molecular, biochemical and genetic techniques are available for this aim, such as real-time semiquantitative / quantitative reverse transcription PCR (qRT-PCR), which could be used to measure transcript level of the target gene (Burch-Smith et al., 2004; Rotenberg et al., 2006). High performance liquid chromatography (HPLC) is another molecular technique that is used to determine the silencing efficiency of various reporter genes. HPLC can be used to separate the various substances as well as to identify and quantify the specific chemical compounds. Based on this principle, HPLC is used to confirm the silencing efficiency of PDS by measuring the amount of phytoene in the tissue of the infected plants (Holzberg et al., 2002).

Table 1. List of plant genes with visual silencing phenotypes that were used as reporter genes in VIGS experiments.

Reporter genes	**Abbreviation**	**Description**	**Reference**
Phytoene desaturase	*pds*	Impairs carotenoid and chlorophyll biosynthesis	(Ruiz et al., 1998)
H subunit of the *Magnesium chelatase*	*chlH*	Prevents chlorophyll synthesis (Chlorose)	(Hiriart et al., 2002)
Cloroplastos alterados 1	*CLA1*	Involved in chloroplast development (albino phenotype)	(Estévez et al., 2000)
Actin	*Actin*	Stunting (yellowing)	(Ding et al., 2006)
β7 subunit of the 20S proteasome complex	*20S-β7*	Spontaneous cell death (Necrosis)	(Tai et al., 2005)
chalcone synthase	*CHS*	Highly dissected mosaic pattern	(Chen et al., 2004)

1.5. Aims of the study

The aim of the investigation carried out within this work was to develop a new vector systems for establishing VIGS in sugar beet based on the *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV). Infectious full-length cDNA clones are indispensable as a starting material for the methodical approach to develop a VIGS systems. Full-length cDNA clones of BNYVV (A-type) and BSBMV under control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) in a binary vector (pDIVA) for *rhizobium*-mediated infection have been successfully generated by an isothermal *in vitro* recombination (Manuscript 1). In order to estimate possible interaction between BNYVV and BSBMV during mixed infection, both cDNA clones should be used to create BNYVV/BSBMV reassortants in *N. benthamiana* (Manuscript 1).

Additionally, various reporter genes such as *monomeric red fluorescent protein* (*mrfp*) or *green fluorescent protein* (*gfp*) should be cloned into the existing full-length cDNA clones of the RNA2 of BNYVV and BSBMV, respectively (Manuscript 2). After rhizoinoculation of plants, the presence of the modified RNAs in the primary or systemically infected plant parts can be detected indirectly by fluorescence microscopy. This is to show the ability of RNA2 modification of BNYVV or BSBMV (insertion of foreign genes) to impact particle formation and the viral protein function such as replication, movement and symptom induction. Furthermore, the fluorescent labeled viruses (BNYVV and BSBMV) were used to study the interaction between both viruses as closely related viruses and two unrelated viruses, namely *Tobacco rattle virus* (TRV) and *Potato virus X* (PVX), in co-infection and super-infection experiments (Manuscript 2).

The replication and propagation of altered RNAs are the prerequisite for an efficient VIGS system. For this approach, parts of both *phytoene desaturase* (*pds*) gene from *N. benthamiana* and *magnesium chelatase* (*chlH*) gene from *Nicotiana tabacum* were amplified as model genes for the silencing experiments. The generated fragments were integrated by restriction enzyme digest and ligation into the modified BNYVV and BSBMV RNA2. *N. benthamiana* plants that were inoculated with *pds* and *chlH* fragments in the background of BNYVV and BSBMV RNA2 displayed an obvious silencing phenotype at 26 dpi (Manuscript 3).

2. Chapter 2: Manuscript 1

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

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2.1. 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 compared to available BNYVV B-type clone. BNYVV and BSBMV RNA1+2 reassortants were viable and capable of viral long-distance movement in *N. benthamiana* with symptoms dependent on the BNYVV type. Small genomic RNAs were exchangeable and moved long-distance in *B. macrocarpa* species. These infectious clones represent a powerful tool for the identification of specific molecular host-pathogen determinants

2.2. 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 well-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 the 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) motifs. 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% sequence 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 species, namely RNA 3 and RNA4, 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 infectious clones were lacking. Initial construction of BNYVV B-type infectious clone for generation of infectious *in vitro* transcripts of RNA2 (Ouillet 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 and binary expression vectors into *Rhizobium radiobacter*.

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 infection cycle including systemic colonisation, 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.

2.3. Materials and methods

2.3.1. 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).

2.3.2. 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 visualized 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).

2.3.3. 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).

2.3.4. 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). 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 (strain DH5α) (Inoue et al., 1990). Viral inserts were sequenced by commercial capillary Sanger sequencing (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 *R. radiobacter* 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. Lower bacterial concentration was applied for *N. benthamiana* due to necrosis induction of suspensions with OD_{600} = 0.5. *R. radiobacter* 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).

2.3.5. 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).

2.3.6. *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. Seven plants per pot were planted into virus-free field soil (six pots in total) 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.

2.4. Results

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

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. Following GA *in vitro* recombination into pDIVA (Acc. No. KX665539), the presence of viral cDNA inserts within plasmids was verified by means of appropriate restriction enzyme digestions and cDNA inserts were sequenced. The 35S-promoter cDNA and *Hepatitis delta virus* (HDV) ribozyme junction sequences were particularly verified. Sequencing results showed that GA had worked precisely, without introducing any deletions or insertions between viral cDNA and regulatory sequences. After *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).

To gain knowledge about degree of similarity against published sequences of the characterized 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), sequence comparisons at nucleotide and amino acid levels were performed (Table S2 and Table S3, see Supporting Information). RNA4 described by Lee et al. (2001) represents a non-functional deleted form of genomic species deficient for vector transmission (D'Alonzo et al 2012). Therefore, this sequence (Acc. no. NC_003508.1) was omitted. Sequence similarity of isolate BSBMV-CA at nucleotide level over all RNA components was closer to isolate MRM06 than EA suggesting a close relation between BSBMV-CA and –MRM06. (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. A 100% similarity was found for all RNA2 encoded proteins and 99.95% similarity was retrieved for the 239K polyprotein. Furthermore, 99.48% and 99.22% similarities were retrieved for RNA4-encoded P32 and RNA3-encoded P29, respectively. CA and EA isolates differ in the RNA1-encoded P239 and RNA2-encoded RT proteins (97.68% and 99.71% similarity, respectively; Table S3).

The genome sequence of the Japanese A-type isolate (BNYVV-S) is now supplemented with the nucleotide sequence of BNYVV Yu2 isolate produced in this work (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. The complete genome sequence (RNA1-3) of Yu2 and RNA4 from Italy was compared with those of major A- (BNYVV-S), B- (BNYVV-B; isolate F2/13) and P-type (BNYVV-Pithiviers) sequences (Bouzoubaa et al., 1985; Bouzoubaa et al., 1986; Bouzoubaa et al., 1987; Gilmer et al., unpublished; Klein et al., 2007; Schirmer and Gilmer, unpublished). The RNA1, -2 and -3 highest nucleotide sequence homologies (99.41%; 98.69% and 98.76, respectively) were found between Yu2 and Pithiviers isolates while RNA4 was closer to isolate F2 with 99.85% similarity (Table S4). At the amino acid level, homologies between the two A-types were high for RNA1+2 encoded proteins (237K 99.38%, 21K 100%, 75K 98.39%, 42K 99.74%, 13K 98.31%, 15K 98.48%, 14K 95.42%) and for proteins encoded by smaller genomic RNAs (25K 94.52% and 31K 96.81%). Only RNA2-encoded 14K

protein was identical for Yu2 and Pithiviers. The 42K sequences were similar among isolates (99.74%). Overall, the B-type isolate was the most distant except for RNA4-encoded 31K protein (99.65%). The remaining proteins encoded by RNAs 3 and 4 displayed lower similarity versus isolate S and Pithiviers, respectively (Table S5, see Supporting Information). To summarize, the ORF sequence comparison confirmed the closer relationship between the two A-type isolates compared to the P-type isolate.

2.4.2. Proof of infectivity

To obtain evidence that cDNA clones of both viral species are able to generate *in vivo* transcripts that carry out a complete infection cycle, each cDNA clone was transformed into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) (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*. 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) (Fig. 1a). Necrosis appeared 22 dpi. The same approach performed with 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).

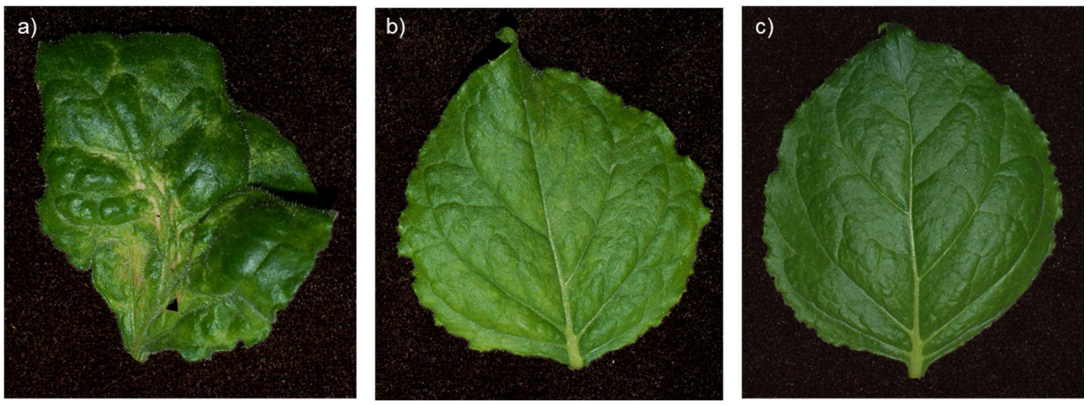

Fig. 1 Symptom expression on *Nicotiana benthamiana* systemically infected leaves obtained after agroinoculation of (a) BSBMV cDNA clones RNA1-4 and (b) BNYVV cDNA clones RNA1-4 compared to (c) mock-inoculated healthy control at 22 dpi.

The experimental Benyvirus local lesion host *C. quinoa* turned out to be resistant towards agroinoculation (data not shown). Therefore, sap from systemically infected tissues issued of agroinoculated *N. benthamiana* was used for rub-inoculations. Both viruses induced typical species specific local lesions at 7-10 dpi with BSBMV lesions developing to necrotic spots quite rapidly comparable to wild-type viruses (data not shown). 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 (data not shown). Systemic spread and symptoms development occurred at 25-33 dpi (BSBMV) and 19-22 dpi (BNYVV), respectively (Fig. 7, c, h, see below). BSBMV or BNYVV agroinoculation of *B. vulgaris* leaf tissue of three weeks old plants (BBCH 12) resulted in local lesions formation inside the infiltrated patch at 13 and 18 dpi, respectively (data not shown). Lesions increased in size and yellow blotches developed at 25 dpi, spreading to leaf veins, indicative for slow basipetal movement (data not shown). However systemic spread associated with virus symptoms in newly emerging leaves was not observed with any of the two viruses. This observation was in accordance with mechanical leaf inoculation of the wild-type viruses (data not shown). Additionally, variation of agroinoculation methods like vacuum-infiltration, root-dipping or vortex-inoculation of *B. vulgaris* roots with *A. tumefaciens* suspensions did not result in development of systemic infection (data not shown). To analyse if missing an effective root infection by agrobacteria was responsible for this absence of systemic infectivity, vortex-inoculation of 12 days old seedlings was applied according to Bornemann and Varrelmann (2011) using sap from *C. quinoa* local lesions obtained as described above. Following this approach, first systemic viral symptoms were observed on newly emerging leaves 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 derived (Fig. 2a) and wild-type (Fig. 2b) BSBMV inocula induced yellow blotches and bands. Typical necrotic yellow veins and chlorotic parenchymatic leaf tissue were observed with cDNA derived (Fig. 2c) or wild-type BNYVV (Fig. 2d). Viral accumulation within *B. vulgaris* lateral roots was assayed using species-specific antisera and ELISA based virus detection. Mean absorbance (A_{405nm}) values were similar between cDNA derived and wild-type virus (0.40 vs 0.44 for BSBMV and 1.18 vs 1.46 for BNYVV). To prove the presence of replicating viral RNAs transcribed from the cDNA clones, total RNA was extracted from systemically infected leaf tissue of *N. benthamiana*, *B. macrocarpa* (agroinoculated) and *B. vulgaris* (vortex-inoculated), displaying clear viral symptoms. RT-PCR with specific

primers detected all BSBMV or BNYVV RNA-components according to infiltrated or inoculated combinations.

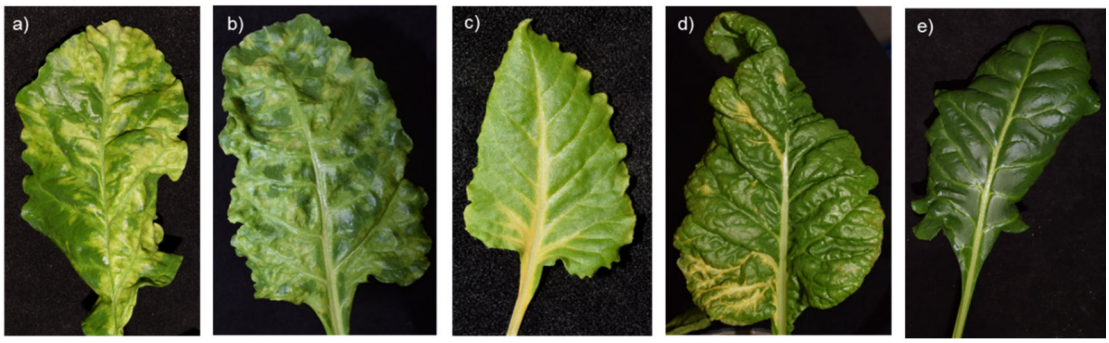

Fig. 2 Systemic symptom expression on *Beta vulgaris* systemically infected leaves produced after mechanical root vortex-inoculation with plant sap from *C. quinoa* local lesions infected with (a) BSBMV cDNA clones RNA1-4, (b) wild-type BSBMV, (c) BNYVV cDNA clones RNA14 and (d) wild-type BNYVV compared to (e) healthy control at 48 dpi.

2.4.3. Electron microscopy

N. benthamiana leaf tissues, systemically infected following agroinoculation of BSBMV and BNYVV, were used for negative contrast transmission electron microscopy (TEM). Rod shaped viral particles of varying lengths displaying a central core were observed from both samples (Fig. 3). Clear decoration was observed when specimens were treated with specific antisera supporting that particles observed indeed represented virions of BNYVV and BSBMV origin.

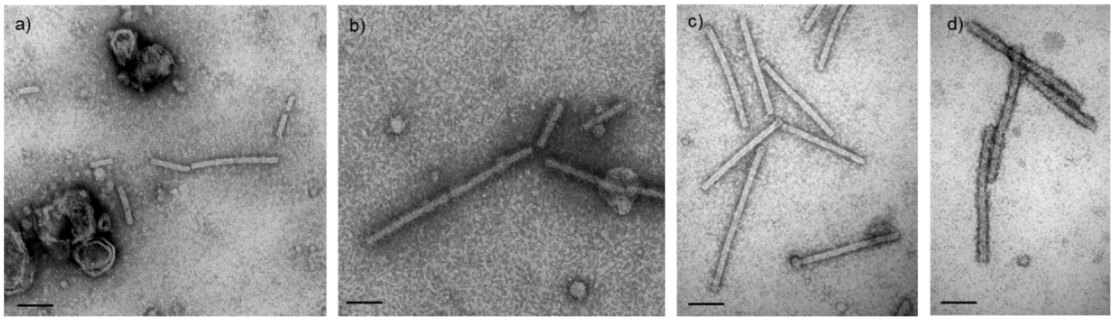

Fig. 3 (a) Transmission electron microscopy (TEM) of *Beet soil-borne mosaic virus* (BSBMV) and (c) *Beet necrotic yellow vein virus* (BNYVV) particles from systemically infected *N. benthamiana* leaf tissue and (b) BSBMV and (d) BNYVV particles, respectively, decorated with virus specific antisera.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

2.4.4. *Polymyxa betae* transmission of recombinant viruses

Having shown the abilities of BSBMV and BNYVV cDNA clones to initiate virus replication, followed by cell-to-cell and long distance movement as well as particle assembly, we verified their ability to be vectored by *P. betae.* To serve as source for the transmission, *N. benthamiana* plants were agroinoculated with the full-length clone RNA1-4 and sap used for sugar beet mechanical vortex inoculation. Plants displaying systemic symptoms were transplanted into the vector-containing soil to allow *P. betae* virus loading from rootlets. 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).

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

Previous work evidenced the exchangeability of small genomic RNA3+4 of BSBMV in B-type BNYVV RNA1+2 background, suggesting possible interaction of both viral species during mixed infection (D`Alonzo et al., 2012; Ratti et al., 2009). 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 in this experimental host (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 (BNa12, BNb12 and BS12). BNb1BS2 did not result in visible symptoms while BS1BNb2 displayed severe necrosis 21-23 dpi (Fig. 4 and Table 1).

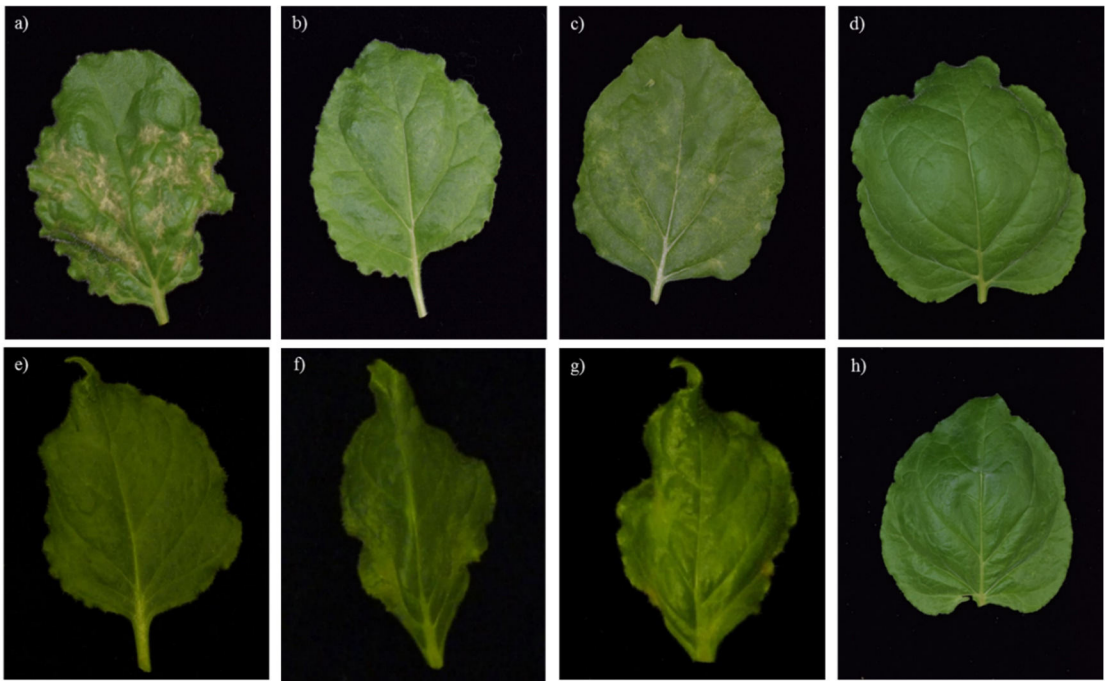

Fig. 4 Systemic symptom expression on *N. benthamiana* systemically infected leaves at 17 days post agroinoculation of (a) BSBMV cDNA clones RNA1-2 (BS12), (b) BNYVV A-type cDNA clones RNA1-2 (BNa12) and reassortants consisting of (c) BNYVV RNA1+BSBMV RNA2 cDNA clones (BNa1BS2) and (d) BSBMV RNA1+BNYVV RNA2 cDNA clones (BS1BNa2), (e) BNYVV B-type cDNA clones RNA1-2 (BNb12) and reassortants 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.

BNa12, BNb12 and BS12 infections appeared systemic 12-16 dpi while pseurorecombinants 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. Leaf tissues were harvested at 29 dpi for ELISA based detection of BSBMV and BNYVV (Table 1). High ELISA values were obtained for all plants displaying systemic symptoms as well as some symptomless plants inoculated by BNb12 and BN1bBS2

combinations. Symptomless plants inoculated with BS1BNa2 produced ELISA values similar to the healthy control. BS12, BNa1BS2 and BNb1BS2 resulted in mean ELISA absorbance (A_{405nm}) values of 0.41, 0.43 and 0.40, respectively, when assayed with BSBMV specific antiserum. When a BNYVV specific antibody was used, mean values of 0.91 (BNa12), 0.94 (BNb12), 0.83 (BS1BNa2) and 0.86 (BS1BNb2) were obtained.

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 reassortants (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

2.4.6. 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. 5). BNa12 and BNb12 lesions were faint whereas BS12 lesions quickly developed necrosis. Lesions produced by reassortants BNa1BS2 (Fig. 5 c) were comparable to BS12 whereas BNb1BS2 (Fig. 5 f) displayed a more faint and BS1BNb2 (Fig. 5 g) showed necrotic appearance. Interestingly, BS1BNa2 sap from infiltrated leaves applied for rub-inoculation of *C. quinoa* leaves did not produce

local lesions (Fig. 5 d). There was no consistent specific RNA component effect on local lesion phenotype formation.

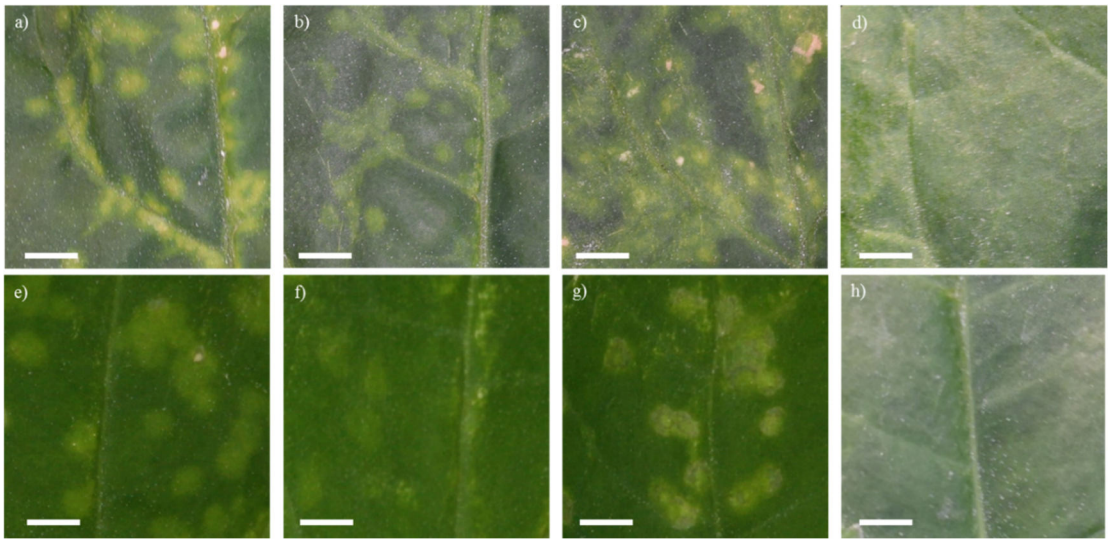

Fig. 5 Local lesions in *C. quinoa* inoculated leaves (7 dpi) obtained by rub-inoculation of sap from *N. benthamiana* leaves infiltrated by (a) BS12, (b) BNa12, (c) BNa1BS2, (d) BS1BNa2, (e) BNb12, (f) BNb1BS2, (g) BS1BNb2, (h) healthy. Bars represent 5 mm.

2.4.7. 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 BNYVV and BSBMV RNA species specific probes (Flobinus et al., 2016). ImageJ software (Schneider et al., 2012) was used to quantify the accumulation of viral RNAs within each local lesion with was normalized to the RNA loading (ribosomal RNAs). Image processing highlighted a higher accumulation of both genomic RNAs in BNb1BS2 reassortants when compared to BS1BNb2 and BNb12 or BS12 combinations (Fig. 6).

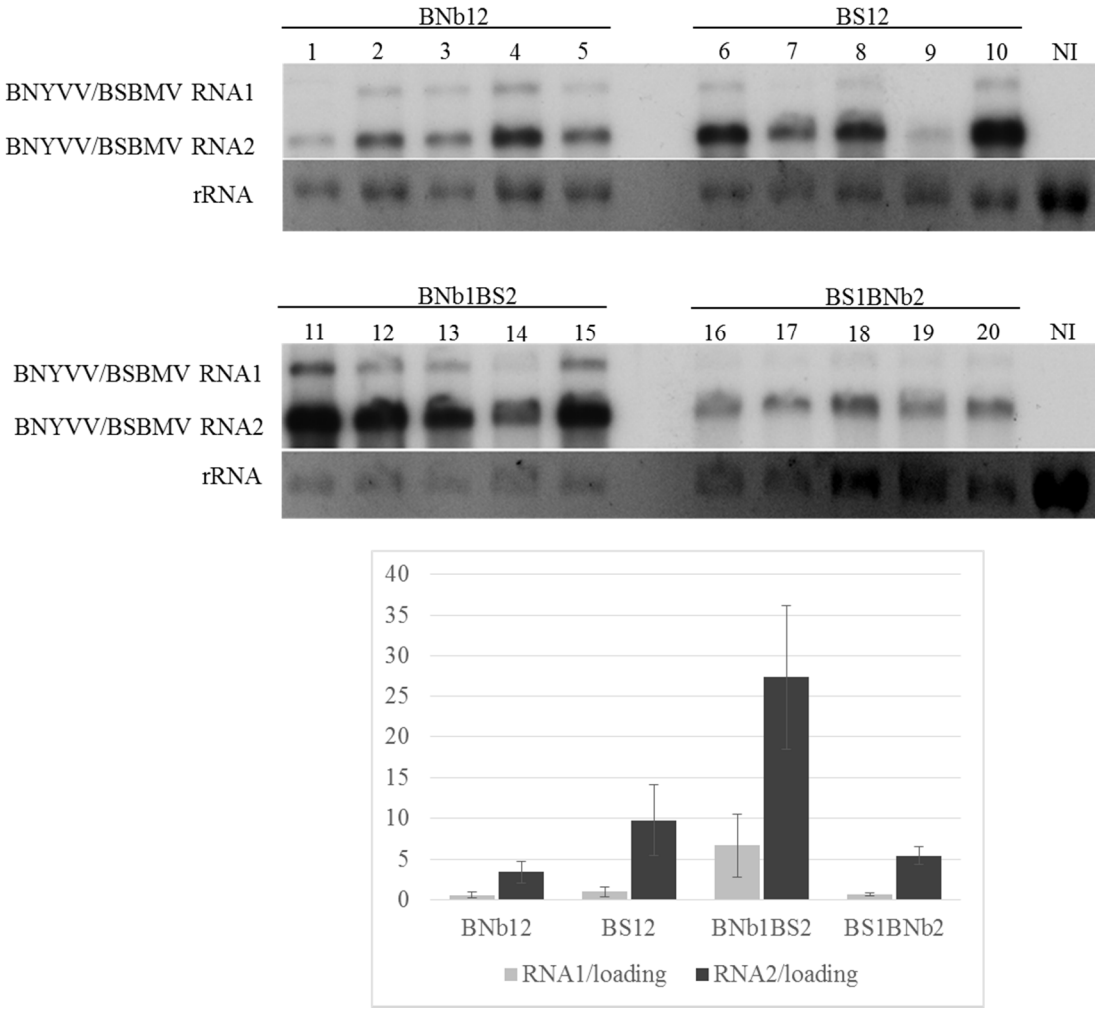

Fig. 6 Northern blot analysis of RNA extracted from local lesions of *C. quinoa* described in Fig. 5. BNYVV and BSBMV RNAs 1 and 2 were detected using specific ^{32}P labeled RNA probes while ribosomal RNAs (rRNA) have been used as loading control. Blotting image has been analyzed by the Imagej software 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).

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

In the USA, mixed infections occur between viral species coexisting in the soil, namely between A-type BNYVV and BSBMV. As BNYVV RNA3 is required for long-distance movement in *B. macrocarpa* (Lauber et al., 1998; Peltier et al., 2012) and BSBMV RNA3 species can substitute for this function (Ratti et al., 2009), 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. 7a, f, Tab. 2). Local lesions that were formed in the inoculated patch did not increase in size and no systemic viral movement and associated symptoms were observed (data not shown). 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). However, when RNA3 was supplemented to the inocula, systemic movement occurred with different kinetics. 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). Systemic symptoms produced by BS1-3 or BNa1-3 were comparable to those formed in *B. vulgaris*. BSBMV infection induced yellow blotches and bands (Fig. 7b, c) while BNYVV mainly provoked vein yellowing (Fig. 7g, h). Systemic infection using BSBMV RNA1-2+BNYVV RNA3 reassortants (BS12+BNa3, Fig. 7d) 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 7i) appeared delayed (33-39 dpi) with symptoms similar to those formed by BS1-3 (Fig. 7b) and BS1-4 (Fig. 7c). When RNA4 was added to the three genomic species combinations, no differences were observed on the phenotype or infection kinetics (Fig. 7e, 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).

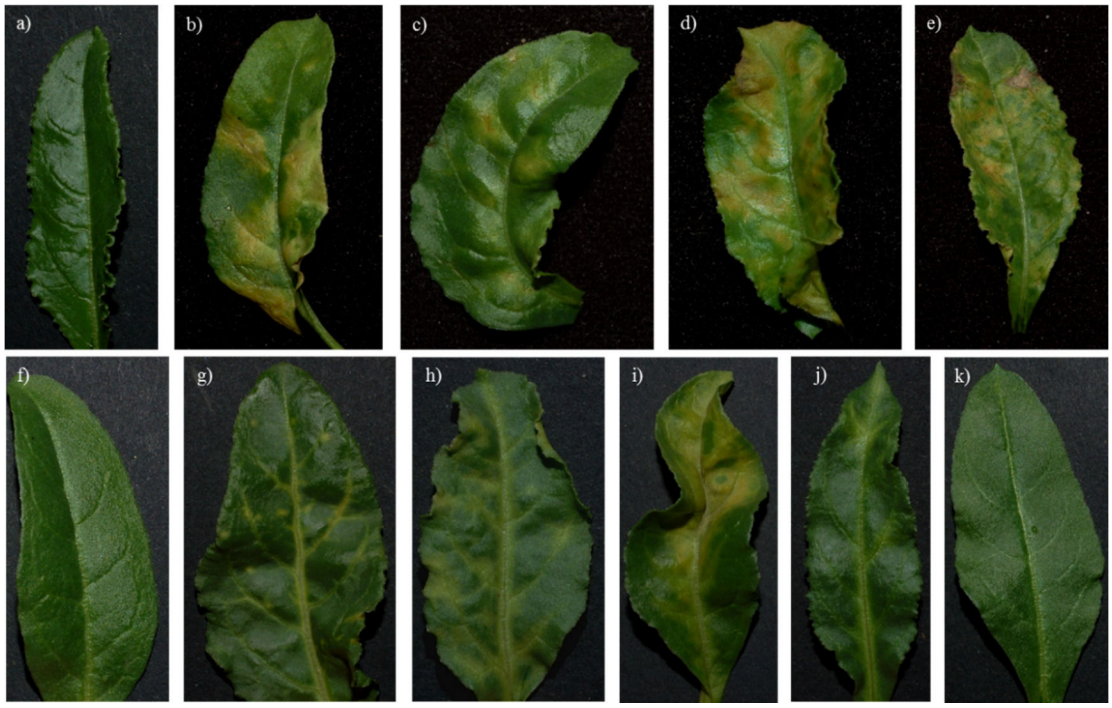

Fig. 7 Symptom expression in *Beta macrocarpa* systemically infected leaves obtained after agroinoculation of cDNA clones a) BSBMV RNA1+2, b) BSBMV RNA1-3, c) BSBMV RNA1-4, d) BSBMV RNA1+2 plus BNYVV RNA3, e) BSBMV RNA1-3 plus BNYVV RNA4, f) BNYVV RNA1+2, g) BNYVV RNA1-3, h) BNYVV RNA1-4, i) BNYVV RNA1+2 plus BSBMV RNA3, j) BNYVV RNA1-3 plus BSBMV RNA4, compared to k) healthy control at 43 dpi.

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 reassortants.

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)
BNa12BS3	6-9	33-39	80 (8/10)
BNa1-3BS4	6-9	19-22	100 (10/10)

2.5. Discussion

This study describes successful application of the GA single step *in vitro* recombination technique for the generation of agroinfectious cDNA clones of a multipartite RNA virus. Using GA lead to successful assembly of three cDNA fragments in the case of BSBMV RNA1 (6683 nucleotides total cDNA) and two fragments for BNYVV RNA1 (6746 nucleotides) without any detectable functional errors. It can be speculated that GA will speed up the establishment of plant viral reverse genetic systems. Using this approach, we reported the generation of fully infectious cDNA clones of a Californian BSBMV and a European A-type BNYVV isolates. Identical properties were found when artificial 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 agroinfiltration. Indeed, agroinoculation of *C. quinoa* leaves failed and no systemic infection occurred after root agroinoculation of *B. vulgaris*. Flores Solís et al. (2003) and Komari (1990) reported difficulties to transform *C. quinoa* by *A. tumefaciens*. Furthermore, in the natural host plant *B. vulgaris* leaf infection either using rub-inoculation (Chiba et al., 2008) or agroinoculation (this study) does not lead to systemic infection. Besides the impossibility to initiate a systemic infection from *B. vulgaris* leaves whatever the method employed, agroinfection failure 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. Alternatively, BSBMV or BNYVV viral combinations could be loaded through *B. macrocapra* agroinoculated plants and transmitted through the vector *P. betae* to the natural host if RNA3 and 4 species are supplemented to the inoculum (Delbianco et al., 2013, D'Alonzo et al., 2012). 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). Similar observation applied as well for the RNA4 species for their involvement in vector transmission. The infectious cDNA clones described in this study offer many possibilities to investigate the rhizomania disease complex in different *B.* species hosts either BNYVV-susceptible or tolerant such as *Rz1* or *Rz2* plus combinations of both. While some RNA segment exchanges were performed in our study, all reassortment 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 will provide more precise information about the molecular basis of resistance breaking found in certain strains by analysis of viral amplification and plant response depending on the host genotype (Bornemann and Varrelmann, 2013 and 2011; Koenig et al., 2009).

The initial work performed by Ratti et al (2009) illustrated the compatibility of BSBMV RNA3 with BNYVV viral replication, movement and packaging machineries. Benyvirus (BSBMV and BNYVV) RNA1 and 2 reassortment 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. From a phylogenetic point of view, sequence similarity between BNYVV and BSBMV suggests the evolution from a common ancestral virus species. 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. Detection of reassorted BSBMV/BNYVV in sugar beet has never been described in the USA where both viruses could infect the same plant. However, if such situation occurs, a reassorted virus displaying properties highlighted in this study would not be selected (Willemsen et al., 2017). Besides this hypothesis, exclusion from the same cell in mixed infection as well as super-infection exclusion is another possibility that requires further analysis. It is known that some monopartite potyvirus species colonise plants in mixed infections with other species from the same family in spatial separation but mix with viruses from separate families (Dietrich and Maiss, 2003). Understanding if members of the genus *Benyvirus* follow a similar colonisation strategy, will require labeling of individual genome components including RNA2 for future investigations. 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.

2.6. 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.

2.7. Supporting information

Table S2. Oligonucleotides used for viral full-length cDNA cloning

genome component and primer name	sequence (5'- 3')
BSBMV RNA1	
RNA1-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTTCCC ATTCGCCATCATTG
RNA1-low1	CGATCTGACCAAGTGATACCCTT
RNA1-up2	TGTTGGAGAAGTTGATGAAG
RNA1-low2	CATAATAGTAGCCTCCAAAA
RNA1-up3	GCTGATAGTGGTGTGTCTCCAAC
RNA1-low3	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTT TTATATCAATA
BNYVV RNA1	
RNA1-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCGATTC TTCCCATTC
RNA1-low1	GTGTAGGAATTTTCTGATGTACACCTATTAAC
RNA1-up2	GTTAATAGGTGTACATCAGAAAATTCCTACAC
RNA1-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTT TTATATCAATATAC
BSBMV RNA2	
RNA2-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTAATT ATTATCTCCATTG
RNA2-low1	GAAGACACGTCTAATCTTTCTACTA
RNA2-up2	CGGCAATTAAGTTGGATATAGTAG
RNA2-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTT TTCAATAAACT
BNYVV RNA2	
RNA2-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTAACT ATTATCTCC
RNA2-low1	CATTTATACCCATCCTCTACTAGTGTTTTCTC
RNA2-up2	GAGAAAACACTAGTAGAGGATGGGTATAAATG
RNA2-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTT TTCAATATACTG
BSBMV RNA3	
RNA3-up	AGGAAGTTCATTTCATTTGGAGAGGAAATTTAAATC TATCACCACATT
RNA3-low	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTT TTCTTCAATAT
BNYVV RNA3	
RNA3-up1	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAAT TTACCATTACATATTG
RNA3-low1	CGAGGGAAATTTGTTGCATTAGGC
RNA3-up2	GCCTAATGCAACAAATTTCCCTCG
RNA3-low2	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTT TTGTCAATATACTGAC
BNYVV RNA4	

RNA4-up	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAACTCAAAAATATAA
RNA4-low	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTAATAAACTG
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

3. Chapter 3: Manuscript 2

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

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Keywords: BNYVV, BSBMV, co-infection exclusion, fluorescent labelling, super-infection

3.1. Abstract

Infectious full-length clones of *Beet soil-borne mosaic virus* (BSBMV) and *Beet necrotic yellow vein virus* (BNYVV) were used for fluorescent labelling with the aim to study the interaction between both viruses in co-infection and super-infection experiments. Labelling was achieved by replacement of a part of the read-through domain on RNA2 with either the *gfp* or the *mrfp* gene. This resulted in a fusion protein made of the coat protein and the fluorescent marker protein. The labeled recombinant viruses were infectious, replicated to wild-type virus levels and moved systemically in *Nicotiana benthamiana*, as well as in *Beta macrocarpa*, producing wild-type like symptoms. 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. Co-infection experiments revealed a spatial separation of differentially labeled populations of BSBMV after agroinoculation of *N. benthamiana*. A

similar observation was made when BNYVV and BSBMV were co-infected. In contrast, co-infections of BSBMV and *Potato virus X* (PVX) or *Tobacco rattle virus* (TRV) showed massively co-infected cells with only marginal spatial separation. Microprojectile co-bombardment of *N. benthamiana* leaves with differently labeled populations of TRV revealed that both viral populations co-infect only a few cells before they start to separate. In super-infection experiments, BSBMV and BNYVV could not establish a secondary infection in *N. benthamiana* plants that have been previously infected with BNYVV or BSBMV as primary infecting viruses. Only the two unrelated viruses PVX and TRV were able to establish a secondary infection.

3.2. 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 (Gilmer and Ratti, 2012). BNYVV is the causal agent of rhizomania disease that is world-wide distributed in nearly all sugar beet growing areas (Peltier et al., 2008) whereas BSBMV is restricted to the United States (Heidel et al., 1997; Lee et al., 2001). Both viruses are transmitted by the soil-borne plasmodiophoromycete *Polymyxa betae* Keskin, which produce resting spores that allow the virus to persist in soil for decades (Keskin 1964; Tamada and Kondo 2013). Although both viruses are closely related species with the same host-range (Heidel et al., 1997), symptoms caused in the natural host sugar beet (*Beta vulgaris*) differ considerably. Roots infected with BSBMV appear asymptomatic whereas light yellow vein banding, mottling, mosaic patterns and slight leaf distortion can be observed on the foliar (Heidel et al., 1997). 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 (reviewed in Peltier et al., 2008). Since BNYVV and BSBMV share the same host-range and vector species, mixed infections in sugar beet plants have been found in different fields (Workneh et al., 2003). Moreover, reassortment experiments revealed that BSBMV RNA3 can even be trans-replicated and trans-encapsidated by BNYVV (Ratti et al., 2009).

In general, mixed infections of related or unrelated viruses can occur after co-infection or super-infection depending on the interval between two viruses infect the same host plant (reviewed in Syller, 2012; Syller and Grupa, 2016). The term co-infection is used when

a host plant is infected by two viruses simultaneously. In contrast, the term super-infection is used when a host, that has been previously systemically infected by a primary virus, is subsequently infected a secondary virus (after an incubation time). The outcome of an interaction after super-infection of two viruses can be either synergistic or antagonistic. Synergistic interaction occurs between more unrelated viruses leading to enhanced symptom development and virus replication as shown for different *Potyviruses* with *Potato virus X* (PVX) (González-Jara et al., 2004; Vance, 1991). Furthermore, two viral species interacting in a synergistic manner after super-infection are able to replicate within the same cells as recently shown for *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) (Tatineni and French, 2016). 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. Similar to co-infection, the exclusion mechanisms seem to be strongly driven by the degree of relationship. Folimonova et al. (2010) showed that super-infection exclusion of *Citrus tristeza virus* (CTV) occurred only between isolates of the same strain and not between isolates of different strains. Mahmood and Rush (1999) showed very early that super-infection exclusion can also occur between BNYVV and BSBMV indicating an antagonistic interaction between both viruses.

Similarly, to super-infection co-infection of two different viruses can either results in a synergistic or non-synergistic interaction (Dietrich and Maiss, 2003). On cellular level, two viral populations of one virus remain spatially separated (co-infection exclusion) with only a few doubly infected cells at the border between the two populations. This exclusion mechanism indicates an antagonistic interaction that has been described for a broad range of viruses including *Apple latent spherical virus* (ALSV), *Clover yellow vein virus* (ClYVV), PVX, *Plum pox virus* (PPV), *Tobacco mosaic virus* (TMV), *Tobacco vein mottling virus* (TVMV), *Turnip mosaic virus* (TuMV), TriMV and WSMV (Dietrich and Maiss, 2003; Gutiérrez et al., 2015; Julve et al., 2013; Takahashi et al., 2007; Tatineni and French 2016). The same spatial separation can be also observed when two viral populations of closely related viruses are co-infected as shown for TVMV and PPV (Dietrich and Maiss, 2003). However, co-infection can also lead to synergistic interaction with massive doubly infected cells without spatial separation when two distant related viruses are co-infected. This interaction has been described for mixed infections with *Potato virus X* and another distant related virus like *Plum pox virus*, *Tobacco vein mottling virus* and *Clover yellow vein virus* (Dietrich and Maiss, 2003). On

the whole-organisms level, Wisler et al. (2003) could show in greenhouse experiments that BNYVV suppress BSBMV in mixed infections. However, it is not known whether both viruses also remain spatially separated on a cellular level.

Previous studies investigating the interaction between viruses in either co-infection or super-infection experiments used fluorescent labeled full-length clones (Dietrich and Maiss, 2003; Folimonova, 2012; González-Jara et al., 2009; Julve et al., 2013; Takahashi et al., 2007; Tatineni and French 2016). With this approach, the distribution of differentially labeled populations from one or two viruses can be easily visualized using confocal laser scanning microscopy (CLSM). However, it requires flexible viral genomes that allow the integration of additional coding sequences and expression of fluorescent proteins. BNYVV and BSBMV have four single-stranded positive-sense RNAs with a similar genome organisation. 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 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 important for transmission by the fungal vector *Polymyxa betae* (Tamada and Kusume, 1991). The next three overlapping ORFs, named triple gene block (TGB1-3), are responsible for cell-to-cell movement (Gilmer et al., 1992) and the last ORF encodes the viral suppressor of RNA silencing (Chiba et al., 2013). BNYVV RNA3 is involved in long distance movement (Lauber et al., 1998; Peltier et al., 2012) and encodes the pathogenicity factor P25 (Chiba et al., 2008; Koenig et al., 1991). BSBMV RNA3 is also involved in long distance movement and encodes a P29 protein that is probably responsible for virus pathogenicity (Ratti et al., 2009). The RNA4-encoded BNYVV P31 and BSBMV P32 proteins are responsible for vector transmission (D`Alonzo et al., 2012; Tamada and Abe, 1989). In previous studies, fluorescent labelling of BNYVV and BSBMV was achieved by co-infection with viral replicons based on RNA3, expressing fluorescent marker genes (Erhardt et al., 2000; Ratti et al., 2009) or RNA5 (Schmidlin et al., 2005). Erhardt et al. (2001) integrated the GFP gene into the RNA2 of BNYVV by replacing a part of the RT domain of the P75. Infectivity was only confirmed in the local lesion host *Chenopodium quinoa* but infection of a host that allows systemic movement was not studied. Based on previous results, it was assumed that CP-RT is required for efficient

virus assembly (Schmitt et al., 1992). Furthermore, Haeberlé et al. (1994) and Erhardt et al. (2001) showed that CP-RT is located at the extremities of wild-type BNYVV particles. In this study, labelling of BNYVV and BSBMV full-length clones was achieved by nearly a complete 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 in different host plants. Furthermore, the influence of fluorescent reporter protein fusions on particle formation was investigated. Finally, co-infection and super-infection experiments including BNYVV and BSBMV as well as two unrelated viruses, namely *Potato virus X* (PVX) and *Tobacco rattle virus* (TRV), were conducted in *N. benthamiana*. Based on these results, the interaction between two closely related *Benyviruses* on the cellular level is revealed.

3.3. Methods

3.3.1. Construction of labeled full-length clones

Both BSBMV and BNYVV RNA2 cDNA clones for agroinoculation (Laufer et al., submitted) were modified to express the monomeric red fluorescent protein (mRFP) (Campbell et al., 2002), soluble modified red-shifted green fluorescent protein (smRS-GFP) (Davis & Vierstra,1998) and the UV-excited green fluorescent protein (GFPuv) (Baulcombe et al., 1995). Labelling was achieved by replacing the RT part of the *cp-rt* gene, thereby retaining the leaky stop codon of the *cp*-gene and the first two codons (CAATTA) of the RT domain. Replacement of the RT by *mrfp*, smRS-*gfp* and *gfp*uv gene, respectively, was followed by two stop codons (TGATAG) and the remaining 249 nucleotides of the *rt*-gene containing the putative subgenomic (sg) promotor for the 42 kDa triple gene block protein (P42) (Figure 1). This modification resulted in a large read-through protein made of the CP and the fluorescent proteins. The DNA fragments of fluorescent marker genes coding sequences were cloned into linearized plasmids of BNYVV/BSBMV RNA2 clones (Laufer et al., submitted) by means of Gibson assembly (Gibson et al., 2009). For this purpose, the marker genes coding sequences were amplified with Phusion Flash High-Fidelity PCR Master Mix (ThermoScientific, Schwerte, Germany) according to the manufacturer's instructions. Specific primers (Supplementary Table 1) contained 5′- and 3′-extensions overlapping with RNA2 of BNYVV/BSBMV

(25-28nt). The BNYVV/BSBMV RNA2 clones were linearized 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, Dueren, Germany) 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. (1990). 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, Ebersberg, Germany). The resulting clones were named BNYVV-RNA2-mRFP/-smRS-GFP/-GFPuv and BSBMV-RNA2-mRFP/-smRS-GFP/-GFPuv.

To optimize the fluorescence of GFPuv, an alanine to lysine mutation at amino acid position 206, as described by von Stetten et al. (2012), was introduced into the coding sequence of GFPuv. The mutation was generated by amplification of BNYVV-RNA2-GFPuv and BSBMV-RNA2-GFPuv with specific primers for site directed-mutagenesis (Supplementary Table 1). To introduce the 5' phosphate, PCR amplification product was phosphorylated by further reaction with T4 Polynucleotide Kinase (Promega, Mannheim Germany). After ligation with T4 DNA Ligase (Promega, Mannheim Germany), transformation of *in vitro* recombination products was performed and the resulting clones were named BNYVV-RNA2-GFPuv^{A206K} and BSBMV-RNA2-GFPuv^{A206K}.

To study the effect of the RT deletion on particle assembly, the RT coding sequence was deleted from the BSBMV RNA2 cDNA clone. The *cp*-gene 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 clones were named BSBMV-RNA2-deltaRT.

The infectivity of the above mentioned constructs was tested in *Beta macrocarpa* and *Nicotiana benthamiana* using agroinoculation. For this purpose, viral cDNA clones in binary vectors were transformed into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) strain C58C1. Bacterial cultures were prepared according to the method of Voinnet et al. (2003) with an optical density at 600 nm (OD_{600}) of either 0.1 (*N. benthamiana*) or 0.5 (*Beta macrocarpa*). 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 visualize fluorescent labeled full-length clones in systemically infected leaf tissue.

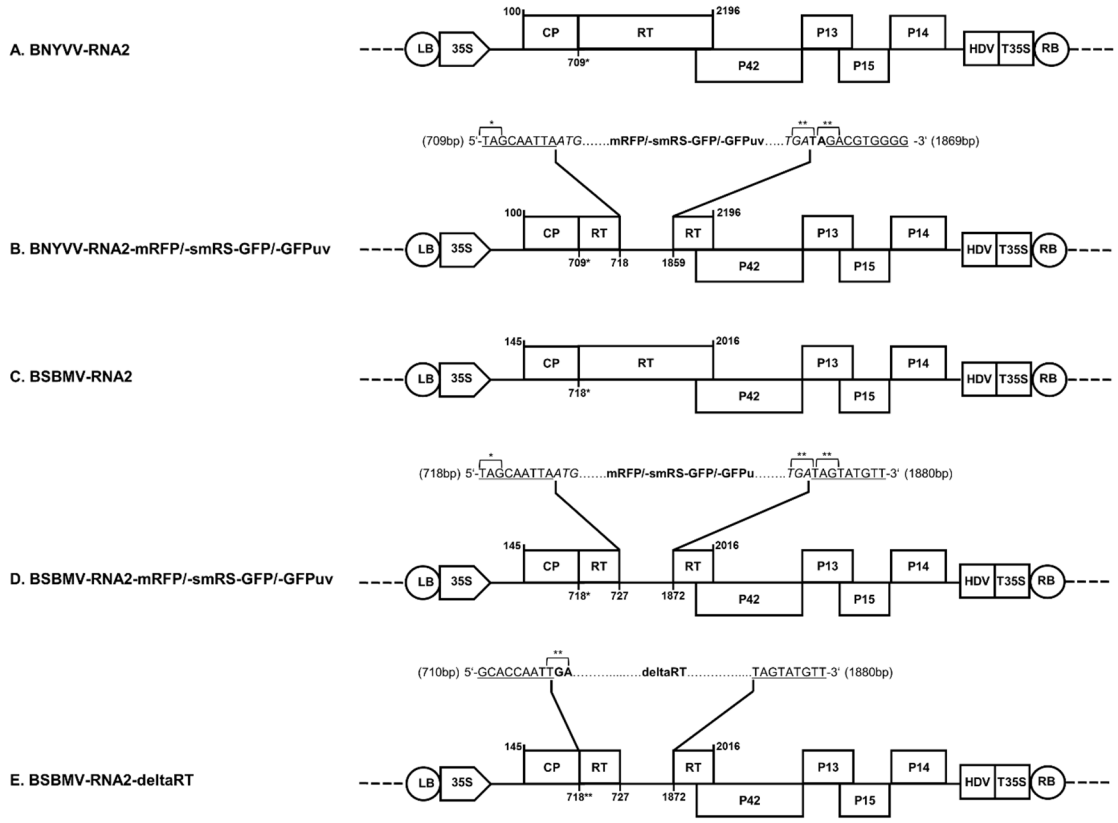

Fig. 1 Schematic representation of infectious full-length cDNA clones of BNYVV-/BSBMV-RNA2 (A, C) and modified variants carrying either a fluorescent marker gene (C and D) or a deletion in the RT-ORF (E). Underlined capital letters highlight the last nine nucleotides of the viral background whereas italic capital letters indicate the start and stop codon of the coding sequence from the fluorescent proteins (mRFP, smRS-GFP, GFPuv). Insertion of specific bases are indicated by bold capital letters. Numbers indicate the nucleotide position in the original sequence of BNYVV-RNA2 (Acc. No. KX665537) and BSBMV-RNA2 (Acc. No. KX352170). 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; smRS-GFP: soluble modified red-shifted green fluorescent protein; GFPuv: UV-excited green fluorescent protein; HDV: *Hepatitis delta virus* ribozyme; T35S: CaMV Terminator 35S; RB: Right border; *: leaky stop codon; **: stop codon.

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

Co-infection exclusion was studied with newly developed fluorescent labeled full-length clones of BNYVV and BSBMV. Additionally, fluorescent labeled full-length clones of the two unrelated viruses PVX and TRV were included in the experiments as controls. PVX expressing either dsRED (Dietrich and Maiss, 2002; PVX201-*optRed*) or GFPuv (Draghici and Varrelmann, 2009; 35S-PVX-GFP) have been described previously. TRV vectors expressing either dsRED or GFP composed of pTRV1 (Liu et al. 2002) and pTRV2-dsRED/pYL156-GFP (Ghazala and Varrelmann, 2007). Two differentially labeled viruses were inoculated simultaneously but in separte leaves of 3 weeks old *N. benthamiana* plants using agroinoculation as described above. After symptom development, virus distribution was visualized in systemically infected leaf tissue by means of CLSM. In addition to agroinoculation, particle bombardment was applied to study the spread of differentially labeled viruses starting from a single doubly infected mesophyll cell. For this purpose, detached leaves from 4- to 5-week-old *N. benthamiana* plants were subjected to microprojectile co-bombardment with a particle inflow gun (Gray et al. 1994) using 10 µl purified plasmid DNA corresponding to each viral RNA component. Detached leaves were placed in a petri dish with watered filter paper and incubated at room temperature in the dark. Virus distribution was visualized with CLSM after 2-5 days.

Super-infection exclusion experiments were performed in *N. benthamiana* plants using BNYVV-GFPuv as primary virus and BNYVV-mRFP, BSBMV-mRFP, TRV-dsRED and PVX-dsRED, respectively, as secondary virus. For this purpose, *N. benthamiana* plants were first infected with BNYVV-GFPuv using agroinoculation as described above. After three weeks, leaves displaying systemic symptoms were mechanically inoculated with the challenging viruses. Prior to the secondary infection, the establishment of the primary infection was confirmed with CLSM. The inoculum for the secondary infection was produced in *N. benthamiana* using agroinfiltration 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 three weeks, the establishment of the secondary infection was checked in inoculated and upper non-inoculated leaves using CLSM. Each variant comprised of 5 repetitions.

3.3.3. Confocal laser scanning microscopy

Systemic infected leaf tissue from *B. macrocarpa* and *N. benthamiana* was harvested and visualized with the TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Excitation/emission wavelengths for the different fluorescent proteins were as follows: mRFP 561 nm/520-540 nm, dsRED 561 nm/520-540 nm, smRS-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).

3.3.4. Transmission electron microscopy

Purified virions were 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, Gauting, Germany). Samples were 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, Eindhoven, and The Netherlands). Summed averaged images of the virions were calculated using RELION. 7000 individual overlapping segments of the virions, respectily were boxed using RELION. The images were sorted by MSA and summed to obtain a class average image of the virions (Van Heel et al., 2016).

3.4. Results

3.4.1. Effect of fluorescent labeling on infectivity of full-length clones

Different strategies were developed to identify a suitable place in the genome of BNYVV/BSBMV to introduce genes of different fluorescence markers. A replacement of the RT ORF by a fluorescence marker gene including a doubling of the sg promoter of P42 failed. Similarly, clones with an insertion between P15 and P14 including doubling of the sg promoter of P42 were not infectious. The replacement of P29 on RNA3 and P32 on RNA4 produced local fluorescence but systemic movement of labelled RNAs was not

observed. However, the replacement of the RT part of the *cp-rt* gene by ORFs from fluorescence markers allowed fluorescence labelling of BNYVV/BSBMV.

In order to verify that fluorescent labelling of BNYVV and BSBMV full-length clones does not negatively interfere with replication, encapsidation and systemic movement, plant infection was investigated by agroinoculation into leaf tissue of *N. benthamiana* and *B. macrocarpa*. For this purpose, a clone of BNYVV or BSBMV RNA1, respectively, was always inoculated together with an unlabeled or labeled clone of RNA2. In *N. benthamiana*, the unmodified full-length clone of BSBMV produced systemic symptoms including chlorotic vein banding, yellow blotches, leaf crinkling and necrosis 16 days a past inoculation (dpi) (Fig. 2A). The deletion of the RT encoding sequence in BSBMV-deltaRT did not affect symptom development and the speed of systemic infection (Fig. 2B). Similarly, the replacement of the RT domain by mRFP had no effect on symptom onset and severity (Fig. 2C). In contrast, plants infected with BSBMV-smRSGFP showed a delay in symptom development and a reduced symptom severity (Fig. 2D). No improvement was observed when smRSGFP was replaced by GFPuv (Fig. E). The unmodified full-length clone of BNYVV caused also systemic symptoms consisting of light yellow chlorosis (Fig. 2F). Also in case of BNYVV RNA2 the replacement of the RT domain by mRFP, smRSGFP or GFPuv had no effect on symptom onset and severity (Fig. 2G-I). In addition to *N. benthamiana* infection, mRFP labeled full-length clones from BNYVV and BSBMV were also inoculated on *B. macrocarpa*, a host for both viral species that requires the presence of RNA1-3 for systemic movement. Both BNYVV and BNYVV-mRFP caused similar symptoms with chlorotic lesions, vein yellowing and leaf crinkling 13 dpi (Fig. 2K-L). Symptoms caused 13 dpi by the unmodified full-length clones of BSBMV were chlorosis, necrotic lesions and leaf crinkling (Fig. 2M). In contrast, plants infected with BSBMV-mRFP developed the first systemic symptoms 30 dpi (Fig. 2N), visualized by means of CLSM of fluorescent proteins in leaf tissue displaying symptoms of a systemic infection. A clear homogeneous mRFP expression was observed in mesophyll cells of *N. benthamiana* plants infected with BSBMV-mRFP (Fig. 3A) and BNYVV-mRFP (Fig. 3E). In contrast, the fluorescence of smRS-GFP expressed by BNYVV or BSBMV was unevenly distributed in small bright clusters (Fig. 3B,F). A similar pattern was observed for both recombinant viruses when the smRS-*gfp* gene was replaced by *gfp*uv (Fig. 3C,G). This indicated a poor solubility of both fluorescent proteins. Therefore, an alanine to lysine mutation at amino acid position 206, as described by von Stetten et al. (2012), was introduced into the coding sequence

of *gfp*uv with the aim to increase the protein solubility. The resulting constructs BNYVV-GFPuv^{A206K} and BSBMV-GFPuv^{A206K} led to a better distribution of the fluorescence but still several small bright clusters remained (Fig. 3D,H). In case of *B*. *macrocarpa*, both BNYVV-mRFP (Fig. 3I) and BSBMV-mRFP (Fig. 3J) displayed a clear homogeneous expression of mRFP in mesophyll cells.

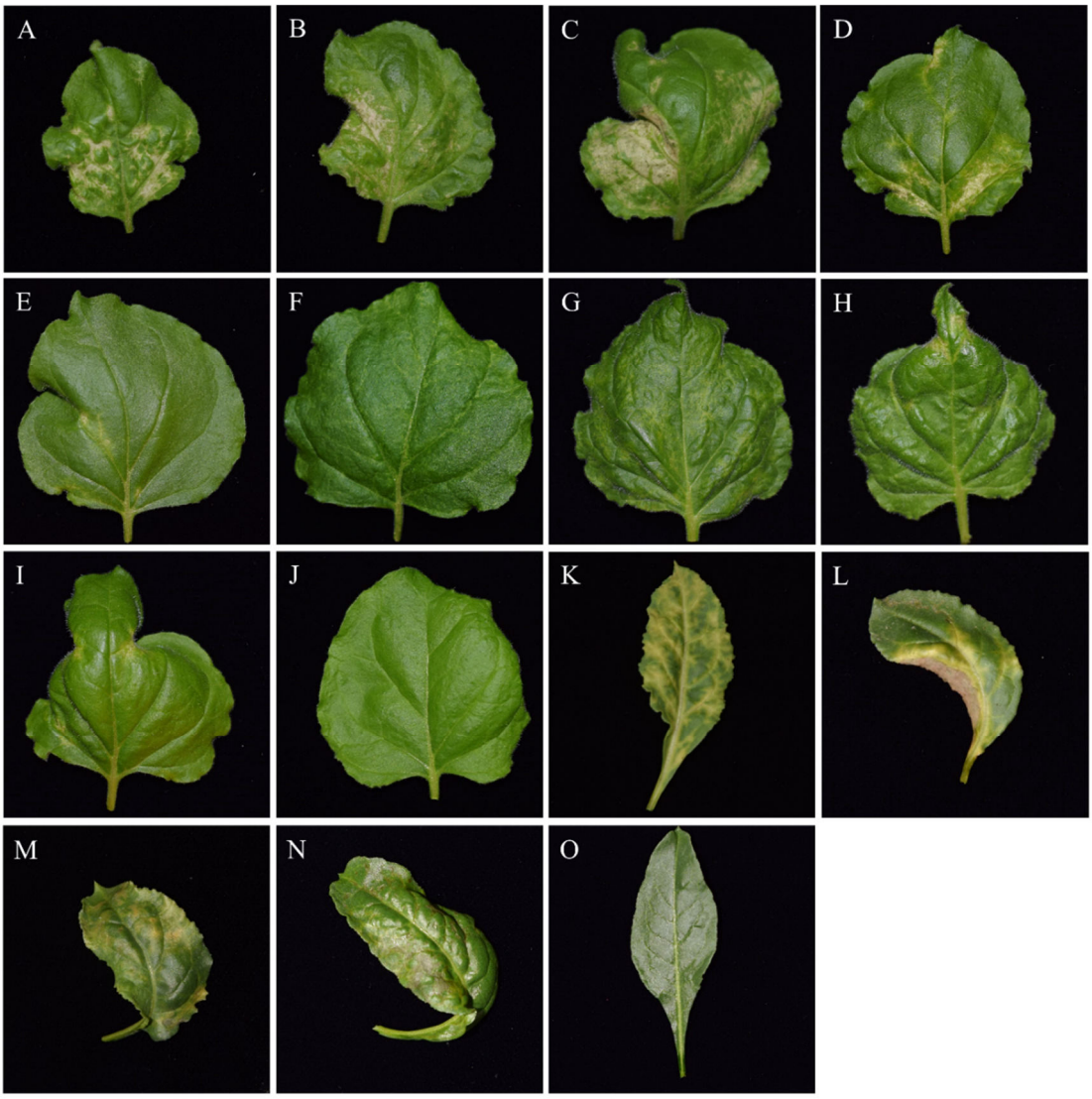

Fig. 2 *N*. *benthamiana* leaves displaying systemic symptoms following agroinfection with RNA1 and 2 cDNA clones of (A) BSBMV at 16 dpi, (B) BSBMV-deltaRT at 16 dpi, (C) BSBMV-mRFP at 16 dpi, (D) BSBMV-smRSGFP at 20 dpi, (E) BSBMV-GFPuv at 20 dpi, (F) BNYVV at 24 dpi, (G) BNYVV-mRFP at 24 dpi, (H) BNYVV-smRSGFP at 24 dpi and (I) BNYVV-GFPuv at 20 dpi in comparison to (J) healthy control. *B*. *macrocarpa* (28-37 dpi) leaves displaying systemic symptoms following agroinfection with RNA1-4 cDNA clones of (K) BNYVV, (L) BNYVV-mRFP, (M) BSBMV and (N) BSBMV-mRFP in comparison to (O) healthy control.

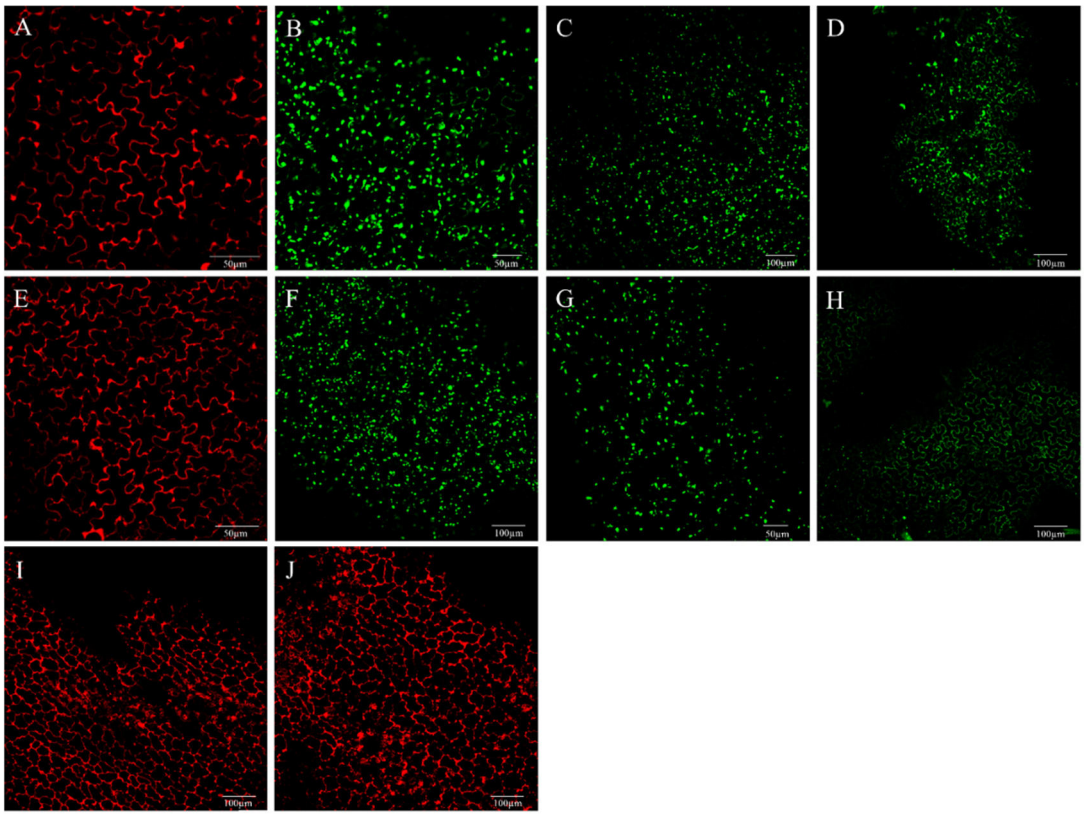

Fig. 3 Confocal imaging of *N. benthamiana* leaf tissue systemically infected with cDNA clones of fluorescently labeled RNA1 and 2 of (A) BSBMV-mRFP, (B) BSBMV- smRS-GFP, (C) BSBMV-GFPuv, (D) BSBMV-GFPuv^{A206K}, (E) BNYVV-mRFP, (F) BNYVV-smRS-GFP, (G) BNYVV-GFPuv and (H) BNYVV-GFPuv^{A206K}. Confocal imaging of *B. macrocarpa* leaf tissue systemically infected with cDNA clones of fluorescently labeled RNA1, 2 and 3 of (I) BNYVV-mRFP and (J) BSBMV-mRFP.

3.4.2. Effect of fluorescent labelling on particle formation of BNYVV and BSBMV

Fluorescent labelling of recombinant BNYVV and BSBMV was achieved by retaining the leaky stop codon in the CP open reading frame on RNA2 leading to fusion of CP and a fluorescent marker protein. Since both CP and CP-RT are part of wild-type virus particles, it was hypothesized that the fusion protein is also incooperated into virus particles. For proof of this hypothesis, particle composition of BSBMV was studied in detail with TEM. The presence of rod shaped virus particles in systemically infected *N. benthamiana* leaf tissue could be confirmed for all BSBMV-derived constructs (Fig. 4A-D) Also in case of BNYVV-derived constructs rod shaped virus particles were

observed (data not shown). Comparison of average particle diameter between the unmodified viruses from the full length-clone BSBMV (20.32 nm) and the fluorescent labeled clones BSBMV-mRFP (21.38nm) and BSBMV-GFPuv (20.36 nm) revealed no significant differences in particle diameter. Similarly, virus particles from BSBMV-deltaRT (20.88 nm) with a complete deletion of the RT domain were not distinguishable from BSBMV particles. Virus particles from BSBMV-GFPuv were also treated with 10 nm colloidal gold-labeled GFP antibodies to localize the GFP protein on the particle surface. A decoration with gold particles could be observed indicating that the read-through protein made of CP and GFP is incorporated over the entire surface of virus particles (Fig. 4E-F).

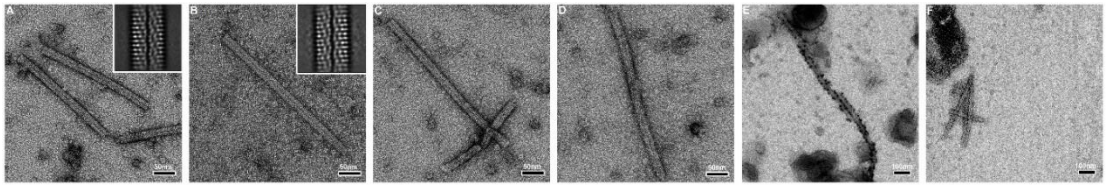

Fig. 4 Electron microscope images after negative staining of virus particles derived from the RNA1 and 2 cDNA clones of (A) BSBMV, (B) BSBMV-deltaRT, (C) BSBMV-GFPuv and (D) BSBMV-mRFP. Virus particles of BSBMV-GFPuv were also treated with 10 nm colloidal gold-labeled GFP antibodies (D and E). All virus particles were isolated from leaf tissue displaying systemic symptoms. mRFP: monomeric red fluorescent protein; GFPuv: UV-excited green fluorescent protein.

3.4.3. Co-infection exclusion of different labeled viruses

Co-infection exclusion of differentially labeled populations from one or two virus species was studied in *N. benthamiana* plants following agroinoculation in systemically infected tissue. Primary infections were initiated in separate leaves and the virus distribution was visualized with CLSM in upper non-inoculated leaves after the expression of systemic symptoms. GFPuv^{A206K} displayed a better distribution of the fluorescence signal but requires a UV laser (405 nm) for visualization that causes fast necrosis of mesophyll cells during specimen evaluation. Therefore, co-infection experiments were performed with BNYVV and BSBMV full-length clones expressing smRS-GFP that could be visualized

at longer wavelengths with less damaging potential (488 nm). Comparison of the results from both fluorescence markers revealed no differences (data not shown).

Depending on the combination of virus populations, the areas of mixed fluorescence varied from marginal overlapping of two different fluorescent cells to large clusters of cells. When two differentially labeled clones of BSBMV (BSBMV-smRSGFP + BSBMV-mRFP) were inoculated, the presence of both virus populations could be confirmed by a clear fluorescence of the two reporter proteins (Fig. 5A-D). Merged images revealed that both virus populations entered 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-smRSGFP (Fig. 5E-H). Thus all interactions between differentially labeled populations from one or two viruses belonging to the genus *Benyvirus* led to a spatial separation. PVX (Genus *Potexvirus*) and TRV (Genus *Tobravirus*) were included in the experiments as two unrelated viruses. BSBMV-mRFP was either co-infected with TRV-GFPuv or with PVX-GFPuv. Confocal imaging revealed that virus populations in the combinations BSBMV-mRFP + TRV-GFPuv (Fig. 5M-O) and BSBMV-mRFP + PVX-GFPuv (Fig. 5Q-S) infected the same areas in systemically infected leaf tissues. Close-ups of co-infected mesophyll cells showed massively co-infected cells with yellow fluorescence indicating that both viruses replicate within the same cell (Fig. 5P, T). In order to identify the viral RNA responsible for the exclusion effect between BNYVV and BSBMV, a reassortants made of BNYVV-RNA1 and BSBMV-mRFP was co-inoculated with BNYVV-smRSGFP (Fig. 5I-L). However, visualization of viral populations in systemically infected leaves showed also a clear separation with a few double infected cells at the border between both populations. A reassortants made of BSBMV-RNA1 and BNYVV-mRFP was not infectious and thus could not be tested.

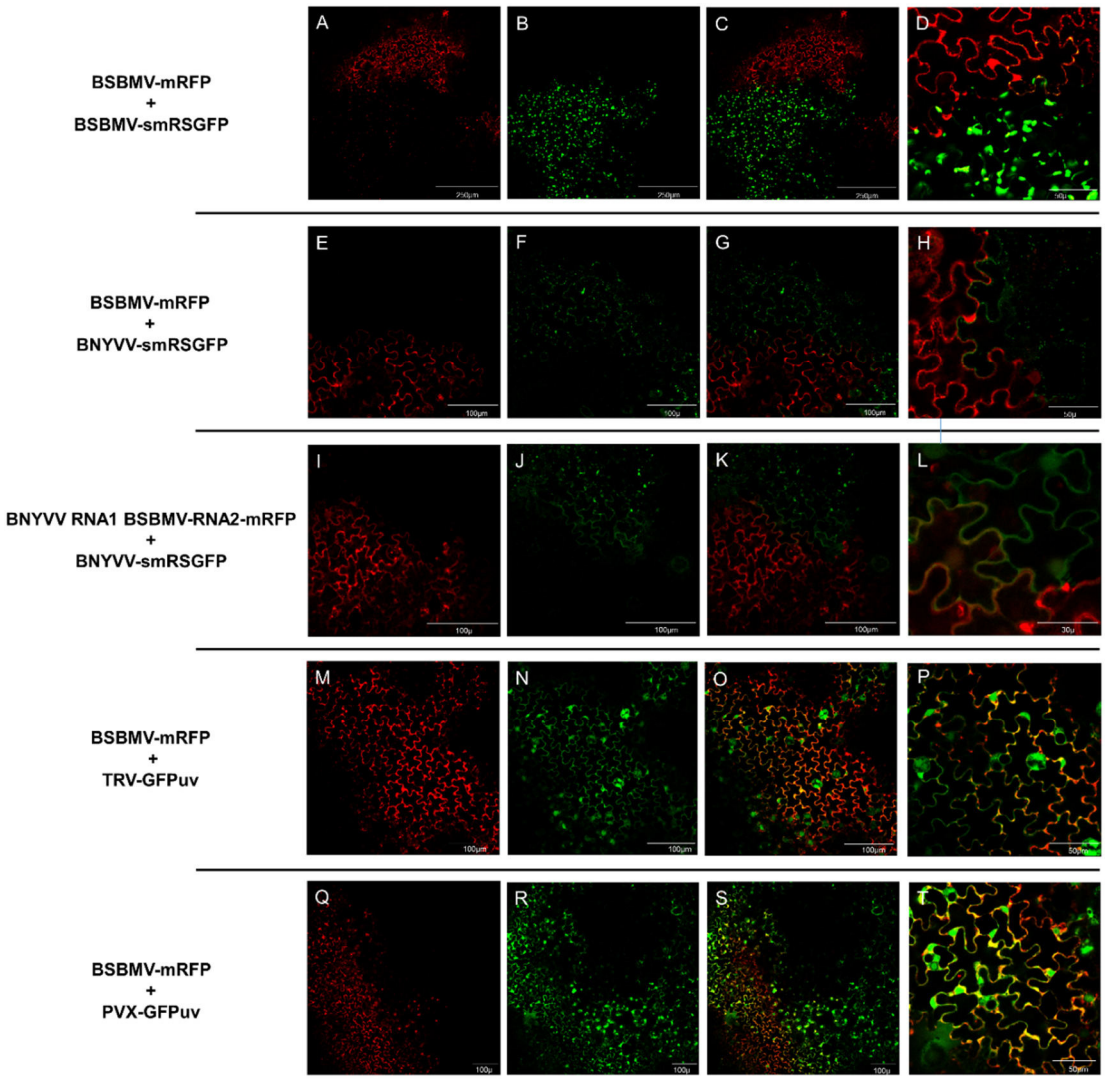

Fig. 5 Virus distribution in systemically infected *N. benthamiana* tissues following agroinfection with cDNA clones of BSBMV-mRFP (A) + BSBMV-smRSGFP (B); BSBMV-mRFP (E) + BNYVV-smRSGFP (F); BNYVV RNA1 BSBMV-RNA2-mRFP (I) + BNYVV-smRSGFP (J); BSBMV-mRFP (M) + TRV-GFPuv (N) and BSBMV-mRFP (Q) + PVX-GFPuv (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 different colored fluorescence cluster (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 population (D, H, L) whereas mixed populations show massive co-infected cells (P, T).

Agroinoculation of differentially labeled populations from one or two viruses revealed spatial separation in systemically infected leaf tissue. It was not possible to show that both viral populations start to separate when the infection is initiated from a doubly infected cell. Therefore, co-infection exclusion was also studied using microprojectile co-bombardment of viral cDNA clones that allows initiating a primary double infection in a single cell. TRV-dsRED and TRV-GFPuv were chosen for this experiment as they displayed the highest infection rate after particle bombardment. When cDNA clones of TRV RNA1, TRV-dsRED and TRV-GFPuv were co-bombarded, both viral populations replicated in distinct areas (Fig. 6C). High resolution imaging of the border between both viral populations showed a clear spatial separation (Fig. 6D). Primary infection sites could be identified by the yellow appearance of mesophyll cells (indicated by arrows) (Fig. 6E-H). Doubly infected cells were surrounded by spatially separated viral populations indicating a rapid onset of co-infection exclusion after co-bombardment. Besides co-infection of differentially labeled populations from one virus, 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 massive co-infected cells with yellow fluorescence (Fig. 6K-L). A similar pattern was observed for TRV-dsRED co-infected with PVX-GFPuv (Fig. 6O-P).

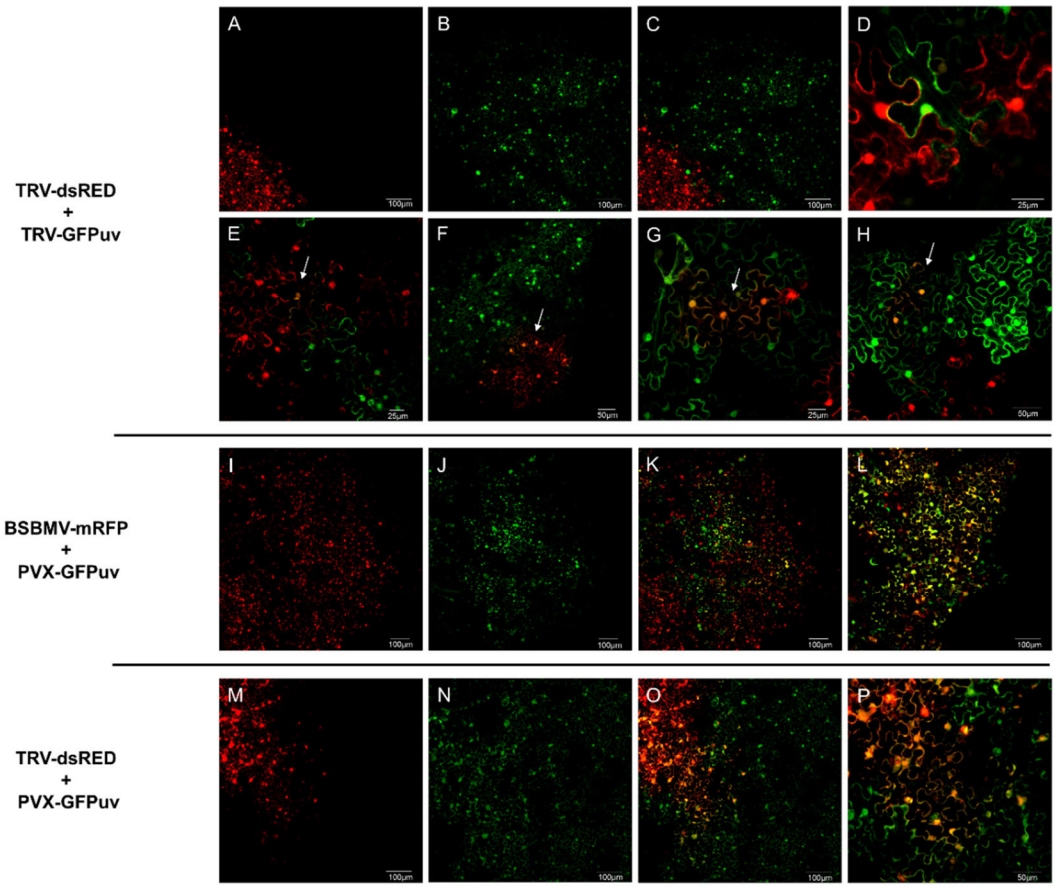

Fig. 6 Virus distribution in detached leaves of *N. benthamiana* after microprojectile co-bombardment with cDNA clones of TRV-dsRED (A) + TRV-GFPuv (B); BSBMV-mRFP (I) + PVX-GFPuv (J) and TRV-dsRED (M) + PVX-GFPuv (N). Confocal images (D-H), (K-L) and (O-P) are merged images of RFP (A, I, M) and GFP (B, J, N) channels. After co-bombardment, differentially labeled 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 lead to mixed populations represented by large yellow clusters (K, O). Close-up of massively co-infected cells are shown in (L) and (P).

3.4.4. Super-infection exclusion of different labeled viruses

Besides co-infection exclusion, differentially labeled full-length clones were applied to study the interaction between BNYVV and BSBMV in super-infection exclusion experiments. For this purpose, *N. benthamiana* plants were inoculated with BNYVV-GFPuv as a primary virus. After 21 dpi, leaves displaying systemic symptoms were mechanically super-inoculated with BNYVV-mRFP, BSBMV-mRFP, PVX-dsRED or TRV-dsRED as secondary viruses. Virus expressed fluorescence was observed in inoculated and upper non-inoculated leaves displaying systemic symptoms 42 dpi (Table 1). When plants were super-infected with BNYVV-mRFP or BSBMV-mRFP as secondary virus, only a green fluorescence belonging to BNYVV-GFPuv could be observed in upper non-inoculated leaves, however, small fluorescence clusters of BNYVV-mRFP and BSBMV-mRFP were observed in super-infected leaves. Thus, 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 indicating that the secondary virus could successfully establish an infection and moved systemically in non-inoculated upper leaves. The infectivity of the inoculum used for super-infection was confirmed in parallel by mechanical inoculation of *N. benthamiana* plants.

Table 2. Evaluation of super-infection experiments with fluorescently labeled full-length clones BNYVV-GFPuv/-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 viruses. Virus fluorescence was then evaluated in non-inoculated upper leaves after 42 days after the infection 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-GFPuv	BNYVV-mRFP	BNYVV-GFPuv
BNYVV-GFPuv	BSBMV-mRFP	BNYVV-GFPuv
BNYVV-GFPuv	PVX-dsRED	BNYVV-GFPuv + PVX-dsRED[b]
BNYVV-GFPuv	TRV-dsRED	BNYVV-GFPuv + 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

-: No inoculation.

[a]: Plants were inoculated with phosphate buffer.

[b]: Viral populations were mixed indicating no spatial separation

3.5. Discussion

In this study, fluorescently labeled viruses derived from full length-clones of two closely related *Benyviruses*, namely BNYVV and BSBMV, were developed. Labelling was achieved by a nearly complete deletion of the RT domain - despite of removing 249 nts in front of the TGB - and replacement with genes encoding different fluorescent proteins. Fluorescent labelling did not hamper the infectivity as both viruses moved systemically in two different host plants, induced characteristic disease symptoms and formed particles comparable to the unmodified virus. As BSBMV-deltaRT with a mutated leaky stop codon and a complete deletion of the RT region as well was able to infect *N. benthamiana* systemically and formed virus particles, it can be concluded that the RT domain including the P75 minor coat protein is dispensable for particle formation, systemic infection and symptom development. This is in contrast to the observations made in previous studies. Schmitt et al. (1992) and Tamada et al. (1996) analyzed the effect on BNYVV RT deletions on virion formation by means of an encapsidation assay employing exogenous nucleases during RNA extraction. With this assay they were able to demonstrate that all, even small in-frame N-terminal deletions negatively affected virion formation. Furthermore, a mutant in which the leaky stop codon was converted to UAA and reinforced by two additional termination codons displayed also impaired packaging (Schmitt et al, 1992). However, electron microscopic analysis was not performed. It may be possible that altered RT proteins with deletions interfere with particle assembly that cannot occur when the whole RT domain is deleted. To clarify this question, a detailed analysis of RT deletion mutants with TEM is required.

In this study, TEM virion analysis and decoration of recombinant BSBMV expressing CP-GFPuv revealed incorporation of the fusion protein along the whole surface of the rod-shaped particle. This was unexpected as Haeberlé et al. (1994) showed by TEM and immunogold labelling with an RT-specific antiserum that CP-RT/P75 is located only at the extremities of the wild-type BNYVV particles. Incorporation of the RT protein near one extremity of virus particles has been also shown for potato mop-top virus (PMTV; Cowan, et al., 1997). We cannot say if the smaller size of GFP (27 kDa) compared to the RT-domain (54 kDa) allows the CP-GFP fusion to be encapsidated over the entire particle surface, if recognition of specific CP-RT epitopes by the antiserum only permitted the RT-detection at particle extremities or if the RT domain contains specific sequences that are required for an integration of P75 at the extremity of virus particles. Nevertheless, as

no effect of CP-GFP on particle diameter could be detected, it seems that the CP-GFP overcoat is only partial, which is in accordance to the fact that suppression of the leaky stop codon occurs at a rate of 10% when ribosomes encounter it (Schmitt et al., 1992). Similarly, the particle diameter of the virus from construct BSBMV-deltaRT did not differ from the unmodified virus.

The fluorescence of mRFP expressed by BNYVV and BSBMV was clear and homogeneously distributed throughout the cytoplasma of infected cells whereas the fluorescence of smRS-GFP and GFPuv was unevenly distributed in small bright clusters indicating a poor solubility of both fluorescent proteins fused to the viral CP. The alanine to lysine mutation in BNYVV-GFPuv^{A206K} and BSBMV-GFPuv^{A206K} led to a better distribution of the fluorescence but several small bright clusters remained. It was suspected that the clusters represent virus particles that localize to mitochondria as reported by Erhardt et al. (2000). Co-localization studies with mitochondrial markers, however, could not confirm this hypothesis (data not shown). It was also reported by Erhardt et al. (2000) that at later times during infection virus particles relocated to semiordered clusters in the cytoplasm. However, it is also possible that the CP-smRSGFP and –GFPuv fusion interfered with the encapsidation process. This might also explain why both fluorescent proteins when expressed by the virus caused a delay in symptom development. Nevertheless, it was assumed that this had no effect on the spatial separation of viral populations in co- and super-infection experiments.

The results from co-infection experiments showed that populations of identical, but differentially labeled viruses of *Benyvirus* species BNYVV and BSBMV replicated predominantly in discrete areas and remained separately. A similar spatial separation was observed when BNYVV and BSBMV were co-inoculated. The contact of differentially labeled virus populations from BNYVV and BSBMV were restricted to a small number of cells at the border of different fluorescent cell clusters indicating that both viral species in principle can replicate within the same cell. A similar observation was reported in infected tissues with populations of identical but differentially labeled monopartite potyviruses (Dietrich and Maiss, 2003). A reassortants viral population consisting of BNYVV-RNA1, BSBMV-RNA2-mRFP and BNYVV-smRSGFP showed also a clear spatial separation indicating that RNA1 is not required to induce spatial separation of the two viruses. 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 show massive co-infected cells (Dietrich and Maiss, 2003; Takahashi et al., 2007). An identical pattern was observed after particle bombardment of detached leaves, which confirms a previous observation that spatial separation occurs in primary and systemically infected leaves (Dietrich and Maiss, 2003). During systemic movement, it is supposed that virus genotypes move together and are individually isolated during the first phase of leaf infection (Gutiérrez et al., 2015). In our study, co-bombardment of *N. benthamiana* leaves with differentially labeled populations of TRV revealed that two viral populations co-infect only a few cells before they start to separate. This indicates an efficient mechanism that induce a rapid spatial separation of two viral populations, even though the molecular basis is still unknown.

The exclusion pattern of BNYVV and BSBMV in super-infection experiments was similar to the co-infection experiments. Neither BNYVV-mRFP nor BSBMV-mRFP could establish a secondary infection in *N. benthamiana* plants that have been previously infected with BNYVV-GFPuv, even though small fluorescence clusters of the challenging viruses could be observed in super-infected leaves. This indicates that virus replication was possible in primary infected cells but cell-to-cell movement was inhibited by the secondary virus. Only the two unrelated viruses PVX and TRV were able to establish a secondary infection. Different mechanisms have been proposed for the exclusion of two viral species after super-infection but experimental evidence is restricted to a few plant viruses. It was shown very early that super-infection exclusion between two closely related plant viruses can be elicited by RNA silencing (Ratcliff et al., 1997). More recently, it was shown that super-infection exclusion by CTV requires the production of the viral protein p33 (Folimonova, 2012). This protein mediates super-infection exclusion at the whole organism level but is not required for exclusion at the cellular level (Bergua et al., 2014). Similarly, Tatineni and French (2016) demonstrated that WSMV- and TriMV-encoded CP and NIa-Pro proteins trigger super-infection exclusion independently of each other. However, further studies will be needed to clarify whether co-and super-infection exclusion of *Benyvriuses* shares similar features with previously reported mechanism.

The data presented here provides the first evidence that BNYVV and BSBMV remain also spatially separated in their natural host sugar beet. 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 viral population within in a host, also referred to as quasi species, is a collection of related viral variants subjected to a

continuous process of genetic variation, competition, and selection. The ability of viral variants to exclude each other in mixed infections eliminates the competition for the host resources. Furthermore, it has great implications for the stability of viral sequences and the genetic structure of a virus population (Folimonova, 2012). Newly emerging viral variants have a benefit by favouring uninfected cells rather than already infected host cells (Syller and Grupa, 2016). However, replication of two or more viral genomes in one cell increase 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 reassortants of BNYVV and BSBMV in mixed infections seems to be very low due to the spatial separation on the cellular level. Moreover, both viral species seem to have developed different infection strategies since BNYVV infections are mainly restricted to the root and BSBMV cause more systemic foliar symptoms (Heidel et al., 1997). The fluorescence labeled viruses derived from full length-clones developed in this study represent versatile tools to address this question in the natural host sugar beet.

3.6. Supporting information

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-smRSGFP	BSBMV2-smRSGFP-s	CGTTCCACGCACCAATTAGCAATTAATGAGTAAAGGAGAAGAACT
	BSBMV2-smRSGFP-as	AACACCACGTGTTCGTAACATACTATCATTTGTATAGTTCATCCA
BSBMV-RNA2-GFPuv	BSBMV2_GFPuv_s	CGTTCCACGCACCAATTAGCAATTAATGAGTAAAGGAGAAGAAC
	BSBMV2_GFPuv_as	AACACCACGTGTTCGTAACATACTATCATTTGTATAGTTCATCCATGC
BSBMV-RNA2-deltaRT	BSBMV-deltaRT-fw	ATGGCGCGCCTCA ATTGGTGCGTGGAACGGCAGGAGTAACACCCC
	BSBMV-deltaRT-rv	GATTAATTAA TAGTATGTTACGAACACGTGGTGTTAGTAATA
Linearization of BSBMV-RNA2	BSBMV2-s	TAGTATGTTACGAACACGTGGTGTTAGTAATA
	BSBMV2-as	TAATTGCTAATTGGTGCGTGGAACGGCAGGAG
BNYVV-RNA2-mRFP	BNYVV2-mRFP-s	GAACCAGTCCACCCGGACAATAGCAATTAATGGCCTCCTCCGAGGAC
	BNYVV2-mRFP-as	GAAGAACCAGCCCCACGTCTATCAGGCGCCGGTGGAGTGGCG
BNYVV-RNA2-smRSGFP	BNYVV2-smRSGFP-s	GAACCAGTCCACCCGGACAATAGCAATTAATGAGTAAAGGAGAAGAAC
	BNYVV2-smRSGFP-as	GAAGAACCAGCCCCACGTCTACTATTTGTATAGTTCATCCATGCC
BNYVV-RNA2-GFPuv	BNYVV2_GFPuv_s	GAACCAGTCCACCCGGACAATAGCAATTAATGAGTAAAGGAGAAGAAC
	BNYVV2_GFPuv_as	GAAGAACCAGCCCCACGTCTATCATTTGTATAGTTCATCCATGC
BNYVV-RNA2-GFPuv^{A206K}	BNYVV2- GFPuvA206K_s	CAATCTAAGCTTTCGAAAGATCCCAACGAAAAG
	BNYVV2- GFPuvA206K_as	TGTGGACAGGTAATGGTTGTCTGGTAAAAG
Linearization of BNYVV-RNA2	BNYVV2_s	ACGTGGGGCTGGTTCTTC
	BNYVV2_as	TTGTCCGGGTGGACTGGTTC

4. Chapter 4: Manuscript 3

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

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4.1. 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* and *Nicotiana benthamiana*. Therefore, we tested if in infectious full-length clones of both viruses the RTD can be substituted by untranslatable cDNA fragments from *magnesium chelatase H subunit* (*chIH*) and *phytoene desaturase* (*pds*) to create tools for virus-induced gene silencing (VIGS). Agroinfection 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* and *pds*. Quantitative real-time PCR displayed significant reductions in *pds* (59-77%) and *chIH* (67-85%) transcripts in the photobleached leaves of both BSBMV/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.

RNA interference (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 defense (Brodersen and Voinnet, 2006; Li and Ding, 2006; Meister and Tuschl, 2004). During RNAi, dsRNA from different sources 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). Virus induced gene silencing (VIGS) is a powerful technique adapted from the RNA-mediated antiviral defense mechanism (Kumagai et al., 1995; Lindbo et al., 1993). In the last twenty years several DNA and RNA viral vectors have been successfully constructed for silencing different genes such as *magnesium chelatase* (*chlH*) or *phytoene desaturase* (*pds*) in different plants (Hiriart et al., 2002; Ratcliff et al., 2001; Robertson, 2004). To verify if two *Benyviruses* are in general suitable tools for VIGS, RNA2 of BNYVV and BSBMV was successfully equipped with the appropriate restriction sites to insert different target genes and finally tested in *N. benthamiana.*

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 naturally transmitted by zoospores of the plasmodiophorid *Polymyxa betae* (Adams et al., 2001). Both viruses possess a similar genome organisation and morphological structure 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. In addition, some BNYVV isolates contain a fifth RNA (Miyanishi et al., 1999). 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, 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, cell-to-cell movement, replication and suppression of posttranscriptional gene silencing (PTGS) (Dunoyer et al., 2002; Richards and Tamada, 1992). BNYVV and BSBMV contain beside RNA1 and RNA2 two additional smaller RNAs: RNA 3 and RNA 4. RNA 3 is important for the development of rhizomania symptoms in roots of sugar beet, whereas RNA 4 is involved in virus transmission by *Polymyxa betae* (Chiba et al., 2008; Jupin et al., 1992). In principle RNA 1 and RNA 2 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 agroinoculated *Beta macrocarpa* and *N. benthamiana* (Laufer et al., submitted). Moreover, the RTD can be replaced by different genes encoding fluorescent proteins allowing the construction of fluorescent labeled cDNA clones. 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 *magnesium chelatase H subunit* (*chlH*) of *Nicotiana tabacum* and *phytoene desaturase* (*pds*) of *N. benthamiana*. Furthermore, both VIGS-constructs should be optimized 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 ChlHNb 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). All primers used to amplify *chlH* and *pds* were extended with *AscI* (5′-GGCGCGCC-3′) and *PacI* (5′-TTAATTAA-3′) recognition sites. To produce a 578 bp

PDSNb sense and antisense fragment, primers were designed according to the *N. benthamiana pds* sequence (GenBank accession number 93117609) (Table 1).

Table 1. Oligonucleotide primer sequences used in this study. Restriction sites are underlined.

Primer	Sequence (5′-3′)
S-ChlHNb-AscI-for	5′-TTGGCGCGCCGAATCTCCTTGACCGAGCAGTC-3′
S-ChlNb-PacI-rev	5′-CGTTTAATTAAATGCCTTCATACCACTTGGGGT-3′
AS-ChlNb-PacI-for	5′-GATTAATTAAGAATCTCCTTGACCGAGCAGTC-3′
AS-ChlNb-AscI-rev	5′-TTGGCGCGCCATGCCTTCATACCACTTGGGGT-3′
S-PDSNb-AscI-for	5′-TTGGCGCGCCTTGTTATTGCTG-3′
S-PDSNb-PacI-rev	5′-GATTAATTAAAGTTCAAAGCAATCAAAATGCA-3′
AS-PDSNb-PacI -for	5′-CGTTTAATTAAGTTCAAAGCAATCAAAATG-3′
AS-PDSNb-AscI-rev	5′-TTGGCGCGCCTTGTTATTGCTGGTGCAGG-3′
BN-RNA2-AscI-rev	5′-ATGGCGCGCCTTGTCCGGGTGGACTGGTTC-3′
BN-RNA2-PacI-for	5′-GATTAATTAATGATAGACGTGGGGCTGGTTCTTC-3′
BS-RNA2-AscI-rev	5′-ATGGCGCGCCTCAATTGGTGCGTGGAACGGCAGGAGTAACACCCC-3′
BS-RNA2-PacI-for	5′-GATTAATTAATAGTATGTTACGAACACGTGGTGTTAGTAATA-3′
PDSNb-for	5′-TTCTTTTGCCTGAAGACTGGAAA-3′
PDSNb-rev	5′-GAACTCCCACTAGCTTCTCCAACT-3′
ChlHNb-for	5′-AGCGTGACCTTGTGGTAGGAA-3′
ChlHNb-rev	5′-TGGAGGTTCACCAATGATGTGA-3′
60S-Nb-for	5′-AAGGATGCCGTGAAGAAGATGT-3′
60S-Nb-rev	5′-GCATCGTAGTCAGGAGTCAACC-3′
FBOX-Nb-for	5′-GGCACTCACAAACGTCTATTTC-3′
FBOX-Nb-rev	5′-ACCTGGGAGGCATCCTGCTTAT-3′
PDSNb-1for	5′-CGAGCTGAATGAGGATGGAAGTG-3′
PDSNb-1rev	5′-GCACCTTCCATTGAAGCCAAG-3′
ChlHNb-1for	5′-GCTGATGCAGTTCTCCACTTTGG-3′
ChlHNb-1rev	5′-GACGGAGCAGCTCCACATCTC-3′
Act-Nb-1for	5′-GGCAGGTCGTGACCTCACTG-3′
Act-Nb-1rev	5′-CACCACTGAGCACTATGTTTCCG-3′

The PDS PCR products of ChlH were digested with AscI and PacI restriction enzymes, purified from agarose gels with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and cloned into RNA2 cDNA clones of BNYVV and BSBMV, respectively (Laufer et al., submitted). For this purpose, these clones were firstly re-amplified, thereby deleting the RTD but leaving 249 bp upstream of the TGB1 ORF retaining the subgenomic promotor and creating the single restriction enzyme recognition sites *AscI* and *PacI* followed by two stop codons (TGATAG) to facilitate the cloning of *chlH* or *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 DH5 α) (Inoue et al., 1990). Subsequently all constructs (Table S2) were electroporated into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) strain GV2260 via heat shock transformation (Panja et al., 2008) and inoculated into 2-3 leaves of 3-4 week old *N. benthamiana* plants as described by Voinnet et al. (1998)

Non-inoculated plants and plants inoculated with the empty vector developed no systemic symptoms (Fig. 1A and B; Fig. 2A and B). All plants inoculated with the wild-type BNYVV RNA1+ BNYVV-RNA2 (BN1BN2) without the *pds* or *chlH* insert showed only typical systemic BNYVV symptoms in form of a chlorosis and necrotic yellow veins (Fig. 1C). Similarly, plants inoculated with the wild-type BSBMV RNA1+ BSBMV-RNA2 (BS1BS2) displayed mosaic patterns and yellow blotches (Fig. 2C) without a PDS or ChlH silencing phenotype. In contrast, all plants inoculated with BNYVV/BSBMV as tools for VIGS showed a typical PDS or ChlH silencing phenotype. First symptoms of photobleaching caused by BNYVV RNA1+ BNYVV 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 apparent 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 color 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 color 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).

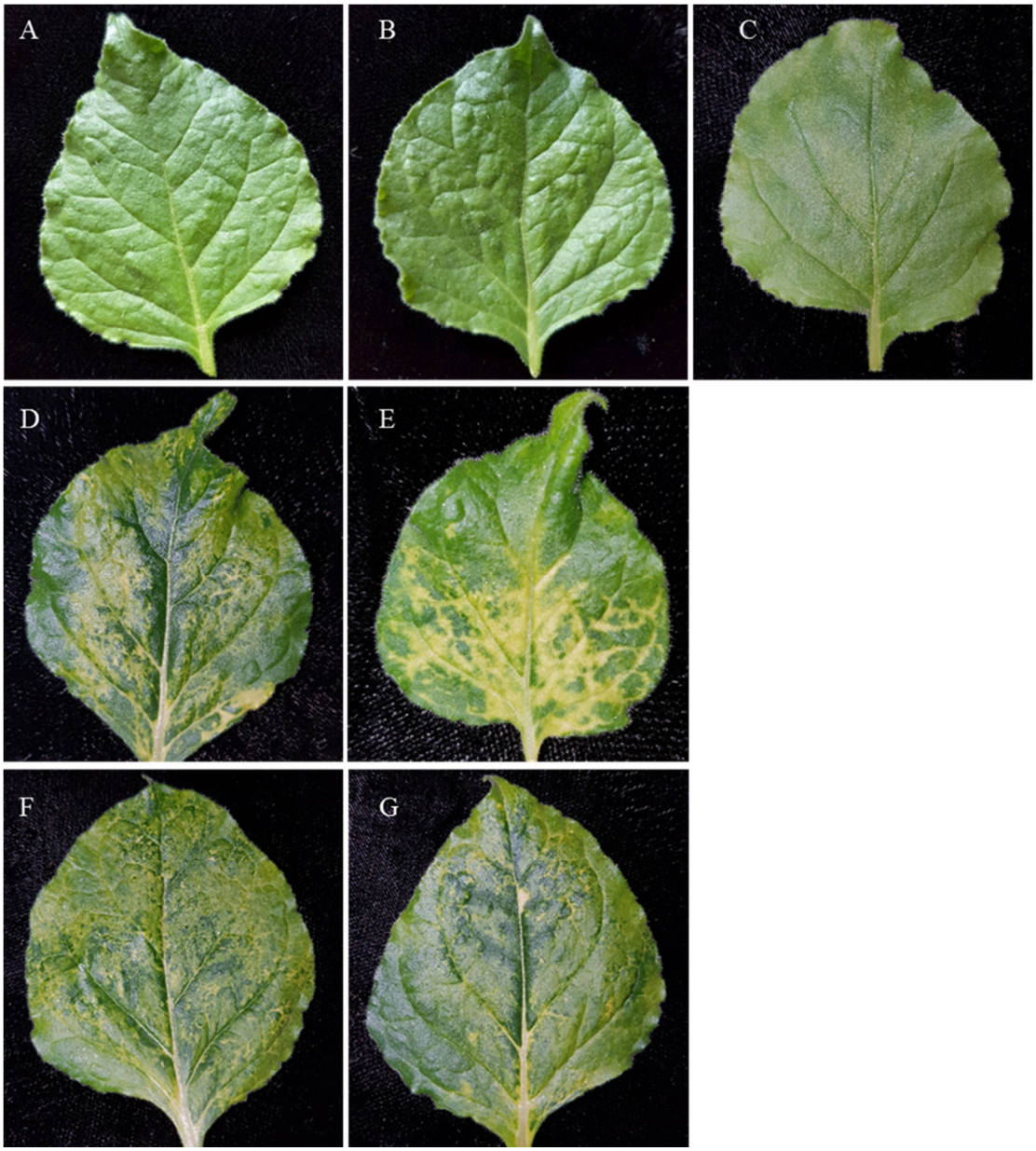


Fig. 1 Silencing of endogenous *phytoene desaturase* (*pds*) and *magnesium chelatase H subunit* (*chlH*) genes in *Nicotiana benthamiana* after agroinfiltration with different VIGS-vectors from BNYVV (31dpi). A) Healthy *N. benthamiana* plants. B) Plants inoculated only with infiltration buffer containing 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. F) An intensive white/yellow photobleaching occurring at 31 dpi in upper non-inoculated leaves of plants infected with BN1BN2-ChlH-s and G) with BN1BN2-ChlH-as.

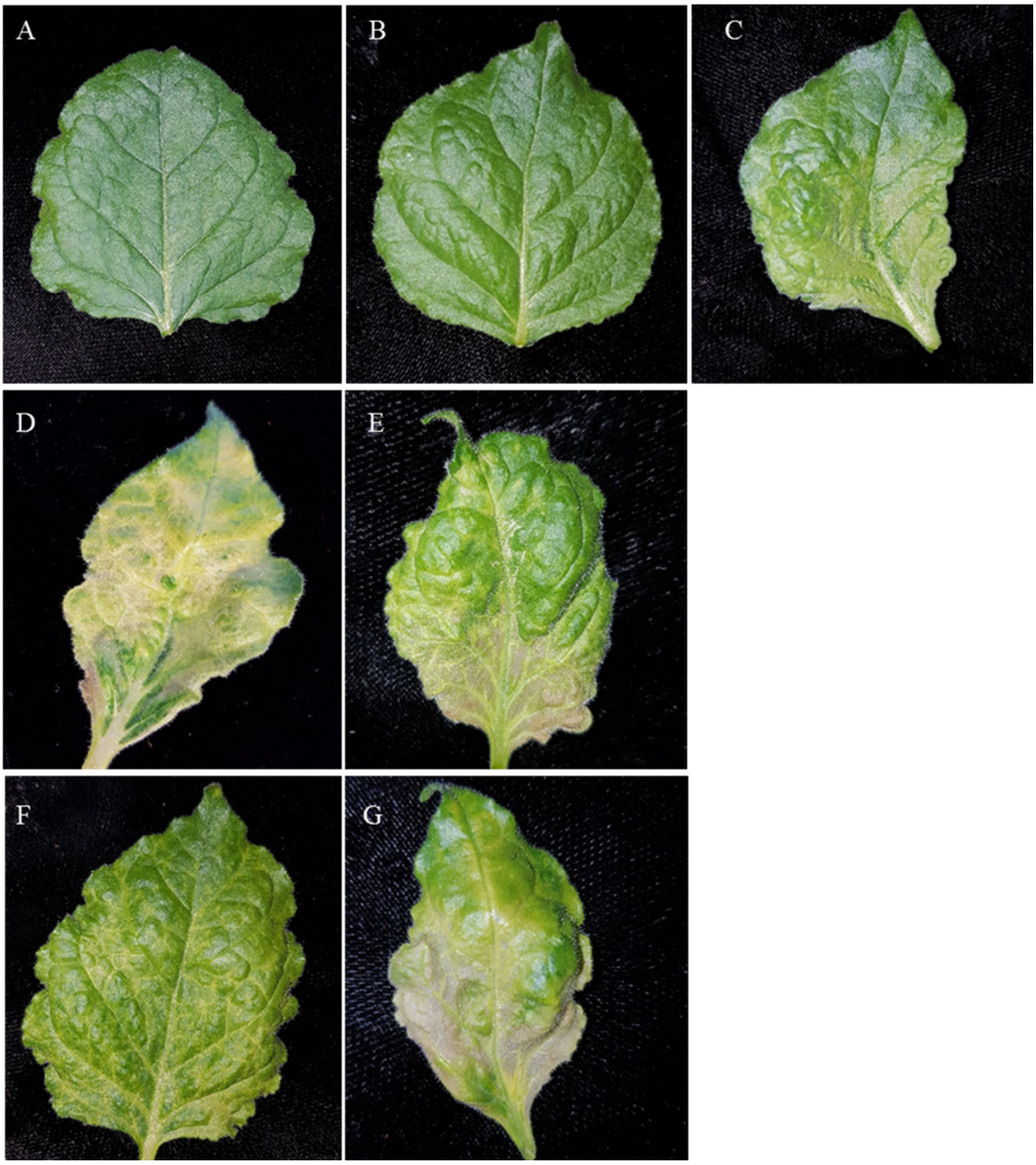


Fig. 2 Silencing of endogenous *phytoene desaturase* (*pds*) and *magnesium chelatase H subunit* (*chlH*) genes in *N. benthamiana* after agroinfiltration with different VIGS-vectors from BSBMV (31dpi). A) Healthy *N.benthamiana* plants. B) Plants inoculated only with infiltration buffer containing only *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. F) An intensive white/yellow photobleaching occurring at 31 dpi in upper non-inoculated leaves of plants infected with BS1BS2-ChlH-s and G) with BS1BS2-ChlH-as.

In the next step, the silencing effect on transcript levels of *chlH* and *pds* was analyzed 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 synthesized 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 normalization (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 *pds* gene expression was reduced by 7% and *chlH* gene 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*, there were no significant differences between sense (85%) and antisense (86%) constructs (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).

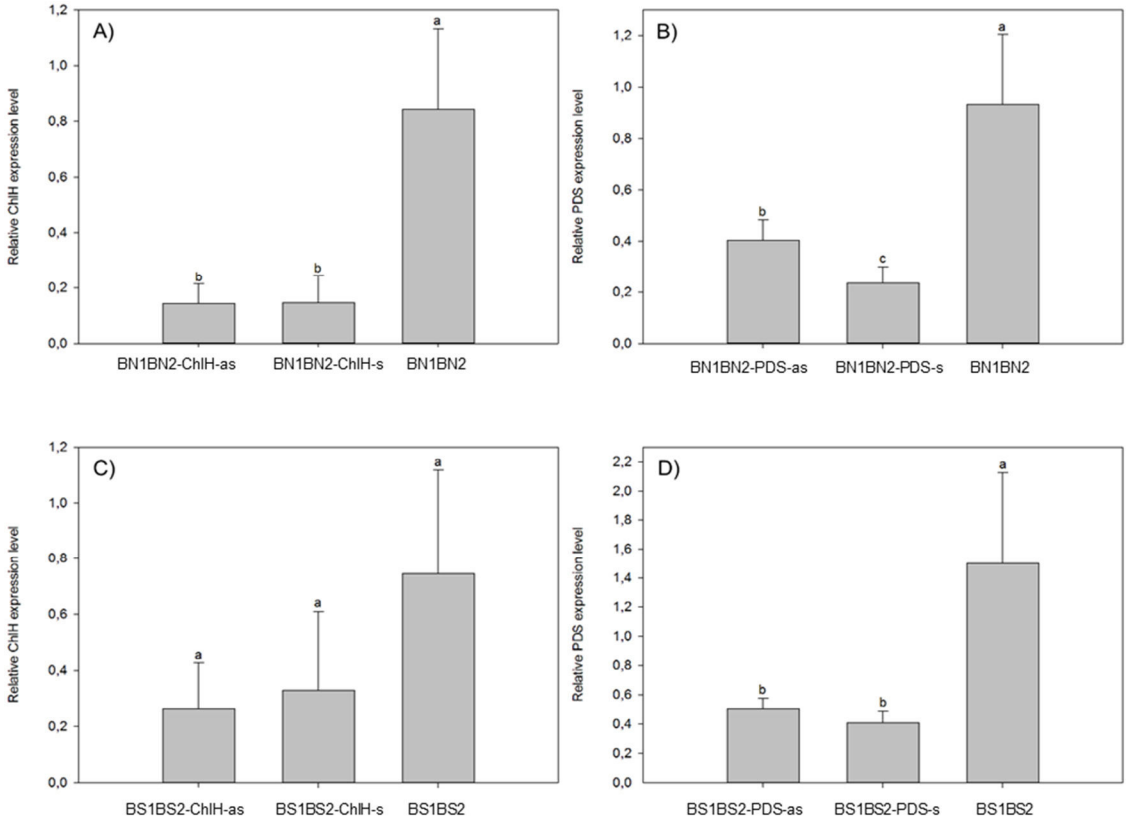


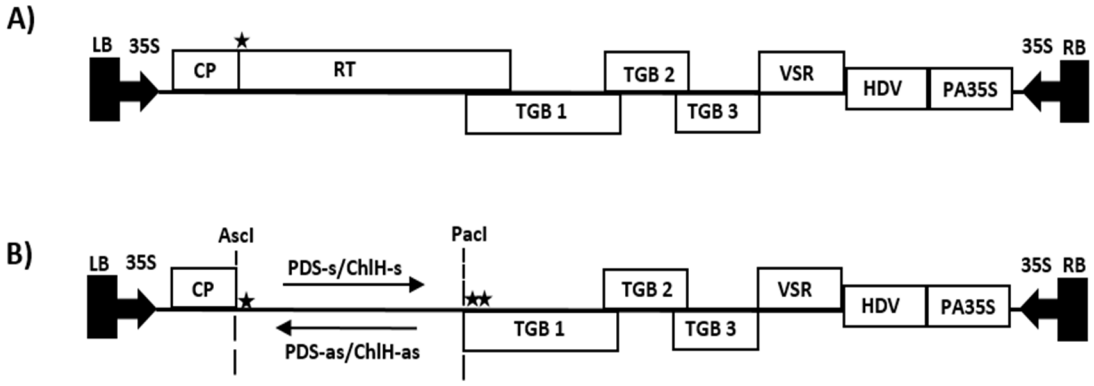
Fig. 3 *Magnesium chelatase* (*chlH*) and *phytoene desaturase* (*pds*) expression levels for different silencing constructs of BNYVV (A and B) and BSBMV (C and D). Fold change values are mean values of five *N. benthamiana* plants calculated relative to the mock-inoculated plants. Error bars indicate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

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). This corroborates the presumption that the initiator of PTGS is the dsRNA represented by a replicative intermediate of the viral RNA

(Montgomery et al., 1998; Morel and Vaucheret, 2000; Waterhouse et al., 1998). However, many approaches have been proposed to insert a target gene into a VIGS-vector. Among other, inserting a fragment of the *pds* gene between the large coat protein (L-CP) and movement protein (MV) 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, 2011b). 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 our best knowledge, BNYVV and BSBMV are the first *Benyviruses* modified for efficient VIGS. These VIGS-constructs can be utilized as a simple tool to determining the gene function in reverse genetics analyses. Moreover, the VIGS-systems should be optimized to be suitable in their natural hosts *B. vulgaris* and *B. macrocarpa.*

4.2. Supporting information



Supplementary Fig. 1 Schematic representation of BNYVV/BSBMV-RNA2 cDNA infectious clones and genomic modifications. **A**, Organization 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.

Supplementary Table 1. List of plasmids and VIGS constructs

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-ChlH-s	ChlH-s (549 bp)	BSBMV	BS2-ChlH-s
pDIVA:BSBMV-RNA2-CP-ChlH-as	ChlH-as (549 bp)	BSBMV	BS2-ChlH-as

5. GENERAL DISCUSSION

Infectious full-length cDNA clones of plant viruses provide valuable knowledge about the replication cycle of viruses, viral functionality and pathogenicity in diverse host plants using *in vitro* mutagenesis (insertions, substitutions and deletions) or by labeling with various reporter genes for replication and expression studies. In addition, infectious full-length clones are useful in the exploration of the genesis of satellite RNA, mechanisms of defective interfering RNA, as well as of induced or natural recombination (Nagyová and Šubr, 2007). Last, but not least, the infectious clones are considered as essential source for the investigation and analysis of antiviral strategies, as well as a raw material for generation and development of the new viral vectors (Bujarski and Miller, 1992). In this work, infectious full-length cDNA clones of BNYVV (A-type) and BSBMV under control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) in a binary vector (pDIVA) for rhizoinfection have been successfully constructed (Manuskript 1). For this purpose we have used Gibson Assembly (GA) as a single step *in vitro* recombination technique (Gibson et al., 2009). GA provides a fast, flexible and reliable alternative to the other conventional DNA cloning procedures. Firstly for the construction of an infectious cDNA full-length clone, GA was applied for a *Potyvirus* genome (Bordat et al., 2015) as well as of *Tomato blistering mosaic virus* (ToBMV) (Blawid and Nagata, 2015). In this study, the GA strategy has been applied to the generation of infectious cDNA full-length clones of multipartite plant RNA viruses (BNYVV and BSBMV). Furthermore, the newly produced infectious full-length clones were tested for their viral functions like replication, local or systemic propagation, and ability to form viral particles on different plants such as *N. benthamiana*, *B. vulgaris*, *B. macrocarpa* and *C. quinoa*. In comparison with the wild-type viruses, the prepared infectious cDNA clones of both BNYVV and BSBMV induce similar symptoms on the tested plants, which indicate their ability for replication, encapsidation and movement.

In order to transfect plants with cDNA full-length clones of BNYVV or BSBMV, several possibilities were investigated in this study. Especially, rhizoinoculation was used as a fast and uncomplicated method for *N. benthamiana* (Kang et al., 2015; Nagyová and Subr, 2007). On the other hand, our study demonstrated that the usage of this method can be restricted, since rhizoinoculation of *B. vulgaris* roots and *C. quinoa* leaves did not lead to an infection and any systemic symptoms. Therefore, the mechanical root inoculation

(vortex-inoculation) with sap from rhizoinfected *B. macrocarpa* plants was required to produce infections of *B. vulgaris* root system, since the other methods of leaf inoculation, such as mechanical inoculation (Chiba et al., 2008) or rhizoinoculation (Manuscript1) did not cause systemic infection. However, previous attempts to transform *B. vulgaris* using *Rhizobium* in both transient and stable transformation systems were only of limited success (Chiba et al., 2008; Hisano et al., 2004; Yang et al., 2005). Various factors might be the reason for this phenomenon. In contrast to other dicotyledonous plants, sugar beet is especially recalcitrant to transformation needing a laborious, costly and time consuming procedure to recieve an acceptable number of plants that are successfully transformed and regenerated (Kifle et al., 1999; Yang et al., 2005). Thus, the recalcitrance of transformed sugar beet plants may be caused by a small number of morphogenic cells and perhaps also by a limited access to such cells if they are surrounded by high numbers of non-morphogenic cells (Joersbo, 2007). In addition, further studies demonstrated, that beside a general lower ability of *Rhizobium* to transform root cells, also the number of bacterial cells infiltrated in the test plants play a key role in the efficiency of transformation; a low concentrations of *Rhizobium* cells cause a reduction in the frequency of T-DNA transfer, whereas an excessive number can lead to stress and the inoculated plant cells have a reduced regeneration ability (Costa et al., 2002; Grevelding et al., 1993). Strongly dependent upon genotype of plants, both the regeneration rate and transformation frequency of T-DNA after rhizo-mediated transformation of sugar beet cells are very low (Fry et al., 1991; Jacq et al., 1993; Kifle et al., 1999). Alternatively, the silencing level in leaves of some experimental plants (e.g. *N. benthamiana* and *A. thaliana*) is higher than those in root cells, which especially increase the ability of some soil-borne viruses to initiate the infection with high efficiency in roots than leaves (Andika et al., 2016). For example, BNYVV suppress RNA silencing in roots of *N. benthamiana* more effectively than leaves, which contributes to a high BNYVV RNA genome and lower siRNA accumulation in root cells compared to leaf cells (Andika et al., 2016; Andika et al., 2005). In addition, BNYVV p31 display high efficiency RNA silencing suppression in roots compared to leaves that leads to a successful transmission of virus into roots by *P. betae* (Andika et al., 2016; Chiba et al., 2013; Rahim et al., 2007; Zhang et al., 2005). Unlike *R. radiobacter*, *A. rhizogenes* transformation of sugar beet as an alternative method leads to hairy root cultures (Hamill et al., 1987). To overcome this issue, disarmed *A. rhizogenes* have been used to successfully transform sugar beet roots (Ehlers et al., 1991; Mankin et al., 2007; Pavli and Skaracis, 2010). Alternatively, a novel

method of rhizoinoculation, called ‘agrodrench’ may be used to transform very young seedlings of sugar beet where the leaf inoculation method is not possible (Ryu et al., 2004).

The modified full-length cDNA clones of BNYVV/BSBMV have been applied for the generation of VIGS-constructs suitable to produce RNA silencing in different experimental plants (Manuscript 3). In order to achieve this aim, BNYVV-/BSBMV-RNA2 was equipped with fragments of the *pds* (578 bp) and the *chlH* (549 bp) genes in sense and in antisense orientation. Rhizoinfection of *N. benthamiana* with BNYVV or BSBMV constructs containing *chlH or pds* genes in different orientations produced an obvious photobleaching phenotype and a reduction in the mRNA accumulation of *pds* and *chlH* genes. Many previous studies already described the use of *pds* and *chlH* as marker genes in silencing experiments in the background of different viruses, like *Tobacco rattle virus* (TRV), *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) (Guo et al., 2016; Lange et al., 2013; Ma et al., 2015; Thomas et al., 2001; Yuan et al., 2011). In this study, the visual silencing phenotypes (yellow/white color) caused by suppression of *chlH* and *pds* gene expression in *N. benthamiana* after inoculation with different PDS or ChlH VIGS-constructs seem to be similar to those observed in the preceding studies (Liu et al., 2016; Voinnet, 2001; Zhao et al., 2016). Both PDS and ChlH-constructs were not able to produce photobleaching on the whole infected plant. The silencing effect appears mainly on new and middle non-inoculated leaves causing yellowing on 60-70% of the leaf's area. Generally, symptoms induced by VIGS in many experimental plants show a patchy distribution, probably because parts of the plant tissue remain not silenced at all or only poorly silenced, due to a limited spread of the virus or incomplete distribution of the silencing signal (Dong et al., 2007; Mustafa et al., 2015; Rotenberg et al., 2006; Unver and Budak, 2009).

The obtained results were verified using the qRT-PCR technique. The conducted qRT-PCR demonstrated a significant lower mRNA expression level of *pds* or *chlH* genes in most of the tested plants infiltrated with BNYVV-/BSBMV-RNA1+RNA2 equipped with the target genes in comparission with healthy non-infected or only with BNYVV/BSBMV (wild-type) infected plants. However, one exceptional case was observed with the BSBMV-ChlH construct. This construct displays a visually clear photobleaching phenotyp in the target plants, however, no significant difference in *chlH* expression level could be confirmed with qRT-PCR compared to control plants infected

only with BSBMV (wild-type). Nevertheless, we might bypass this by increasing the number of plants per test to convert the trend into a statistically significant result.

In this work, we could not determine any visual differences in the silencing phenotype or any significant differences in mRNA expression level measured by qRT-PCR between antisense and sense PDS- or ChlH-constructs. This points out that the development of ChlH- or PDS-VIGS is independent of the insert orientation in both BNYVV and BSBMV. Indeed, early studies demonstrated similar results in *N. benthaiana* and *N. tabacum* after infection with a PDS-construct in sense and antisense orintation in the background of TMV and PVX (Gosselé et al., 2002; Kumagai et al., 1995; Ruiz et al., 1998). Nevertheless, the BN2-PDS-s construct showed via qRT-PCR a higher silencing effect than the BN2-PDS-as construct. The reason for the BN2-PDS-as lower silencing efficacy in *N. benthamiana* encountered in the work presented here is unclear but might be due to a difference in silencing induction caused by differences in viral RNA accumulation that affect the mRNA accumulation of the target gene. In similar way, Ruiz et al. (1998) assumed a correlation between VIGS efficiency of GFP and the reduction in PVX genomic RNA accumulation in *N. benthamiana*-16c plants after infection with a PVX construct including a part of the GFP sequence. On the other hand, the correlation between the level of virus accumulation and VIGS efficiency in target plants might be dependent on the replication cycle of the virus itself as well as on the virus-host interaction (Ramegowda et al., 2014; Senthil-Kumar and Mysore, 2011b).

Both VIGS reporter genes used in this study (*pds* and *chlH)* display visually similar silencing phenotypes but have slightly different quantitative effects in the test plants. QRT-PCR results indicated that plants infiltrated with ChlH-constructs in the background of both viruses produced higher silencing levels than those infiltrated with PDS-constructs. It was previously shown that the 5` end of the *pds* gene coding region displayed little PDS silencing in *N. benthamiana*, whereas the middle to 3` end PDS part resulted in higher silencing effects (Igarashi et al., 2009; Zhang et al., 2010). This underlines an important role of the selected sequence part on the silencing efficacy (Bruun-Rasmussen et al., 2007; Mei et al., 2016; Pflieger et al., 2008). Studies corroborate that in many target plants RNAi has an inconstant effect, which could be due to siRNA molecules, which are not able to bind to the target mRNA, since the target region is occupied with a protein or is not reachable due to secondary structures (Ogita et al., 2004; Ossowski et al., 2008; Tomari and Zamore, 2005). Furthermore, the silencing efficiency

might be also influenced by the viral suppressors of RNA silencing (VSR) of BNYVV and BSBMV (p14) (Andika et al., 2012; Andika et al., 2016; Zhang et al., 2005). Chiba et al. (2013) postulated that the VSR is involved in the downregulation of the DICER activity and the primary siRNA creation, probably because of an intervention with AGO-siRNA loading, which produces a mild silencing suppression.

This work has established efficient BNYVV/BSBMV based VIGS systems that can be used successfully to silence endogenous genes in *N. benthamiana* within 21 days. Additionally, these VIGS-systems may serve as a simple tool for determining the functions of many candidate genes in reverse genetics analyses. To our knowledge, BNYVV and BSBMV are the first *Benyviruses* modified to be used as VIGS-vectors. Furthermore, the VIGS-systems could be optimized to be applicable in their natural hosts *B. vulgaris*.

As a further step in the optimization of the silencing concept, the infectious full-length cDNA clones of both viruses have been used to produce a number of BNYVV/BSBMV RNA 1+2 reassortants in *N. benthamiana* (data not shown). In this study we indicated that both *in vitro* reassortants were viable and capable of viral systemic infection in the target plants. Remarkably, the plants infiltrated with both reassortants showed a difference in symptom severity, where symptoms occurred later compared to the wild-type (BNYVV/BSBMV) combinations. Interestingly, BSBMV-RNA1 with BNYVV-RNA2 A-type produces very mild symptoms on the experimental host *N. benthamiana*, which may allow for a better and quicker recognition of silencing phenotypes. According to Ratti and colleagues (2009) RNA3 of BSBMV is supported by BNYVV in terms of viral replication and systemic movement. Our BNYVV/BSBMV reassortants analyses gave a clear evidence for the important role of genome segments in symptoms expression and development in *N. benthamiana*, which seem to be mostly regulated by the RNA2 segment or by proteins encoded thereof. However, wild-types of both viruses display species-specific symptoms on different host and test plants (e.g. *B. vulgaris*, *B. macrocarpa* and *N. benthamiana*). We could also confirm that RNA1+2 of BSBMY and BNYVV (A-type) derived from the generated cDNA clones are sufficient to initiate systemic movement and propagation throughout different tissues in *N. benthamiana* (Chiba et al., 2013; Rahim et al., 2007). This supports the functional similarities of RNA1 and RNA2 for BNYVV (A-type) and BSBMV. Sequence and genomic homologies between BSBMV and BNYVV hypothesize the possibility that both viruses evolved from

a common ancestral virus species. Conversely, this study and other reports demonstrated that both BNYVV and BSBMV RNA3 is involved in long distance movement in *Beta* species for both wild-type and reassortants (Lauber et al., 1998; Peltier et al., 2012; Ratti et al., 2009).

In addition to the developed VIGS-vectors based on RNA2 of BNYVV and BSBMV, this study further used full-length clones of both viruses for fluorescent labeling. The labeled viruses were investigated to study the interaction between both viruses in super-infection and co-infection experiments in different host plants. Firstly, the labeled BSBMV and BNYVV constructs were achieved by almost a complete replacement of the RT domain on RNA2 with various reporter genes. Here, many clones with the green fluorescent protein (GFPuv; GFPuv^{A206K} and smRSGFP) or the monomeric red fluorescent protein (mRFP) based on RNA2 of BNYVV or BSBMV were successfully generated. The labeled BNYVV and BSBMV clones were then inoculated with *R. radiobacter* into different host plants such as *B. macrocarpa* and *N. benthamiana*. The inoculated plants displayed typical but delayed BNYVV or BSBMV disease symptoms after 23 and 30 dpi, respectively. This indicates that the labeled clones of both BNYVV and BSBMV are infectious and able to move systemically through the phloem in different host plants. These results demonstrate that p75 minor coat protein is not required for efficient virus assembly. This might be explained by the mutated RT proteins with deletions in different positions in the C- or N-terminal parts interfering with particle formation, whereas that can not take place when the whole RTD is deleted. Nevertheless, it has been shown in previous studies that p75 is needed for viral particle formation and successful transmission by the fungal vector *P. betae* (Haeberle et al., 1994; Schmitt et al., 1992; Tamada and Kusume, 1991; Tamada et al., 1996). Particularly, it has been assumed that the RT domain of p75 consists of two different subdomains: the N-terminal half contains sequences implicated in virion assembly, while the C-terminal portion carries sequences involved in interactions with the fungal vector (Adams et al., 2001; Tamada and Kusume, 1991; Tamada et al., 1996). Remarkably, any type of mutations in the N-terminal part of p75 can adversely affect or inhibit production of viral particles (Schmitt et al., 1992; Tamada et al., 1996). Many informations are still missing about the exact role of p75 in virus assembly, however, the most plausible probability is that the protein intervenes in the assembly initiation (Schmitt et al., 1992; Tamada et al., 1996). In this study, we could confirm a successful encapsidation of viral RNA by the presence of BNYVV or BSBMV characteristic rod shaped virus particles in systemically infected leaves of *N. benthamiana*

via electron microscopy. In addition, these results showed that the fluorescent labeling of BNYVV or BSBMV with different reporter genes (*gfp*uv; *gfp*uv ^{A206K}; smRS-*gfp* and *mrfp*) have no impact on viral particle diameter and did not interfere with symptom development and systemic movement in two different host plants.

Remarkably, the fluorescence of smRS-GFP and GFPuv expressed by BSBMV and BNYVV was unequally distributed in spherical shaped clusters whereas mRFP displayed a strong fluorescence signal evenly distributed throughout the cytoplasm. Several observations proposed that GFP is monomeric and homogeneous at low concentrations, while it tends to dimerization at high protein expression level, which might cause a kind of artefacts in microscopy experiments (von Stetten et al., 2012; Yang, 1997). To ensure a truly monomeric state of GFPuv, the alanine to lysine mutation at position 206 (A206K) has been introduced into BSBMV-GFPuv^{A206K} and BNYVV-GFPuv^{A206K}, with marginal effect on the spectroscopic properties. The mutated GFPuv^{A206K} was visualised by a homogeneous and well distributed green fluorescence signal, however, many spherical shaped clusters remained. This might be explained probably by virus particles addressed to the cytosolic surfaces of mitochondria, since these places are known as sites of virion assembly (Erhardt et al., 2000; Valentin et al., 2005). Furthermore, plants infected with GFPuv and smRSGFP in the background of BNYVV or BSBMV showed a delay in symptom development compared to plants infected only with wild-type viruses. This might be explained by the GFPuv- and smRSGFP-CP fusion protein developed in this study that presumably interfered with virus encapsidation.

Additionally, co-infection and super-infection experiments based on recently constructed fluorescent labeled clones of BSBMV and BNYVV were performed in *N. benthamiana*. The results from co-infection experiments presented in this work showed that double infections of identical, but differently labeled viruses of BSBMV and BNYVV revealed a discrete colonisation and separate fluorescent clusters. These discrete clusters with different fluorescence (red and green) were detected in both primary and systemically infected leaf tissue in co-inoculated plants independently of the inoculation method (particle bombardment or rhizoinoculation). CLSM images of mesophyll cells confirmed that both fluorescence signals were recognizable only in a few number of cells at the border region of two adjacent, different fluorescent cell clusters. Taken together, these results indicate that both viral populations can principally co-replicate simultaneously within the same cell. Several studies reported a similar spatial separation effect in infected

leaves with identical but different labeled *potyviruses* (monopartite viruses) or with *cheraviruses* (bipartite viruses) (Dietrich and Maiss, 2003; Takahashi et al., 2007). In the same manner, a reassortant of double infections including BNYVV-RNA1, BNYVV-smRSGFP and BSBMV-RNA2-mRFP displayed an obvious spatial separation pattern. This means that RNA1 does not play a key role to induce spatial separation of BNYVV and BSBMV. On the other hand, double infections of different virus populations (e.g. BSBMV with two unrelated viruses TRV and PVX) pointed out that both viruses were able to replicate and distribute simultaneously in the same infected leaf tissue. Hence, our data are consistent with several prior reports, which demonstrated that distantly related viruses of different families show clear co-infected cells whereas closely related viruses belonging to the same genus or family remain spatially distributed (Aaziz and Tepfer, 1999; Dietrich and Maiss, 2003; Masuta et al., 1998; Takahashi et al., 2007). Taken together, such a behaviour of populations of identical virus isolates and strains seems to be a widely distributed common phenomenon, however, the molecular basis of this mechanism is still not clear and only poorly understood.

Our data show that BNYVV and BSBMV act in super-infection experiments in the same manner as in the co-infection experiments. A preexisting viral infection of BNYVV-GFPuv prevents or interferes with the establishment of a secondary infection with the same or a closely related strain (BNYVV-mRFP and BSBMV-mRFP) in *N. benthamiana* plants. In other words, when infected with BNYVV-GFPuv (protecting virus), the test plant becomes resistant to super-infection with BNYVV-mRFP and BSBMV-mRFP (challenging viruses), or disease symptoms induced by the challenging virus are suppressed. However, secondary infection by distantly related viruses (TRV and PVX) can be mostly unaffected. Indeed, previous studies demonstrated similar results in *N. benthamiana* plants and suggested several mechanisms to explain this phenomenon (Lecoq and Raccah, 2001; Syller, 2012; Ziebell and Carr, 2010). These include, the disassembly of the challenging virus, which could be prevented by expression of the CP of the protecting virus (Abel et al., 1986; Bendahmane and Beachy, 1999; Folimonova, 2012). Recently, it was shown that TriMV- and WSMV-encoded NIa-Pro and CP proteins are super-infection exclusion effectors (Tatineni and French, 2016). However, the induction of RNA silencing by the protecting virus is the most rational explanation of this phenomenon, which probably causes a sequence-specific degradation of the challenging virus RNA (Fagoaga et al., 2006; Ratcliff et al., 1999). In addition, precursor exhaustion has also been suggested as a mechanism that may explain this phenomenon. Here it is

hypothesized that the protecting virus using some cell components that were needed by a closely related virus or by occupying of some cellular replication sites that are shared by related viruses but are not required for distantly related viruses (Ziebell and Carr, 2010). Further work is required to better elucidate if super-and co-infection exclusion of *Benyviruses* shares identical properties with earlier observed mechanisms.

In conclusion, this study contributed to improve our knowledge about BNYVV and BSBMV biological features such as host-pathogen interactions, symptom induction, behavior in mixed infections and tissue colonisation strategies. For this purpose, we constructed infectious full-length cDNA clones of an European A type BNYVV (Yu2) and a Californian BSBMV isolate using the GA single step *in vitro* recombination technique. Furthermore, VIGS-constructs based on the newly generated BNYVV/BSBMV clones have been successfully developed. The results presented in this study demonstrated that RNA2 of BNYVV or BSBMV are suitable as tools for VIGS together with BNYVV-/BSBMV-RNA1 in *N. benthamiana*. However, so far these constructs have not been able to produce silencing effects on *B. vulgaris*.

Assuming the VIGS vectors can be adapted for silencing in sugar beet, the exact role and function of various disease resistance genes present in the sugar beet gene pool as well as in the wild-type *Beta* species could be verified. Furthermore, it could be applied to characterize metabolic pathways in sugar beet. Last, but not least VIGS-constructs might be used to uncover host genes associated with virus replication and movement in sugar beet plants.

This work additionally delivered several BNYVV and BSBMV vectors labeled with different fluorescent reporter genes (*mrfp*, smRS-*gfp* and *gfp*uv), which can be helpful for further characterization of the interaction between both viruses in co- and super-infection experiments. Multipartite benyviruses BSBMV and BNYVV tend to be spatially separated during plant colonisation whereas distant related viruses show clear co-infected cells in co- and super-infection experiments. Finally, further work will be needed to further investigate the colonisation strategies in natural mixed infections and the suitability of BNYVV/BSBMV based VIGS-constructs in their natural host sugar beet.

6. REFERENCES

Aaziz, R., and Tepfer, M. (1999). Recombination between genomic RNAs of two cucumoviruses under conditions of minimal selection pressure. *Virology* **263**, 282-289.

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-744.

Acosta-Leal, R., Fawley, M. W., and Rush, C. M. (2008). Changes in the intraisolate genetic structure of Beet necrotic yellow vein virus populations associated with plant resistance breakdown. *Virology* **376**, 60-68.

Adams, M. J., Antoniw, J. F., and Mullins, J. G. (2001). Plant virus transmission by plasmodiophorid fungi is associated with distinctive transmembrane regions of virus-encoded proteins. *Arch Virol* **146**, 1139-53.

Adenot, X., Elmayan, T., Lauressergues, D., Boutet, S., Bouché, N., Gasciolli, V., and Vaucheret, H. (2006). DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Current Biology* **16**, 927-932.

Agrawal, N., Dasaradhi, P., Mohmmed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003). RNA interference: biology, mechanism, and applications. *Microbiology and molecular biology reviews* **67**, 657-685.

Ajayi, A. M., Tanayen, J. K., Magomere, A., and Ezeonwumelu, J. O. (2017). Antinociceptive and anti-inflammatory effects of aqueous extract of Chenopodium opulifolium schrad leaves. *Journal of intercultural ethnopharmacology* **6**, 14.

Akbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., Zhang, P., Gruissem, W., Meins, F., and Hohn, T. (2006). Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic acids research* **34**, 462-471.

Allen, E., Xie, Z., Gustafson, A. M., and Carrington, J. C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**, 207-221.

Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* **113**, 673-676.

Andika, I. B., Kondo, H., Nishiguchi, M., and Tamada, T. (2012). The cysteine-rich proteins of Beet necrotic yellow vein virus and Tobacco rattle virus contribute to efficient suppression of silencing in roots. *Journal of General Virology* **93**, 1841-1850.

Andika, I. B., Kondo, H., and Sun, L. (2016). Interplays between Soil-Borne Plant Viruses and RNA Silencing-Mediated Antiviral Defense in Roots. *Frontiers in Microbiology* **7**.

Andika, I. B., Kondo, H., and Tamada, T. (2005). Evidence that RNA silencing-mediated resistance to Beet necrotic yellow vein virus is less effective in roots than in leaves. *Molecular plant-microbe interactions* **18**, 194-204.

Angell, S. M., and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *The EMBO Journal* **16**, 3675-3684.

Asher, M. (1993). Rhizomania. *In* "The Sugar Beet Crop", pp. 311-346. Springer.

Axtell, M. J. (2013). Classification and comparison of small RNAs from plants. *Annual review of plant biology* **64**, 137-159.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M. A., and Lagrange, T. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes & Development* **24**, 904-915.

Balmori, E., Gilmer, D., Richards, K., Guilley, H., and Jonard, G. (1993). Mapping the promoter for subgenomic RNA synthesis on beet necrotic yellow vein virus RNA 3. *Biochimie* **75**, 517-521.

Bance, V. B. (1991). Replication of potato virus X RNA is altered in coinfections with potato virus Y. *Virology* **182**, 486-494.

Bartley, G. E., and Scolnik, P. A. (1995). Plant carotenoids: pigments for photoprotection, visual attraction, and human health. *Plant Cell* **7**, 1027-38.

Barzen, E., Stahl, R., Fuchs, E., Borchardt, D. C., and Salamini, F. (1997). Development of coupling-repulsion-phase SCAR markers diagnostic for the sugar beet Rr1 allele conferring resistance to rhizomania. *Molecular Breeding* **3**, 231-238.

Baulcombe, D. (2004). RNA silencing in plants. *Nature* **431**, 356-363.

Baulcombe, D. C., Chapman, S., and Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. *The Plant Journal* **7**, 1045-1053.

Baulcombe, D. C. (1999). Fast forward genetics based on virus-induced gene silencing. *Current opinion in plant biology* **2**, 109-113.

Baulcombe, D. C. (2015). VIGS, HIGS and FIGS: small RNA silencing in the interactions of viruses or filamentous organisms with their plant hosts. *Current Opinion in Plant Biology* **26**, 141-146.

Baumberger, N., and Baulcombe, D. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11928-11933.

Bendahmane, M., and Beachy, R. N. (1999). Control of tobamovirus infections via pathogen-derived resistance. *Advances in virus research* **53**, 369-386.

Benedito, V. A., Visser, P. B., Angenent, G. C., and Krens, F. A. (2004). The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. *Genet Mol Res* **3**, 323-341.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Beyene, G., Chauhan, R. D., and Taylor, N. J. (2017). A rapid virus-induced gene silencing (VIGS) method for assessing resistance and susceptibility to cassava mosaic disease. *Virology journal* **14**, 47.

Biancardi, E., Lewellen, R. T., De Biaggi, M., Erichsen, A. W., and Stevanato, P. (2002). The origin of rhizomania resistance in sugar beet. *Euphytica* **127**, 383-397.

Blawid, R., and Nagata, T. (2015). Construction of an infectious clone of a plant RNA virus in a binary vector using one-step Gibson Assembly. *Journal of virological methods* **222**, 11-15.

Bleykasten, C., Gilmer, D., Guilley, H., Richards, K., and Jonard, G. (1996). Beet necrotic yellow vein virus 42 kDa triple gene block protein binds nucleic acid in vitro. *Journal of General virology* **77**, 889-897.

Bologna, N. G., and Voinnet, O. (2014). The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. *Annual review of plant biology* **65**, 473-503.

Bordat, A., Houvenaghel, M.-C., and German-Retana, S. (2015). Gibson assembly: an easy way to clone potyviral full-length infectious cDNA clones expressing an ectopic VPg. *Virology journal* **12**, 89.

Borges, F., and Martienssen, R. A. (2015). The expanding world of small RNAs in plants. *Nature Reviews Molecular Cell Biology*.

Bornemann, K., Hanse, B., Varrelmann, M., and Stevens, M. (2015). Occurrence of resistance-breaking strains of beet necrotic yellow vein virus in sugar beet in northwestern Europe and identification of a new variant of the viral pathogenicity factor P25. *Plant pathology* **64**, 25-34.

Bornemann, K., and Varrelmann, M. (2011). Analysis of the resistance-breaking ability of different Beet necrotic yellow vein virus isolates loaded into a single Polymyxa betae population in soil. *Phytopathology* **101**, 718-724.

Bornemann, K., and Varrelmann, M. (2013). Effect of sugar beet genotype on the Beet necrotic yellow vein virus P25 pathogenicity factor and evidence for a fitness penalty in resistance-breaking strains. *Mol Plant Pathol* **14**, 356-64.

Bouzoubaa, S., Guilley, H., Jonard, G., Richards, K., and Putz, C. (1985). Nucleotide sequence analysis of RNA-3 and RNA-4 of beet necrotic yellow vein virus, isolates F2 and G1. *Journal of General Virology* **66**, 1553-1564.

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G. t., and Richards, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *Journal of General Virology* **68**, 615-626.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K., and Jonard, G. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. *Journal of General Virology* **67**, 1689-1700.

Boyer, J.-C., and Haenni, A.-L. (1994). Infectious transcripts and cDNA clones of RNA viruses. *Virology* **198**, 415-426.

Brigneti, G., Martín-Hernández, A. M., Jin, H., Chen, J., Baulcombe, D. C., Baker, B., and Jones, J. D. (2004). Virus-induced gene silencing in Solanum species. *The Plant Journal* **39**, 264-272.

Brodersen, P., and Voinnet, O. (2006). The diversity of RNA silencing pathways in plants. *Trends Genet* **22**, 268-80.

Bruun-Rasmussen, M., Madsen, C. T., Jessing, S., and Albrechtsen, M. (2007). Stability of Barley stripe mosaic virus-induced gene silencing in barley. *Molecular Plant-Microbe Interactions* **20**, 1323-1331.

Bujarski, J. J., and Miller, W. A. (1992). Use of in vitro transcription to study gene expression and replication of spherical, positive sense RNA plant viruses.

Burch-Smith, T. M., Anderson, J. C., Martin, G. B., and Dinesh-Kumar, S. P. (2004). Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J* **39**, 734-46.

Burgyán, J., and Havelda, Z. (2011). Viral suppressors of RNA silencing. *Trends in Plant Science* **16**, 265-272.

Cai, D., Kleine, M., Kifle, S., Harloff, H.-J., Sandal, N. N., Marcker, K. A., Klein-Lankhorst, R. M., Salentijn, E. M., Lange, W., and Stiekema, W. J. (1997). Positional cloning of a gene for nematode resistance in sugar beet. *Science* **275**, 832-834.

Campbell, L. G. (2002). Sugar beet quality improvement. *Journal of crop production* **5**, 395-413.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proceedings of the National Academy of Sciences* **99**, 7877-7882.

Canova, A. (1959). Appunti di patologia della barbabietola. *Informatore Fitopatologico* **9**, 390-396.

Canova, A. (1966). Si studia la rizomania della bietola. *Informatore fitopatologico* **10**, 235-239.

Carrillo-Tripp, J., Shimada-Beltrán, H., and Rivera-Bustamante, R. (2006). Use of geminiviral vectors for functional genomics. *Current opinion in plant biology* **9**, 209-215.

Castel, S. E., and Martienssen, R. A. (2013). RNA interference (RNAi) in the nucleus: roles for small RNA in transcription, epigenetics and beyond. *Nature Reviews. Genetics* **14**, 100.

Castelfranco, P. A., and Jones, O. T. (1975). Protoheme turnover and chlorophyll synthesis in greening barley tissue. *Plant Physiol* **55**, 485-90.

Chen, H.-M., Chen, L.-T., Patel, K., Li, Y.-H., Baulcombe, D. C., and Wu, S.-H. (2010). 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proceedings of the National Academy of Sciences* **107**, 15269-15274.

Chen, J.-C., Jiang, C.-Z., Gookin, T., Hunter, D., Clark, D., and Reid, M. (2004). Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. *Plant molecular biology* **55**, 521-530.

Chiba, S., Hleibieh, K., Delbianco, A., Klein, E., Ratti, C., Ziegler-Graff, V., Bouzoubaa, S., and Gilmer, D. (2013). The benyvirus RNA silencing suppressor is essential for long-distance movement, requires both zinc-finger and NoLS basic residues but not a nucleolar localization for its silencing-suppression activity. *Mol Plant Microbe Interact* **26**, 168-81.

Chiba, S., Kondo, H., Miyanishi, M., Andika, I. B., Han, C., and Tamada, T. (2011). The evolutionary history of Beet necrotic yellow vein virus deduced from genetic variation, geographical origin and spread, and the breaking of host resistance. *Molecular Plant-Microbe Interactions* **24**, 207-218.

Chiba, S., Miyanishi, M., Andika, I. B., Kondo, H., and Tamada, T. (2008). Identification of amino acids of the beet necrotic yellow vein virus p25 protein required for induction of the resistance response in leaves of Beta vulgaris plants. *Journal of General Virology* **89**, 1314-1323.

Cogoni, C., and Macino, G. (1997). Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa. *Proceedings of the National Academy of Sciences* **94**, 10233-10238.

Cogoni, C., and Macino, G. (2000). Post-transcriptional gene silencing across kingdoms. *Current opinion in genetics & development* **10**, 638-643.

Commandeur, U., Jarausch, W., Li, Y., Koenig, R., and Burgermeister, W. (1991). cDNAs of beet necrotic yellow vein virus RNAs 3 and 4 are rendered biologically active in a plasmid containing the cauliflower mosaic virus 35S promoter. *Virology* **185**, 493-495.

Costa, M., Otoni, W., and Moore, G. (2002). An evaluation of factors affecting the efficiency of Agrobacterium-mediated transformation of Citrus paradisi (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Reports* **21**, 365-373.

Cowan, G., Torrance, L., and Reavy, B. (1997). Detection of potato mop-top virus capsid readthrough protein in virus particles. *Journal of General Virology* **78**, 1779-1783.

Csorba, T., Kontra, L., and Burgyán, J. (2015). Viral silencing suppressors: tools forged to fine-tune host-pathogen coexistence. *Virology* **479**, 85-103.

Cuperus, J. T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R. T., Takeda, A., Sullivan, C. M., Gilbert, S. D., Montgomery, T. A., and Carrington, J. C. (2010). Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis. *Nature structural & molecular biology* **17**, 997-1003.

D'Alonzo, M., Delbianco, A., Lanzoni, C., Autonell, C. R., Gilmer, D., and Ratti, C. (2012). Beet soil-borne mosaic virus RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through Polymyxa betae. *Virology* **423**, 187-94.

Darissa, O., Willingmann, P., and Adam, G. (2010). Optimized approaches for the sequence determination of double-stranded RNA templates. *Journal of virological methods* **169**, 397-403.

Davis, S. J., and Vierstra, R. D. (1998). Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant molecular biology* **36**, 521-528.

De Biaggi, M. (1987). Methodes de selection–un cas concret. *Proc IIRB* **50**, 157-161.

Delbianco, A., Lanzoni, C., Klein, E., Rubies Autonell, C., Gilmer, D., and Ratti, C. (2013). Agroinoculation of beet necrotic yellow vein virus cDNA clones results in plant systemic infection and efficient Polymyxa betae transmission. *Molecular plant pathology* **14**, 422-428.

Dietrich, C., and Maiss, E. (2003). Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected Nicotiana benthamiana plants. *Journal of General Virology* **84**, 2871-2876.

Dietrich, C., and Maiss, E. (2002). Red fluorescent protein DsRed from Discosoma sp. as a reporter protein in higher plants. *Biotechniques* **32**, 286, 288-90, 292-3.

Ding, X. S., Schneider, W. L., Chaluvadi, S. R., Mian, M. R., and Nelson, R. S. (2006). Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. *Molecular Plant-Microbe Interactions* **19**, 1229-1239.

Dohm, J. C., Minoche, A. E., Holtgräwe, D., Capella-Gutiérrez, S., Zakrzewski, F., Tafer, H., Rupp, O., Sörensen, T. R., Stracke, R., and Reinhardt, R. (2014). The genome of the recently domesticated crop plant sugar beet (Beta vulgaris). *Nature* **505**, 546-549.

Dong, K., Wang, Y., Zhang, Z., Chai, L. X., Tong, X., Xu, J., Li, D., and Wang, X. B. (2016). Two amino acids near the N-terminus of Cucumber mosaic virus 2b play critical roles in the suppression of RNA silencing and viral infectivity. *Molecular plant pathology* **17**, 173-183.

Dong, Y., Burch-Smith, T. M., Liu, Y., Mamillapalli, P., and Dinesh-Kumar, S. P. (2007). A ligation-independent cloning tobacco rattle virus vector for high-throughput virus-induced gene silencing identifies roles for NbMADS4-1 and-2 in floral development. *Plant physiology* **145**, 1161-1170.

Douchkov, D., Nowara, D., Zierold, U., and Schweizer, P. (2005). A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Molecular Plant-Microbe Interactions* **18**, 755-761.

Draghici, H.-K., and Varrelmann, M. (2009). Evidence that the linker between the methyltransferase and helicase domains of potato virus X replicase is involved in homologous RNA recombination. *Journal of virology* **83**, 7761-7769.

Drake, J. W., and Holland, J. J. (1999). Mutation rates among RNA viruses. *Proceedings of the National Academy of Sciences* **96**, 13910-13913.

Draycott, A. P. (2008). "Sugar beet," John Wiley & Sons.

Du, Z., Chen, A., Chen, W., Liao, Q., Zhang, H., Bao, Y., Roossinck, M. J., and Carr, J. P. (2014). Nuclear-cytoplasmic partitioning of cucumber mosaic virus protein 2b determines the balance between its roles as a virulence determinant and an RNA-silencing suppressor. *Journal of virology* **88**, 5228-5241.

Duke, J., Atchley, A., and Christie, B. (1984). The handbook of Plant Science in Agriculture. CRC Press Inc.

Dunoyer, P., Brosnan, C. A., Schott, G., Wang, Y., Jay, F., Alioua, A., Himber, C., and Voinnet, O. (2010). Retracted: An endogenous, systemic RNAi pathway in plants. *The EMBO journal* **29**, 1699-1712.

Dunoyer, P., Himber, C., and Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nature genetics* **37**, 1356-1360.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O., and Richards, K. E. (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *The Plant Journal* **29**, 555-567.

Eamens, A. L., Smith, N. A., Curtin, S. J., Wang, M.-B., and Waterhouse, P. M. (2009). The Arabidopsis thaliana double-stranded RNA binding protein DRB1 directs guide strand selection from microRNA duplexes. *Rna* **15**, 2219-2235.

Ehlers, U., Commandeur, U., Frank, R., Landsmann, J., Koenig, R., and Burgermeister, W. (1991). Cloning of the coat protein gene from beet necrotic yellow vein virus and its expression in sugar beet hairy roots. *Theoretical and applied genetics* **81**, 777-782.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *nature* **411**, 494-498.

Elvira-Matelot, E., and Martínez, Á. E. (2017). Diversity of RNA Silencing Pathways in Plants. *Plant Gene Silencing: Mechanisms and Applications* **5**, 1.

Endo, Y., Iwakawa, H. o., and Tomari, Y. (2013). Arabidopsis ARGONAUTE7 selects miR390 through multiple checkpoints during RISC assembly. *EMBO reports* **14**, 652-658.

Erhardt, M., Morant, M., Ritzenthaler, C., Stussi-Garaud, C., Guilley, H., Richards, K., Jonard, G., Bouzoubaa, S., and Gilmer, D. (2000). P42 movement protein of Beet necrotic yellow vein virus is targeted by the movement proteins P13 and P15 to punctate bodies associated with plasmodesmata. *Molecular plant-microbe interactions* **13**, 520-528.

Erhardt, M., Vetter, G., Gilmer, D., Bouzoubaa, S., Richards, K., Jonard, G., and Guilley, H. (2005). Subcellular localization of the Triple Gene Block movement proteins of Beet necrotic yellow vein virus by electron microscopy. *Virology* **340**, 155-166.

Estévez, J. M., Cantero, A., Romero, C., Kawaide, H., Jiménez, L. F., Kuzuyama, T., Seto, H., Kamiya, Y., and León, P. (2000). Analysis of the expression of CLA1, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-C-methyl-D-erythritol-4-phosphate pathway in Arabidopsis. *Plant physiology* **124**, 95-104.

Fagoaga, C., López, C., de Mendoza, A. H., Moreno, P., Navarro, L., Flores, R., and Peña, L. (2006). Post-transcriptional gene silencing of the p23 silencing suppressor of Citrus tristeza virus confers resistance to the virus in transgenic Mexican lime. *Plant molecular biology* **60**, 153-165.

Fang, X., and Qi, Y. (2016). RNAi in plants: an argonaute-centered view. *The Plant Cell*, TPC2015-00920-REV.

Fantini, E., and Giuliano, G. (2016). Virus-Induced Gene Silencing as a Tool to Study Tomato Fruit Biochemistry. *Plant Signal Transduction: Methods and Protocols*, 65-78.

FAO (2009). Sugar beet—white sugar. *Food and Agriculture Organization of the United Nations (ed), Rome*.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *nature* **391**, 806-811.

Flobinus, A., Hleibieh, K., Klein, E., Ratti, C., Bouzoubaa, S., and Gilmer, D. (2016). A Viral Noncoding RNA Complements a Weakened Viral RNA Silencing Suppressor and Promotes Efficient Systemic Host Infection. *Viruses* **8**, 272.

Folimonova, S. Y. (2012). Superinfection exclusion is an active virus-controlled function that requires a specific viral protein. *Journal of virology* **86**, 5554-5561.

Folimonova, S. Y., Robertson, C. J., Shilts, T., Folimonov, A. S., Hilf, M. E., Garnsey, S. M., and Dawson, W. O. (2010). Infection with strains of Citrus tristeza virus does not exclude superinfection by other strains of the virus. *Journal of virology* **84**, 1314-1325.

Fry, J., Barnason, A., and Hinchee, M. (1991). Genotype-independent transformation of sugar beet using Agrobacterium tumefaciens in molecular biology and plant development. *In* "Third international congress of the ISPMB, Tucson, USA", pp. 84.

Fu, D. Q., Zhu, B. Z., Zhu, H. L., Jiang, W. B., and Luo, Y. B. (2005). Virus-induced gene silencing in tomato fruit. *The Plant Journal* **43**, 299-308.

Fukunaga, R., and Doudna, J. A. (2009). dsRNA with 5′ overhangs contributes to endogenous and antiviral RNA silencing pathways in plants. *The EMBO journal* **28**, 545-555.

Gao, J., Deng, F., Zhai, H., Ling, X., and Liu, Y. (1983). The occurrence of sugar beet rhizomania caused by beet necrotic yellow vein virus in China. *Acta Phytopathologica Sinica* **13**, 1-4.

García-Arenal, F., Fraile, A., and Malpica, J. M. (2003). Variation and evolution of plant virus populations. *International Microbiology* **6**, 225-232.

Garcia-Asua, G., Lang, H. P., Cogdell, R. J., and Hunter, C. N. (1998). Carotenoid diversity: a modular role for the phytoene desaturase step. *Trends in plant science* **3**, 445-449.

Ghazala, W., and Varrelmann, M. (2007). Tobacco rattle virus 29K movement protein is the elicitor of extreme and hypersensitive-like resistance in two cultivars of Solanum tuberosum. *Molecular plant-microbe interactions* **20**, 1396-1405.

Ghildiyal, M., and Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nature reviews. Genetics* **10**, 94.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature methods* **6**, 343-345.

Gillet, F.-X., Cattoni, D. I., Petiot-Bécard, S., Delalande, F., Poignavent, V., Brizard, J.-P., Bessin, Y., Van Dorsselaer, A., Declerck, N., and Sanglier-Cianférani, S. (2013). The RYMV-encoded viral suppressor of RNA silencing P1 is a zinc-binding protein with redox-dependent flexibility. *Journal of molecular biology* **425**, 2423-2435.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. *Virology* **189**, 40-7.

Gilmer, D., and Ratti, C. (2011). Genus Benyvirus. *Virus Taxonomy: Classification and Nomenclature. Ninth Report of the International Committee on Taxonomy of Viruses*, 1163-1168.

Gilmer, D., Ratti, C., and Ictv, R. C. (2017). ICTV Virus Taxonomy Profile: Benyviridae. *The Journal of general virology*.

Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J. J., and Burgyán, J. (2010). Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *PLoS pathogens* **6**, e1000996.

González-Jara, P., Fraile, A., Canto, T., and García-Arenal, F. (2009). The multiplicity of infection of a plant virus varies during colonization of its eukaryotic host. *Journal of virology* **83**, 7487-7494.

González-Jara, P., Tenllado, F., Martínez-García, B., Atencio, F., Barajas, D., Vargas, M., Díaz-Ruíz, J., and Díaz-Ruíz, J. (2004). Host-dependent differences during synergistic infection by Potyviruses with potato virus X. *Molecular plant pathology* **5**, 29-35.

Gosselé, V., Faché, I., Meulewaeter, F., Cornelissen, M., and Metzlaff, M. (2002). SVISS–a novel transient gene silencing system for gene function discovery and validation in tobacco plants. *The Plant Journal* **32**, 859-866.

Grevelding, C., Fantes, V., Kemper, E., Schell, J., and Masterson, R. (1993). Single-copy T-DNA insertions in Arabidopsis are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. *Plant Molecular Biology* **23**, 847-860.

Grimmer, M., Trybush, S., Hanley, S., Francis, S., Karp, A., and Asher, M. (2007). An anchored linkage map for sugar beet based on AFLP, SNP and RAPD markers and QTL mapping of a new source of resistance to Beet necrotic yellow vein virus. *Theoretical and applied genetics* **114**, 1151-1160.

Grünewald, K., Eckert, M., Hirschberg, J., and Hagen, C. (2000). Phytoene desaturase is localized exclusively in the chloroplast and up-regulated at the mRNA level during accumulation of secondary carotenoids in Haematococcus pluvialis (Volvocales, Chlorophyceae). *Plant Physiology* **122**, 1261-1268.

Guo, Q., Liu, Q., A Smith, N., Liang, G., and Wang, M.-B. (2016). RNA Silencing in Plants: Mechanisms, Technologies and Applications in Horticultural Crops. *Current Genomics* **17**, 476-489.

Gupta, B., Saha, J., Sengupta, A., and Gupta, K. (2014). Recent advances on Virus Induced Gene silencing (VIGS): Plant functional genomics. *Journal of Plant Biochemistry & Physiology* **2013**.

Gutiérrez, S., Pirolles, E., Yvon, M., Baecker, V., Michalakis, Y., and Blanc, S. (2015). The multiplicity of cellular infection changes depending on the route of cell infection in a plant virus. *Journal of virology* **89**, 9665-9675.

Haaksma, J., and Vecchiettini, M. (1988). Beet vinasse-a protein source in animal nutrition. *In* "51. Winter Congress of the International Institute for Sugar Beet Research, Bruxelles (Belgium), 10-11 Feb 1988". IIRB. Secretariat General.

Haeberle, A., and Stussi-Garaud, C. (1995). In situ localization of the non-structural protein P25 encoded by beet necrotic yellow vein virus RNA 3. *Journal of general virology* **76**, 643-650.

Haeberle, A. M., Stussi-Garaud, C., Schmitt, C., Garaud, J. C., Richards, K. E., Guilley, H., and Jonard, G. (1994). Detection by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Arch Virol* **134**, 195-203.

Hamill, J. D., Prescott, A., and Martin, C. (1987). Assessment of the efficiency of cotransformation of the T-DNA of disarmed binary vectors derived from Agrobacterium tumefaciens and the T-DNA of A. rhizogenes. *Plant molecular biology* **9**, 573-584.

Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. *The EMBO journal* **21**, 4671-4679.

Hamilton, A. J., and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952.

Härtl, K., Kalinowski, G., Hoffmann, T., Preuss, A., and Schwab, W. (2017). RNAi-mediated endogene silencing in strawberry fruit: detection of primary and secondary siRNAs by deep sequencing. *Plant biotechnology journal* **15**, 658-668.

He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., and Hannon, G. J. (2005). A microRNA polycistron as a potential human oncogene. *Nature* **435**, 828-833.

Hehn, A., Bouzoubaa, S., Bate, N., Twell, D., Marbach, J., Richards, K., Guilley, H., and Jonard, G. (1995). The small cysteine-rich protein P14 of beet necrotic yellow vein virus regulates accumulation of RNA 2 in cis and coat protein in trans. *Virology* **210**, 73-81.

Hehn, A., Fritsch, C., Richards, K., Guilley, H., and Jonard, G. (1997). Evidence for in vitro and in vivo autocatalytic processingof the primary translation product of beet necrotic yellowvein virus RNA 1 by a papain-like proteinase. *Archives of virology* **142**, 1051-1058.

Heidel, G., and Rush, C. (1994). Distribution of beet necrotic yellow vein virus, beet distortion mosaic virus, and an unnamed soilborne sugar beet virus in Texas and New Mexico. *Plant disease* **78**, 603-606.

Heidel, G., Rush, C., Kendall, T., Lommel, S., and French, R. C. (1997). Characteristics of beet soilborne mosaic virus, a furo-like virus infecting sugar beet. *Plant disease* **81**, 1070-1076.

Heijbroek, W. (1989). The development of rhizomania in two areas of the Netherlands and its effect on sugar-beet growth and quality. *Netherlands Journal of Plant Pathology* **95**, 27-35.

Heijbroek, W., Musters, P., and Schoone, A. (1999). Variation in pathogenicity and multiplication of beet necrotic yellow vein virus (BNYVV) in relation to the resistance of sugar-beet cultivars. *European Journal of Plant Pathology* **105**, 397-405.

Henderson, I. R., Zhang, X., Lu, C., Johnson, L., Meyers, B. C., Green, P. J., and Jacobsen, S. E. (2006). Dissecting Arabidopsis thaliana DICER function in small RNA processing, gene silencing and DNA methylation patterning. *Nature genetics* **38**, 721-725.

Hileman, L. C., Drea, S., Martino, G., Litt, A., and Irish, V. F. (2005). Virus-induced gene silencing is an effective tool for assaying gene function in the basal eudicot species Papaver somniferum (opium poppy). *The Plant Journal* **44**, 334-341.

Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H., Seki, M., Shinozaki, K., and Fukuhara, T. (2005). Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in Arabidopsis thaliana. *Plant molecular biology* **57**, 173-188.

Hiriart, J. B., Lehto, K., Tyystjarvi, E., Junttila, T., and Aro, E. M. (2002). Suppression of a key gene involved in chlorophyll biosynthesis by means of virus-inducing gene silencing. *Plant Mol Biol* **50**, 213-24.

Hisano, H., Kimoto, Y., Hayakawa, H., Takeichi, J., Domae, T., Hashimoto, R., Abe, J., Asano, S., Kanazawa, A., and Shimamoto, Y. (2004). High frequency Agrobacterium-mediated transformation and plant regeneration via direct shoot formation from leaf explants in Beta vulgaris and Beta maritima. *Plant Cell Reports* **22**, 910-918.

Holzberg, S., Brosio, P., Gross, C., and Pogue, G. P. (2002). Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal* **30**, 315-327.

ICTV (2017). International Committee on Taxonomy of Viruses.

Igarashi, A., Yamagata, K., Sugai, T., Takahashi, Y., Sugawara, E., Tamura, A., Yaegashi, H., Yamagishi, N., Takahashi, T., Isogai, M., Takahashi, H., and Yoshikawa, N. (2009). Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, Arabidopsis thaliana, cucurbits, and legumes. *Virology* **386**, 407-416.

Ikegami, M., Kon, T., and Sharma, P. (2016). RNA silencing and viral encoded silencing suppressors. *RNAi Technology*, 209.

Iki, T., Tschopp, M.-A., and Voinnet, O. (2017). Biochemical and genetic functional dissection of the P38 viral suppressor of RNA silencing. *RNA* **23**, 639-654.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. *Gene* **96**, 23-28.

Ipsaro, J. J., and Joshua-Tor, L. (2015). From guide to target: molecular insights into eukaryotic RNA-interference machinery. *Nature structural & molecular biology* **22**, 20-28.

Jacq, B., Lesobre, O., Sangwan, R. S., and Sangwan-Norreel, B. S. (1993). Factors influencing T-DNA transfer in Agrobacterium-mediated transformation of sugarbeet. *Plant cell reports* **12**, 621-624.

Jiang, L., Wei, C., and Li, Y. (2012). Viral suppression of RNA silencing. *Science China Life Sciences* **55**, 109-118.

Joersbo, M. (2007). IV. 4 Sugar Beet. *Edited by T. Nagata (Managing Editor) H. Lörz*, 355.

Johansson, E. (1985). Rizomania in sugar beet-a threat to beet growing that can be overcome by plant breeding. *Sveriges Utsaedesfoerenings Tidskrift (Sweden)*.

Julve, J. M., Gandía, A., Fernández-del-Carmen, A., Sarrion-Perdigones, A., Castelijns, B., Granell, A., and Orzaez, D. (2013). A coat-independent superinfection exclusion rapidly imposed in Nicotiana benthamiana cells by tobacco mosaic virus is not prevented by depletion of the movement protein. *Plant molecular biology* **81**, 553-564.

Jupin, I., Guilley, H., Richards, K., and Jonard, G. (1992). Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. *The EMBO journal* **11**, 479.

Jupin, I., Tamada, T., and Richards, K. (1991). Pathogenesis of beet necrotic yellow vein virus. *In* "Seminars in Virology", Vol. 2, pp. 121-129.

Kanakala, S., and Ghanim, M. (2016). RNA Interference in Insect Vectors for Plant Viruses. *Viruses* **8**, 329.

Kang, M., Seo, J.-K., Song, D., Choi, H.-S., and Kim, K.-H. (2015). Establishment of an Agrobacterium-mediated inoculation system for cucumber green mottle mosaic virus. *The plant pathology journal* **31**, 433.

Kanno, T., Naito, S., and Shimamoto, K. (2000). Post-transcriptional gene silencing in cultured rice cells. *Plant and Cell Physiology* **41**, 321-326.

Kant, R., and Dasgupta, I. (2017). Phenotyping of VIGS-mediated gene silencing in rice using a vector derived from a DNA virus. *Plant Cell Reports*, 1-12.

Kawai, T., Gonoi, A., Nitta, M., Yamagishi, N., Yoshikawa, N., and Tao, R. (2016). Virus-induced gene silencing in various Prunus species with the Apple latent spherical virus vector. *Scientia Horticulturae* **199**, 103-113.

Kehr, J., and Buhtz, A. (2008). Long distance transport and movement of RNA through the phloem. *Journal of experimental botany* **59**, 85-92.

Keskin, B. (1964). Polymyxa betae n. sp., ein Parasit in den Wurzeln von Beta vulgaris Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. *Archives of Microbiology* **49**, 348-374.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes & development* **15**, 2654-2659.

Kifle, S., Shao, M., Jung, C., and Cai, D. (1999). An improved transformation protocol for studying gene expression in hairy roots of sugar beet (Beta vulgaris L.). *Plant Cell Reports* **18**, 514-519.

Kiguchi, T., Saito, M., and Tamada, T. (1996). Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. *Journal of General Virology* **77**, 575-580.

Kjemtrup, S., Sampson, K. S., Peele, C. G., Nguyen, L. V., Conkling, M. A., Thompson, W. F., and Robertson, D. (1998). Gene silencing from plant DNA carried by a geminivirus. *The Plant Journal* **14**, 91-100.

Klahre, U., Crété, P., Leuenberger, S. A., Iglesias, V. A., and Meins, F. (2002). High molecular weight RNAs and small interfering RNAs induce systemic posttranscriptional gene silencing in plants. *Proceedings of the National Academy of Sciences* **99**, 11981-11986.

Klein, E., Link, D., Schirmer, A., Erhardt, M., and Gilmer, D. (2007). Sequence variation within Beet necrotic yellow vein virus p25 protein influences its oligomerization and isolate pathogenicity on Tetragonia expansa. *Virus research* **126**, 53-61.

Kobayashi, K., and Zambryski, P. (2007). RNA silencing and its cell-to-cell spread during Arabidopsis embryogenesis. *The Plant Journal* **50**, 597-604.

Koch, A., and Kogel, K. H. (2014). New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. *Plant biotechnology journal* **12**, 821-831.

Koenig, R., Haeberlé, A.-M., and Commandeur, U. (1997). Detection and characterization of a distinct type of beet necrotic yellow vein virus RNA 5 in a sugarbeet growing area in Europe. *Archives of Virology* **142**, 1499-1504.

Koenig, R., Jarausch, W., Li, Y., Commandeur, U., Burgermeister, W., Gehrke, M., and Luddecke, P. (1991). Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanically inoculated sugarbeets. *J Gen Virol* **72 (Pt 9)**, 2243-6.

Koenig, R., Kastirr, U., Holtschulte, B., Deml, G., and Varrelmann, M. (2008). Distribution of various types and P25 subtypes of Beet necrotic yellow vein virus in Germany and other European countries. *Arch Virol* **153**, 2139-44.

Koenig, R., and Lennefors, B.-L. (2000). Molecular analyses of European A, B and P type sources of Beet necrotic yellow vein virus and detection of the rare P type in Kazakhstan. *Archives of virology* **145**, 1561-1570.

Koenig, R., Loss, S., Specht, J., Varrelmann, M., Lüddecke, P., and Deml, G. (2009). A single U/C nucleotide substitution changing alanine to valine in the beet necrotic yellow vein virus p25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings. *Journal of general virology* **90**, 759-763.

Koenig, R., Lüddecke, P., and Haeberle, A. (1995). Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *Journal of General Virology* **76**, 2051-2055.

Komari, T. (1990). Transformation of cultured cells of Chenopodium quinoa by binary vectors that carry a fragment of DNA from the virulence region of pTiBo542. *Plant Cell Reports* **9**, 303-306.

Kontra, L., Csorba, T., Tavazza, M., Lucioli, A., Tavazza, R., Moxon, S., Tisza, V., Medzihradszky, A., Turina, M., and Burgyán, J. (2016). Distinct effects of p19 RNA silencing suppressor on small RNA mediated pathways in plants. *PLoS pathogens* **12**, e1005935.

Krens, F. A., Trifonova, A., Keizer, L. P., and Hall, R. D. (1996). The effect of exogenously-applied phytohormones on gene transfer efficiency in sugarbeet (Beta vulgaris L.). *Plant Science* **116**, 97-106.

Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A., Savenkov, I., and Burgermeister, W. (1994). Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. *Journal of General Virology* **75**, 1835-1842.

Kühnel, S., Schols, H. A., and Gruppen, H. (2011). Aiming for the complete utilization of sugar-beet pulp: Examination of the effects of mild acid and hydrothermal pretreatment followed by enzymatic digestion. *Biotechnology for Biofuels* **4**, 14.

Kumagai, M., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences* **92**, 1679-1683.

Kutluk Yilmaz, N., Meunier, A., Schmit, J., Stas, A., and Bragard, C. (2007). Partial nucleotide sequence analysis of Turkish isolates of Beet necrotic yellow vein virus (BNYVV) RNA-3. *Plant pathology* **56**, 311-316.

Lacomme, C., and Hrubikova, K. (2003). Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *The Plant Journal* **34**, 543-553.

Lange, M., Yellina, A. L., Orashakova, S., and Becker, A. (2013). Virus-induced gene silencing (VIGS) in plants: an overview of target species and the virus-derived vector systems. *Virus-Induced Gene Silencing: Methods and Protocols*, 1-14.

Lauber, E., Guilley, H., Tamada, T., Richards, K., and Jonard, G. (1998). Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA 3 sequence domain rather than a gene product. *Journal of general virology* **79**, 385-393.

Lawrence, R. J., and Pikaard, C. S. (2003). Transgene-induced RNA interference: A strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *The Plant Journal* **36**, 114-121.

Lecoq, H., and Raccah, B. (2001). Cross-protection: interactions between strains exploited to control plant virus diseases. *Biotic interactions in plant pathogen associations. CAB International, Wallingford*, 177-192.

Lee, J., Cao, D.-V., Kim, J., Pamplona, R. S., Ahn, J., Cho, S.-K., Yang, S.-W., Riu, K.-Z., and Boo, K.-H. (2017). Development of a virus-induced gene silencing (VIGS) system for Spinacia oleracea L. *In Vitro Cellular & Developmental Biology-Plant* **53**, 97-103.

Lee, L., Telford, E., Batten, J., Scholthof, K.-B., and Rush, C. (2001). Complete nucleotide sequence and genome organization of Beet soilborne mosaic virus, a proposed member of the genus Benyvirus. *Archives of virology* **146**, 2443-2453.

Lennefors, B.-L., Savenkov, E. I., Bensefelt, J., Wremerth-Weich, E., van Roggen, P., Tuvesson, S., Valkonen, J. P., and Gielen, J. (2006). dsRNA-mediated resistance to Beet Necrotic Yellow Vein Virus infections in sugar beet (Beta vulgaris L. ssp. vulgaris). *Molecular breeding* **18**, 313-325.

Lennefors, B. L., Savenkov, E. I., Mukasa, S. B., and Valkonen, J. P. (2005). Sequence divergence of four soilborne sugarbeet-infecting viruses. *Virus Genes* **31**, 57-64.

Letschert, J., Lange, W., Frese, L., and Van Den Berg, R. (1994). Taxonomy of Beta section Beta. *Journal of sugar beet research* **31**, 69-85.

Lewellen, R. (1988). Selection for resistance to rhizomania in sugar beet. *In* "Proc 5th International Congress Plant Pathology. Kyoto, Japan".

Lewellen, R., and Biancardi, E. (1990). Breeding and performance of rhizomania resistant sugar beet. *In* "International Institute for Sugar Beet Research, 53rd winter congress, Brussels, Belgium, 14-15 February 1990.", pp. 69-87. International Institute for Sugar Beet Research.

Li, F., and Ding, S. W. (2006). Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annu Rev Microbiol* **60**, 503-31.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M., and Dougherty, W. G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *The Plant Cell* **5**, 1749-1759.

Lindsten, K. (1986). Rhizomania-a complicated disease in sugar beets which can occur also in Sweden [soilborne virus, beet necrotic yellow vein virus, Polymyxa betae]. *Vaextskyddsnotiser (Sweden)*.

Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O., and Gilmer, D. (2005). Functional characterization of the Beet necrotic yellow vein virus RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants. *J Gen Virol* **86**, 2115-25.

Liu, D., Shi, L., Han, C., Yu, J., Li, D., and Zhang, Y. (2012). Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. *PLoS One* **7**, e46451.

Liu, H.-Y., and Lewellen, R. (2007). Distribution and molecular characterization of resistance-breaking isolates of Beet necrotic yellow vein virus in the United States. *Plant disease* **91**, 847-851.

Liu, H., and Duffus, J. (1988). The occurrence of a complex of viruses associated with rhizomania of sugarbeet. *Phytopathology* **78**, 1583.

Liu, L., and Chen, X. (2016). RNA quality control as a key to suppressing RNA silencing of endogenous genes in plants. *Molecular plant* **9**, 826-836.

Liu, N., Xie, K., Jia, Q., Zhao, J., Chen, T., Li, H., Wei, X., Diao, X., Hong, Y., and Liu, Y. (2016). Foxtail mosaic virus-induced gene silencing in monocot plants. *Plant physiology*, pp. 00010.2016.

Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. (2002). Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *The Plant Journal* **30**, 415-429.

Loel, J., Kenter, C., Märländer, B., and Hoffmann, C. M. (2014). Assessment of breeding progress in sugar beet by testing old and new varieties under greenhouse and field conditions. *European journal of agronomy* **52**, 146-156.

Lu, R., Maduro, M., Li, F., Li, H., Broitman-Maduro, G., Li, W., and Ding, S. (2005). Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. *Nature* **436**, 1040-1043.

Luna, G. R., Reyes, C. A., Peña, E. J., Ocolotobiche, E., Baeza, C., Borniego, M. B., Kormelink, R., and García, M. L. (2017). Identification and characterization of two RNA silencing suppressors encoded by ophioviruses. *Virus Research* **235**, 96-105.

Ma, X., Nicole, M.-C., Meteignier, L.-V., Hong, N., Wang, G., and Moffett, P. (2015). Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants. *Journal of experimental botany* **66**, 919-932.

MacFarlane, S. A., and Popovich, A. H. (2000). Efficient expression of foreign proteins in roots from tobravirus vectors. *Virology* **267**, 29-35.

Machado, J. P. B., Calil, I. P., Santos, A. A., and Fontes, E. P. (2017). Translational control in plant antiviral immunity. *Genetics and molecular biology* **40**, 292-304.

Mahmood, T., and Rush, C. M. (1999). Evidence of Cross-Protection Between Beet Soilborne Mosaic Virus and Beet Necrotic Yellow Vein Virus in Sugar Beet. *Plant Disease* **83**, 521-526.

Mankin, S. L., Hill, D. S., Olhoft, P. M., Toren, E., Wenck, A. R., Nea, L., Xing, L., Brown, J. A., Fu, H., and Ireland, L. (2007). Disarming and sequencing of Agrobacterium rhizogenes strain K599 (NCPPB2659) plasmid pRi2659. *In Vitro Cellular & Developmental Biology-Plant* **43**, 521-535.

Masuta, C., Ueda, S., Suzuki, M., and Uyeda, I. (1998). Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses. *Proceedings of the National Academy of Sciences* **95**, 10487-10492.

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P., and Zamore, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**, 607-620.

Matthew, L. (2004). RNAi for plant functional genomics. *Comparative and Functional Genomics* **5**, 240-244.

Matzke, M., Primig, M., Trnovsky, J., and Matzke, A. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *The EMBO Journal* **8**, 643.

Matzke, M. A., and Birchler, J. A. (2005). RNAi-mediated pathways in the nucleus. *Nature Reviews Genetics* **6**, 24-35.

Mei, Y., Zhang, C., Kernodle, B. M., Hill, J. H., and Whitham, S. A. (2016). A Foxtail mosaic virus Vector for Virus-Induced Gene Silencing in Maize. *Plant Physiol*.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343-349.

Miki, D., Itoh, R., and Shimamoto, K. (2005). RNA silencing of single and multiple members in a gene family of rice. *Plant Physiology* **138**, 1903-1913.

Milne, R., and Lesemann, D. (1984). Immunosorbent electron microscopy in plant virus studies. *Methods in Virology*.

Misquitta, L., and Paterson, B. M. (1999). Targeted disruption of gene function in Drosophila by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proceedings of the National Academy of Sciences* **96**, 1451-1456.

Miyanishi, M., Kusume, T., Saito, M., and Tamada, T. (1999). Evidence for three groups of sequence variants of beet necrotic yellow vein virus RNA 5. *Archives of virology* **144**, 879-892.

Moissiard, G., and Voinnet, O. (2004). Viral suppression of RNA silencing in plants. *Molecular plant pathology* **5**, 71-82.

Monteiro, F., Romeiras, M. M., Batista, D., and Duarte, M. C. (2013). Biodiversity assessment of sugar beet species and its wild relatives: linking ecological data with new genetic approaches. *American Journal of Plant Sciences* **2013**.

Montgomery, M. K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences* **95**, 15502-15507.

Montgomery, T. A., Howell, M. D., Cuperus, J. T., Li, D., Hansen, J. E., Alexander, A. L., Chapman, E. J., Fahlgren, N., Allen, E., and Carrington, J. C. (2008). Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* **133**, 128-141.

Morel, J.-B., and Vaucheret, H. (2000). Post-transcriptional gene silencing mutants. *Plant molecular biology* **43**, 275-284.

Mustafa, R., Shafiq, M., Mansoor, S., Briddon, R. W., Scheffler, B. E., Scheffler, J., and Amin, I. (2015). Virus-Induced Gene Silencing in Cultivated Cotton (Gossypium spp.) Using Tobacco Rattle Virus. *Molecular Biotechnology* **58**, 65-72.

Nagyová, A., and Subr, Z. (2007). Infectious full-length clones of plant viruses and their use for construction of viral vectors. *Acta virologica* **51**, 223.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *The plant cell* **2**, 279-289.

Nelson, A. J., and Bushnell, W. R. (1997). Transient expression of anthocyanin genes in barley epidermal cells: potential for use in evaluation of disease response genes. *Transgenic research* **6**, 233-244.

Nielsen, K., Olsen, O., and Oliver, R. (1999). A transient expression system to assay putative antifungal genes on powdery mildew infected barley leaves. *Physiological and molecular plant pathology* **54**, 1-12.

Nielsen, S. L., Nicolaisen, M., Scheel, C., and Dinesen, I. G. (2001). First record of Beet necrotic yellow vein virus in Denmark. *Plant Disease* **85**, 559-559.

Niesbach-Klösgen, U., Guilley, H., Jonard, G., and Richards, K. (1990). Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. *Virology* **178**, 52-61.

Norris, S. R., Barrette, T. R., and DellaPenna, D. (1995). Genetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation. *The Plant Cell* **7**, 2139-2149.

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumlehn, J., and Schweizer, P. (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. *The Plant Cell* **22**, 3130-3141.

Nunes, C. C., and Dean, R. A. (2012). Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies. *Molecular plant pathology* **13**, 519-529.

Oerke, E.-C., and Dehne, H.-W. (2004). Safeguarding production—losses in major crops and the role of crop protection. *Crop protection* **23**, 275-285.

Ogita, S., Uefuji, H., Morimoto, M., and Sano, H. (2004). Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. *Plant molecular biology* **54**, 931-941.

Orzaez, D., Medina, A., Torre, S., Fernández-Moreno, J. P., Rambla, J. L., Fernández-del-Carmen, A., Butelli, E., Martin, C., and Granell, A. (2009). A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. *Plant physiology* **150**, 1122-1134.

Ossowski, S., Schwab, R., and Weigel, D. (2008). Gene silencing in plants using artificial microRNAs and other small RNAs. *The Plant Journal* **53**, 674-690.

Ouillet, L., Guilley, H., Jonard, G., and Richards, K. (1989). In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. *Virology* **172**, 293-301.

Padmanabhan, M., and Dinesh-Kumar, S. P. (2009). Virus-induced gene silencing as a tool for delivery of dsRNA into plants. *Cold Spring Harbor Protocols* **2009**, pdb. prot5139.

Palatnik, J. F., Wollmann, H., Schommer, C., Schwab, R., Boisbouvier, J., Rodriguez, R., Warthmann, N., Allen, E., Dezulian, T., and Huson, D. (2007). Sequence and expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. *Developmental cell* **13**, 115-125.

Panella, L., and Lewellen, R. (2007). Broadening the genetic base of sugar beet: introgression from wild relatives. *Euphytica* **154**, 383-400.

Panja, S., Aich, P., Jana, B., and Basu, T. (2008). How does plasmid DNA penetrate cell membranes in artificial transformation process of Escherichia coli? *Molecular membrane biology* **25**, 411-422.

Panstruga, R. (2004). A golden shot: how ballistic single cell transformation boosts the molecular analysis of cereal–mildew interactions. *Molecular Plant Pathology* **5**, 141-148.

Pant, B. D., Buhtz, A., Kehr, J., and Scheible, W. R. (2008). MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *The Plant Journal* **53**, 731-738.

Papp, I., Mette, M. F., Aufsatz, W., Daxinger, L., Schauer, S. E., Ray, A., Van Der Winden, J., Matzke, M., and Matzke, A. J. (2003). Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant physiology* **132**, 1382-1390.

Park, M. Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R. S. (2005). Nuclear processing and export of microRNAs in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 3691-3696.

Parrish, S., Fleenor, J., Xu, S., Mello, C., and Fire, A. (2000). Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Molecular cell* **6**, 1077-1087.

Pavli, O., and Skaracis, G. (2010). Fast and efficient genetic transformation of sugar beet by Agrobacterium rhizogenes. *Nature Protocol Exchange Year published. doi* **10**.

Pavli, O. I., Stevanato, P., Biancardi, E., and Skaracis, G. N. (2011). Achievements and prospects in breeding for rhizomania resistance in sugar beet. *Field Crops Research* **122**, 165-172.

Peele, C., Jordan, C. V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., and Robertson, D. (2001). Silencing of a meristematic gene using geminivirus-derived vectors. *The Plant Journal* **27**, 357-366.

Pelsy, F., and Merdinoglu, D. (1996). Identification and mapping of random amplified polymorphic DNA markers linked to a rhizomania resistance gene in sugar beet (Beta vulgaris L.) by bulked segregant analysis. *Plant Breeding* **115**, 371-377.

Peltier, C., Hleibieh, K., Thiel, H., Klein, E., Bragard, C., and Gilmer, D. (2008). Molecular biology of the beet necrotic yellow vein virus. *Plant Viruses* **2**, 14-24.

Peltier, C., Klein, E., Hleibieh, K., D'Alonzo, M., Hammann, P., Bouzoubaa, S., Ratti, C., and Gilmer, D. (2012). Beet necrotic yellow vein virus subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. *J Gen Virol* **93**, 1093-102.

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L., and Poethig, R. S. (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. *Genes & development* **18**, 2368-2379.

Pferdmenges, F., and Varrelmann, M. (2009). Breaking of Beet necrotic yellow vein virus resistance in sugar beet is independent of virus and vector inoculum densities. *European journal of plant pathology* **124**, 231-245.

Pflieger, S., Blanchet, S., Camborde, L., Drugeon, G., Rousseau, A., Noizet, M., Planchais, S., and Jupin, I. (2008). Efficient virus-induced gene silencing in Arabidopsis using a 'one-step'TYMV-derived vector. *The Plant Journal* **56**, 678-690.

Pignatta, D., Kumar, P., Turina, M., Dandekar, A., and Falk, B. W. (2007). Quantitative analysis of efficient endogenous gene silencing in Nicotiana benthamiana plants using Tomato bushy stunt virus vectors that retain the capsid protein gene. *Molecular plant-microbe interactions* **20**, 609-618.

Poulsen, C., Vaucheret, H., and Brodersen, P. (2013). Lessons on RNA silencing mechanisms in plants from eukaryotic argonaute structures. *The Plant Cell* **25**, 22-37.

Putz, C., Pinck, M., Fritsch, C., and Pinck, L. (1983). Identification of the 3'-and 5'-ends of beet necrotic yellow vein virus RNAs. *FEBS letters* **156**, 41-46.

Qiu, S., Adema, C. M., and Lane, T. (2005). A computational study of off-target effects of RNA interference. *Nucleic acids research* **33**, 1834-1847.

Qiu, W., and Moyer, J. W. (1999). Tomato spotted wilt tospovirus adapts to the TSWV N gene-derived resistance by genome reassortment. *Phytopathology* **89**, 575-582.

Quadt, R., and Jaspars, E. (1989). RNA polymerases of plus-strand RNA viruses of plants. *Molecular plant-microbe interactions* **2**, 219-223.

Rahim, M. D., Andika, I. B., Han, C., Kondo, H., and Tamada, T. (2007). RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. *Journal of General Virology* **88**, 1611-1619.

Ramanna, H., Ding, X. S., and Nelson, R. S. (2013). Rationale for developing new virus vectors to analyze gene function in grasses through virus-induced gene silencing. *Virus-induced gene silencing: methods and protocols*, 15-32.

Ramegowda, V., Mysore, K. S., and Senthil-Kumar, M. (2014). Virus-induced gene silencing is a versatile tool for unraveling the functional relevance of multiple abiotic-stress-responsive genes in crop plants. *Frontiers in Plant Science* **5**.

Rao, A., and Seo, J.-K. (2017). Methods and molecules for suppression of RNA silencing. Google Patents.

Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. (2001). Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal* **25**, 237-245.

Ratcliff, F. G., MacFarlane, S. A., and Baulcombe, D. C. (1999). Gene silencing without DNA: RNA-mediated cross-protection between viruses. *The Plant Cell Online* **11**, 1207-1215.

Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Autonell, C. R., and Gilmer, D. (2009). Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. *Virology* **385**, 392-9.

Richards, K., Jonard, G., Guilley, H., Ziegler, V., and Putz, C. (1985). In vitro translation of beet necrotic yellow vein virus RNA and studies of sequence homology among the RNA species using cloned cDNA probes. *Journal of general virology* **66**, 345-350.

Richards, K., and Tamada, T. (1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annual review of phytopathology* **30**, 291-313.

Robertson, D. (2004). VIGS vectors for gene silencing: many targets, many tools. *Annu. Rev. Plant Biol.* **55**, 495-519.

Romano, N., and Macino, G. (1992). Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Molecular microbiology* **6**, 3343-3353.

Rotenberg, D., Thompson, T. S., German, T. L., and Willis, D. K. (2006). Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *Journal of virological methods* **138**, 49-59.

Rual, J.-F., Klitgord, N., and Achaz, G. (2007). Novel insights into RNAi off-target effects using C. elegans paralogs. *BMC genomics* **8**, 106.

Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* **10**, 937-946.

Rush, C., and Heidel, G. (1995). Furovirus disease of sugar beets in the United States. *Plant disease* **79**, 868-875.

Rush, C. M. (2003). Ecology and epidemiology of benyviruses and plasmodiophorid vectors. *Annual Review of Phytopathology* **41**, 567-592.

Ryu, C. M., Anand, A., Kang, L., and Mysore, K. S. (2004). Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *The Plant Journal* **40**, 322-331.

Saito, M., Kiguchi, T., Kusume, T., and Tamada, T. (1996). Complete nucleotide sequence of the Japanese isolate S of beet necrotic yellow vein virus RNA and comparison with European isolates. *Archives of virology* **141**, 2163-2175.

Schirmer, A., Link, D., Cognat, V., Moury, B., Beuve, M., Meunier, A., Bragard, C., Gilmer, D., and Lemaire, O. (2005). Phylogenetic analysis of isolates of Beet necrotic yellow vein virus collected worldwide. *Journal of General Virology* **86**, 2897-2911.

Schmidlin, L., Link, D., Mutterer, J. m., Guilley, H., and Gilmer, D. (2005). Use of a Beet necrotic yellow vein virus RNA-5-derived replicon as a new tool for gene expression. *Journal of general virology* **86**, 463-467.

Schmitt, C., Balmori, E., Jonard, G., Richards, K. E., and Guilley, H. (1992). In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. *Proceedings of the National Academy of Sciences* **89**, 5715-5719.

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature protocols* **3**, 1101-1108.

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature methods* **9**, 671-675.

Schnepf, R. D. (2006). European Union biofuels policy and agriculture: An overview. Congressional Research Service, Library of Congress.

Scholten, O. E., and Lange, W. (2000). Breeding for resistance to rhizomania in sugar beet: a review. *Euphytica* **112**, 219-231.

Schramke, V., and Allshire, R. (2004). Those interfering little RNAs! Silencing and eliminating chromatin. *Current opinion in genetics & development* **14**, 174-180.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. *The Plant Cell* **18**, 1121-1133.

Schwarz, D. S., Hutvágner, G., Haley, B., and Zamore, P. D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Molecular cell* **10**, 537-548.

Schweizer, P., Christoffel, A., and Dudler, R. (1999). Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *The Plant Journal* **20**, 541-552.

Scofield, S. R., Huang, L., Brandt, A. S., and Gill, B. S. (2005). Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant Physiology* **138**, 2165-2173.

Scofield, S. R., and Nelson, R. S. (2009). Resources for virus-induced gene silencing in the grasses. *Plant Physiology* **149**, 152-157.

Sen, G. L., and Blau, H. M. (2006). A brief history of RNAi: the silence of the genes. *The FASEB journal* **20**, 1293-1299.

Senthil-Kumar, M., Hema, R., Suryachandra, T. R., Ramegowda, H., Gopalakrishna, R., Rama, N., Udayakumar, M., and Mysore, K. S. (2010). Functional characterization of three water deficit stress-induced genes in tobacco and Arabidopsis: An approach based on gene down regulation. *Plant Physiology and Biochemistry* **48**, 35-44.

Senthil-Kumar, M., and Mysore, K. S. (2011a). Caveat of RNAi in plants: the off-target effect. *RNAi and Plant Gene Function Analysis: Methods and Protocols*, 13-25.

Senthil-Kumar, M., and Mysore, K. S. (2011b). New dimensions for VIGS in plant functional genomics. *Trends Plant Sci* **16**, 656-65.

Senthil-Kumar, M., and Mysore, K. S. (2011c). New dimensions for VIGS in plant functional genomics. *Trends in Plant Science* **16**, 656-665.

Shirako, Y., and Wilson, T. M. A. (1993). Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* **195**, 16-32.

Smith, C., Watson, C., Bird, C., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Molecular and General Genetics MGG* **224**, 477-481.

Smith, N. A., Singh, S. P., Wang, M.-B., Stoutjesdijk, P. A., Green, A. G., and Waterhouse, P. M. (2000). Gene expression: total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320.

Sohi, H., and Maleki, M. (2004). Evidence for presence of types A and B of Beet necrotic yellow vein virus (BNYVV) in Iran. *Virus Genes* **29**, 353-358.

Solís, J. F., Mlejnek, P., Studená, K., and Procházka, S. (2003). Application of sonication-assisted Agrobacterium-mediated transformation in Chenopodium rubrum. *L. Plant Soil Environ* **49**, 255-260.

Stanley, J., Boulton, M. I., and Davies, J. W. (2005). Geminiviridae. *eLS*.

Stevens, M., Liu, H. Y., and Lemaire, O. (2006). Virus diseases. *Sugar Beet*, 256-285.

Sutton, M. D., and Peterson, J. D. (2001). Fermentation of sugarbeet pulp for ethanol production using bioengineered Klebsiella oxytoca strain P2. *Journal of sugar beet research* **38**, 19-34.

Syller, J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular plant pathology* **13**, 204-216.

Syller, J. (2016). Antagonistic within-host interactions between plant viruses: molecular basis and impact on viral and host fitness. *Molecular plant pathology* **17**, 769-782.

Szabó, E. Z., Manczinger, M., Göblös, A., Kemény, L., and Lakatos, L. (2012). Switching on RNA silencing suppressor activity by restoring argonaute binding to a viral protein. *Journal of virology* **86**, 8324-8327.

Tai, Y., Bragg, J., and Edwards, M. C. (2005). Virus vector for gene silencing in wheat. *Biotechniques* **39**, 310.

Takahashi, T., Sugawara, T., Yamatsuta, T., Isogai, M., Natsuaki, T., and Yoshikawa, N. (2007). Analysis of the spatial distribution of identical and two distinct virus populations

differently labeled with cyan and yellow fluorescent proteins in coinfected plants. *Phytopathology* **97**, 1200-1206.

Tamada, T. (2007). Susceptibility and resistance of Beta vulgaris subsp. maritima to foliar rub-inoculation with Beet necrotic yellow vein virus. *Journal of General Plant Pathology* **73**, 76-80.

Tamada, T., and Abe, H. (1989). Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus Polymyxa betae. *Journal of General Virology* **70**, 3391-3398.

Tamada, T., and Kondo, H. (2013). Biological and genetic diversity of plasmodiophorid-transmitted viruses and their vectors. *Journal of general plant pathology* **79**, 307-320.

Tamada, T., and Kusume, T. (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus Polymyxa betae. *J Gen Virol* **72 (Pt 7)**, 1497-504.

Tamada, T., Schmitt, C., Saito, M., Guilley, H., Richards, K., and Jonard, G. (1996). High resolution analysis of the readthrough domain of beet necrotic yellow vein virus readthrough protein: a KTER motif is important for efficient transmission of the virus by Polymyxa betae. *Journal of General Virology* **77**, 1359-1367.

Tamada, T., Uchino, H., Kusume, T., and Saito, M. (1999). RNA 3 deletion mutants of beet necrotic yellow vein virus do not cause rhizomania disease in sugar beets. *Phytopathology* **89**, 1000-1006.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution* **30**, 2725-2729.

Tatineni, S., and French, R. (2016). The coat protein and NIa protease of two Potyviridae family members independently confer superinfection exclusion. *Journal of virology* **90**, 10886-10905.

Thiel, H., Hleibieh, K., Gilmer, D., and Varrelmann, M. (2012). The P25 pathogenicity factor of Beet necrotic yellow vein virus targets the sugar beet 26S proteasome involved in the induction of a hypersensitive resistance response via interaction with an F-box protein. *Molecular plant-microbe interactions* **25**, 1058-1072.

Thiel, H., and Varrelmann, M. (2009). Identification of Beet necrotic yellow vein virus P25 Pathogenicity Factor–Interacting Sugar Beet Proteins That Represent Putative Virus Targets or Components of Plant Resistance. *Molecular plant-microbe interactions* **22**, 999-1010.

Thomas, C. L., Jones, L., Baulcombe, D. C., and Maule, A. J. (2001). Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in Nicotiana benthamiana using a potato virus X vector. *The Plant Journal* **25**, 417-425.

Tijsterman, M., Ketting, R. F., and Plasterk, R. H. (2002). The genetics of RNA silencing. *Annual Review of Genetics* **36**, 489-519.

Tiwari, I. M., Jesuraj, A., Kamboj, R., Devanna, B., Botella, J. R., and Sharma, T. (2017). Host Delivered RNAi, an efficient approach to increase rice resistance to sheath blight pathogen (Rhizoctonia solani). *Scientific Reports* **7**.

Tomari, Y., Matranga, C., Haley, B., Martinez, N., and Zamore, P. D. (2004). A protein sensor for siRNA asymmetry. *Science* **306**, 1377-1380.

Tomari, Y., and Zamore, P. D. (2005). Perspective: machines for RNAi. *Genes & development* **19**, 517-529.

Travella, S., Klimm, T. E., and Keller, B. (2006). RNA interference-based gene silencing as an efficient tool for functional genomics in hexaploid bread wheat. *Plant Physiology* **142**, 6-20.

Turnage, M. A., Muangsan, N., Peele, C. G., and Robertson, D. (2002). Geminivirus-based vectors for gene silencing in Arabidopsis. *The Plant Journal* **30**, 107-114.

Unver, T., and Budak, H. (2009). Virus-induced gene silencing, a post transcriptional gene silencing method. *Int J Plant Genomics* **2009**, 198680.

Valentin, C., Dunoyer, P., Vetter, G., Schalk, C., Dietrich, A., and Bouzoubaa, S. (2005). Molecular basis for mitochondrial localization of viral particles during beet necrotic yellow vein virus infection. *Journal of virology* **79**, 9991-10002.

Van der Krol, A. R., Mur, L. A., Beld, M., Mol, J., and Stuitje, A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *The Plant Cell* **2**, 291-299.

Van Heel, M., Portugal, R. V., and Schatz, M. (2016). Multivariate statistical analysis of large datasets: single particle electron microscopy. *Open Journal of Statistics* **6**, 701.

Van Kammen, A. (1997). Virus-induced gene silencing in infected and transgenic plants. *Trends in Plant Science* **2**, 409-411.

Varrelmann, M. (2007). Occurrence, spread and pathogenicity of different forms of the Rhizomania virus (Beet necrotic yellow vein virus, BNYVV)-review on biology and variability of Rhizomania and on detection of isolates possibly overcoming resistance. *Zuckerindustrie* **132**, 113-120.

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.-L., Bartel, D. P., and Crété, P. (2004). Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Molecular cell* **16**, 69-79.

Verchot-Lubicz, J., Torrance, L., Solovyev, A. G., Morozov, S. Y., Jackson, A. O., and Gilmer, D. (2010). Varied movement strategies employed by triple gene block–encoding viruses. *Molecular plant-microbe interactions* **23**, 1231-1247.

Vetter, G., Hily, J.-M., Klein, E., Schmidlin, L., Haas, M., Merkle, T., and Gilmer, D. (2004). Nucleo-cytoplasmic shuttling of the beet necrotic yellow vein virus RNA-3-encoded p25 protein. *Journal of general virology* **85**, 2459-2469.

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *TRENDS in Genetics* **17**, 449-459.

Voinnet, O. (2005). Induction and suppression of RNA silencing: insights from viral infections. *Nature Reviews Genetics* **6**, 206-220.

Voinnet, O., and Baulcombe, D. C. (1997). Systemic signalling in gene silencing. *Nature* **389**, 553.

Voinnet, O., Lederer, C., and Baulcombe, D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. *Cell* **103**, 157-167.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). Retracted: An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**, 949-956.

Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**, 177-187.

von Stetten, D., Noirclerc-Savoye, M., Goedhart, J., Gadella, T. W., and Royant, A. (2012). Structure of a fluorescent protein from Aequorea victoria bearing the obligate-monomer mutation A206K. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* **68**, 878-882.

Walker, C. J., and Weinstein, J. D. (1991). In vitro assay of the chlorophyll biosynthetic enzyme Mg-chelatase: resolution of the activity into soluble and membrane-bound fractions. *Proc Natl Acad Sci U S A* **88**, 5789-93.

Walkey, D. G. (2012). "Applied plant virology," Springer Science & Business Media.

Wang, J., Lu, Z., Wientjes, M. G., and Au, J. L.-S. (2010). Delivery of siRNA therapeutics: barriers and carriers. *The AAPS journal* **12**, 492-503.

Wang, M.-B., Upadhyaya, N., Brettell, R., and Waterhouse, P. M. (1997). Intron-mediated improvement of a selectable marker gene for plant transformation using Agrobacterium tumefaciens. *Journal of Genetics and Breeding* **51**, 325-334.

Wang, R., Yang, X., Wang, N., Liu, X., Nelson, R. S., Li, W., Fan, Z., and Zhou, T. (2016). An efficient virus-induced gene silencing vector for maize functional genomics research. *The Plant Journal* **86**, 102-115.

Wang, S., Aurora, A. B., Johnson, B. A., Qi, X., McAnally, J., Hill, J. A., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2008). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Developmental cell* **15**, 261-271.

Ward, L., Koenig, R., Budge, G., Garrido, C., McGrath, C., Stubbley, H., and Boonham, N. (2007). Occurrence of two different types of RNA-5-containing beet necrotic yellow vein virus in the UK. *Archives of virology* **152**, 59-73.

Warthmann, N., Chen, H., Ossowski, S., Weigel, D., and Hervé, P. (2008). Highly specific gene silencing by artificial miRNAs in rice. *PLoS One* **3**, e1829.

Waterhouse, P. (2000). Total silencing by intron-spliced hairpin RNAs. Nature 407, 319320Somerville C (2000) Genomics: plant biology 2010. *Science* **290**.

Waterhouse, P. M., Graham, M. W., and Wang, M.-B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences* **95**, 13959-13964.

Waterhouse, P. M., Wang, M.-B., and Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**, 834-842.

Watson, L., and Dallwitz, M. (1999). "The families of flowering plants: descriptions, illustrations, identification, and information retrieval," University of New Orleans.

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D., and Stoutjesdijk, P. A. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal* **27**, 581-590.

Whitham, S. A., Lincoln, L. M., Chowda-Reddy, R., Dittman, J. D., O'Rourke, J. A., and Graham, M. A. (2016a). Virus-Induced Gene Silencing and Transient Gene Expression in Soybean (Glycine max) Using Bean Pod Mottle Virus Infectious Clones. *Current Protocols in Plant Biology*, 263-283.

Whitham, S. A., Qi, M., Innes, R. W., Ma, W., Lopes-Caitar, V., and Hewezi, T. (2016b). Molecular soybean-pathogen interactions. *Annual review of phytopathology* **54**, 443-468.

Willemsen, A., Zwart, M. P., and Elena, S. F. (2017). High virulence does not necessarily impede viral adaptation to a new host: a case study using a plant RNA virus. *BMC evolutionary biology* **17**, 25.

Wilson, R. C., and Doudna, J. A. (2013). Molecular mechanisms of RNA interference. *Annual review of biophysics* **42**, 217-239.

Wintermantel, W., Gulati-Sakhuja, A., Larson, R., Hladky, L., Nunez, A., and Hill, A. (2009). Proteome changes in sugarbeet in response to Beet necrotic yellow vein virus infection. *In* "American Society of Sugar Beet Technologists".

Wisler G.C., L. H.-Y., Duffus J.E. (1994). Beet necrotic yellow vein virus and its relationship to eight sugar beet furo-like viruses from the United States. *Plant Disease*, 995.

Wisler, G., Lewellen, R., Sears, J., Wasson, J., Liu, H.-Y., and Wintermantel, W. (2003). Interactions between Beet necrotic yellow vein virus and Beet soilborne mosaic virus in sugar beet. *Plant disease* **87**, 1170-1175.

Workneh, F., Villanueva, E., Steddom, K., and Rush, C. (2003). Spatial association and distribution of Beet necrotic yellow vein virus and Beet soilborne mosaic virus in sugar beet fields. *Plant disease* **87**, 707-711.

Wozniak, C. A. (1999). Transgenic sugar beet: progress and development. *Appl Plant Biotech* **10**, 301-324.

Xiang, C., Han, P., Lutziger, I., Wang, K., and Oliver, D. J. (1999). A mini binary vector series for plant transformation. *Plant molecular biology* **40**, 711-717.

Xu, P., Zhang, Y., Kang, L., Roossinck, M. J., and Mysore, K. S. (2006). Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiology* **142**, 429-440.

Yang, A., Duan, X., Gu, X., Gao, F., and Zhang, J. (2005). Efficient transformation of beet (Beta vulgaris) and production of plants with improved salt-tolerance. *Plant cell, tissue and organ culture* **83**, 259-270.

Yang, F. (1997). The molecular structure of green fluorescent protein, Rice University.

Yuan, C., Li, C., Yan, L., Jackson, A. O., Liu, Z., Han, C., Yu, J., and Li, D. (2011). A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. *PLoS One* **6**, e26468.

Zhai, J., Zhao, Y., Simon, S. A., Huang, S., Petsch, K., Arikit, S., Pillay, M., Ji, L., Xie, M., and Cao, X. (2013). Plant microRNAs display differential 3' truncation and tailing modifications that are ARGONAUTE1 dependent and conserved across species. *The Plant Cell* **25**, 2417-2428.

Zhang, C., Bradshaw, J. D., Whitham, S. A., and Hill, J. H. (2010). The development of an efficient multipurpose bean pod mottle virus viral vector set for foreign gene expression and RNA silencing. *Plant physiology* **153**, 52-65.

Zhang, L., Wang, Z., Wang, X., Li, D., Han, C., Zhai, Y., and Yu, J. (2005). Two virus-encoded RNA silencing suppressors, P14 ofBeet necrotic yellow vein virus and S6 ofRice black streak dwarf virus. *Chinese Science Bulletin* **50**, 305-310.

Zhang, T., Jin, Y., Zhao, J.-H., Gao, F., Zhou, B.-J., Fang, Y.-Y., and Guo, H.-S. (2016). Host-induced gene silencing of the target gene in fungal cells confers effective resistance to the cotton wilt disease pathogen Verticillium dahliae. *Molecular plant* **9**, 939-942.

Zhao, F., Lim, S., Igori, D., Yoo, R. H., Kwon, S.-Y., and Moon, J. S. (2016). Development of tobacco ringspot virus-based vectors for foreign gene expression and virus-induced gene silencing in a variety of plants. *Virology* **492**, 166-178.

Zheng, Y., Ding, B., Fei, Z., and Wang, Y. (2017). Comprehensive transcriptome analyses reveal tomato plant responses to tobacco rattle virus-based gene silencing vectors. *Scientific Reports* **7**.

Zhong, X., Hou, H., and Qiu, W. (2005). Integrity of nonviral fragments in recombinant Tomato bushy stunt virus and defective interfering RNA is influenced by silencing and the type of inserts. *Molecular plant-microbe interactions* **18**, 800-807.

Ziebell, H., and Carr, J. P. (2010). Cross-protection: a century of mystery. *Advances in virus research* **76**, 211-264.

Ziegler, V., Richards, K., Guilley, H., Jonard, G., and Putz, C. (1985). Cell-free translation of beet necrotic yellow vein virus: readthrough of the coat protein cistron. *Journal of general virology* **66**, 2079-2087.

Zimmermann, T. S., Lee, A. C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M. N., Harborth, J., Heyes, J. A., Jeffs, L. B., and John, M. (2006). RNAi-mediated gene silencing in non-human primates. *Nature* **441**, 111-114.

7. ADDENDUM

Supplementary Fig. 4: Vector map of pDIVA (accession number KX665539).

Tab.2: Characteristics of pDIVA vector

Feature	*Description*	*Position*
oriV		1-630
NptIII	Aminoglycoside resistance	839-1633
TrfA	Plasmid replication initiator protein	1932-3080
T-DNA right border		3235-3259
35S promoter	*Cauliflower mosaic virus* 35S RNA promoter	3330-3757
HDVagrz	Hepatitis delta virusantigenomic "core" ribozyme	3755-3839
PA35s	*Cauliflower mosaic virus* 35S RNA terminator	3875-4079
T-DNA left border		4206-4230

Nucleotides sequence of *N. benthamiana phytoene desaturase* (*pds*) gene; 578 bp

ttgttattgctggtgcaggtttgggtggtttgtctacagcaaaatatctggcagatgctggtcacaaaccgatattgctggagg
caagagatgtcctaggtgggaaggtagctgcatggaaagatgatgatggagattggtacgagactgggttgcacatattctt
tggggcttacccaaatatgcagaacctgtttggagaactagggattgatgatcggttgcagtggaaggaacattcaatgata
tttgcgatgcctaacaagccaggggagttcagccgctttgattttcctgaagctcttcctgcgccattaaatggaattttggcc
atactaaagaacaacgaaatgcttacgtggcccgagaaagtcaaatttgctattggactcttgccagcaatgcttggagggc
aatcttatgttgaagctcaagacggtttaagtgttaaggactggatgagaaagcaaggtgtgcctgatagggtgacagatga
ggtgttcattgccatgtcaaaggcacttaacttcataaaccctgacgagctttcgatgcagtgcattttgattgctttgaac

NCBI Reference Sequence: EU165355.1

Nucleotides sequence of *N. tabacum magnesium-chelatase* subunit (*chlH*) gene; 549 bp

tgaatctccttgaccgagcagtcaagatggttgcagagctcgacgagccagaagaccaaaactacgtcaggaaacatgca
ctagaacaagcaaaaacactcggagttgatgttcgtgaagctgctacaaggatcttctcaaatgcttcaggatcttactcctc
caacattaaccttgctgttgagaattcaacatggaatgatgagaagcaacttcaagacatgtacttgagccgaaagtcatttg
catttgactgtgatgcccctggtgttggcatgactgagaagaggaaagtttttgagatggctcttagcacggctgatgccaca
ttccagaaccttgactcatctgaaatttcattcacagacgtgagtcactacttcgattcagacccaaccaaccttgtgcaaaac
ctcaggaaagacgggaagaagcctagtgcatacattgctgacaccactactgctaatgctcaggtacgtacgttgtctgag
actgtgaggcttgacgcaaggacaaagttgttgaaccccaagtggtatgaaggcat

NCBI Reference Sequence: NM_001325713.1

Nucleotides sequence of *monomeric red fluorescent protein* (*mrfp*) gene; 675 bp

atggcctcctccgaggacgtcatcaaggagttcatgcgcttcaaggtgcgcatggagggctccgtgaacggccacgagtt
cgagatcgagggcgagggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggcgg
ccccctgcccttcgcctgggacatcctgtcccctcagttccagtacggctccaaggcctacgtgaagcaccccgccgacat
ccccgactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtga
ccgtgacccaggactcctccctgcaggacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgac
ggccccgtaatgcagaagaagaccatgggctgggaggcctccaccgagcggatgtaccccgaggacggcgccctgaa
gggcgagatcaagatgaggctgaagctgaaggacggcggccactacgacgccgaggtcaagaccacctacatggcca
agaagcccgtgcagctgcccggcgcctacaagaccgacatcaagctggacatcacctcccacaacgaggactacaccat
cgtggaacagtacgagcgcgccgagggccgccactccaccggcgcc

NCBI Reference Sequence: AF506027.1

Nucleotides sequence of *soluble-modified red-shifted green fluorescent protein (smRSGFP)* gene; 714 bp

atgagtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgt
cagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccat
ggccaacacttgtcactactttcacttatggtgttcaatgcttttcaagatacccagatcatatgaagcggcacgacttcttcaa
gagcgccatgcctgagggatacgtgcaggagaggaccatctctttcaaggacgacgggaactacaagacacgtgctgaa
gtcaagtttgagggagacaccctcgtcaacaggatcgagcttaagggaatcgatttcaaggaggacggaaacatcctcgg
ccacaagttggaatacaactacaactcccacaacgtatacatcacggcagacaaacaaaagaatggaatcaaagctaactt
caaaattagacacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccct
gtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagatcccaacgaaaagagagaccacatggtcct
tcttgagtttgtaacagctgctgggattacacatggcatggatgaactatacaaa

NCBI Reference Sequence: U70496.1

Nucleotides sequence of *green fluorescent protein* (*gfp*) gene; 714 bp

atgagtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgt
cagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccat
ggccaacacttgtcactactttctcttatggtgttcaatgcttttcaagatacccagatcatatgaaacggcatgacttttcaag
agtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaggatgacgggaactacaagacacgtgctgaagtc
aagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaat
tggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattaga
cacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttacc
agacaaccattacctgtccacacaatctgccctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttg
taacagctgctgggattacacatggcatggatgaactatacaaa

NCBI Reference Sequence: U17997.1

Nucleotides sequence of *green fluorescent protein* (*gfp*)_A206K gene; 714 bp

atgagtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgt
cagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccat
ggccaacacttgtcactactttctcttatggtgttcaatgcttttcaagatacccagatcatatgaaacggcatgactttttcaag
agtgccatgcccgaaggttatgtacaggaaagaactatatttttcaaggatgacgggaactacaagacacgtgctgaagtc
aagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaat
tggaatacaactataactcacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattaga
cacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttacc
agacaaccattacctgtccacacaatctaagctttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttg
taacagctgctgggattacacatggcatggatgaactatacaaa

Underlined sequences represent the mutation position

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10. LIST OF PUBLICATIONS

Publications without peer review process

1) Mohammad, H., Pasha, A., Maiss, E. (2017). Labelling of beet mosaic virus (BtMV) with green and red fluorescent proteins and analysis of distribution in *Nicotiana benthamiana*. In: Proceedings of the Sixth Joint Meeting of the DPG working Group"Virus Diseases of Plants" and the "Nederlandse Kring voor Plantevirologie", 27.-28.03. 2017, Rheinische Friedrich-Wilhelms Universität in Bonn

2) Mohammad, H., Laufer, M., Varrelmann, M., Maiss, E. (2016). Development of virus-induced gene silencing (VIGS) based on BNYVV and BSBMV. In: Proceedings of the 60th German Plant Protection Conference, 20.-23.09.2016, Martin-Luther-Universität Halle-Wittenberg

3) Mohammad, H., Laufer, M., Varrelmann, M., Maiss, E. (2016). Development of virus-induced gene silencing (VIGS) based on the *Beet necrotic yellow vein virus* and *Beet soil-borne mosaic virus*. In: Proceedings of the 48th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 07.-08.03.2016, Leibniz Universität Hannover

4) Laufer, M., Mohammad, H., Varrelmann, M., Maiss, E. (2016). Viability of Benyvirus reassortants and co-infection exclusion in *Nicotiana benthamiana*. In: Proceedings of the 48th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 07.-08.03.2016, Leibniz Universität Hannover

5) Mohammad, H., Laufer, M., Varrelmann, M., Maiss, E. (2015). Applications of Benyvirus full-length clones- development of virus induced gene-silencing vectors and study of interactions in mixed infections. In: Proceedings of the 47th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 16.-17.03.2015, Humboldt-Universität zu Berlin

6) Laufer, M., Mohammad, H., Varrelmann, M., Maiss, E. (2015). Construction and fluorescence labeling of infectious full-length cDNA clones of *Beet soil borne mosaic virus* (BSBMV) and *Beet necrotic yellow vein virus* (BNYVV). In: Proceedings of the 47th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 16.-17.03.2015, Humboldt-Universität zu Berlin

Bachelor and Master Thesis

1) Mohammad, H. (2013). Development of a "plant-based" vaccine against the Lungworm (*Dictyocaulus viviparous*) of cattle, Leibniz Universität Hannover

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