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On the track of virus yellows resistance in *Beta vulgaris* – molecular tools and resistance mechanisms for disease management

On the track of virus yellows resistance in *Beta vulgaris* - molecular tools and resistance mechanisms for disease management

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List of abbreviations

3'CITES	3'cap-independent translation enhancers
A / Ade	Adenine
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
A_{405}	Absorption at 405 nm
AA	Amino acid
acc.-no.	Accession number
AD	Activation domain
ADP	Adenosine diphosphate
agroinoculation	*Agrobacterium tumefaciens* mediated inoculation
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Avr	Avirulence effectors
B. vulgaris	*Beta vulgaris* ssp. *vulgaris*
BaYMV	Barley yellow mosaic virus
BChV	Beet chlorosis virus
BCTV	Beet curly top virus
BD	Binding domain
bHLH	Basic helix–loop–helix
bi	Biallelic
BiFC	Bimolecular fluorescence complementation
BLYV	Beet leaf yellowing virus
BMYV	Beet mild yellowing virus
BNYVV	Beet necrotic yellow vein virus

bp	Base pairs
BtMV	Beet mosaic virus
BWYV	Beet western yellows virus
BYV	Beet yellows virus
C	Cytosine
CABYV	Cucurbit aphid-borne yellows virus
CaMV	Cauliflower mosaic virus
CC	Coiled-coil
cDNA	Complementary DNA
CI	Cylindrical inclusion protein
CLSM	Confocal laser scanning microscopy
ClYVV	Clover yellow vein virus
COX	Cytochrome oxidase
CP	Capsid protein
C-terminus	Carboxyl-terminus
CVYV	Cucumber vein yellowing virus
D	Aspartic acid
DAMPs	Damage-associated molecular patterns
DBD	DNA-binding domain
DCL	Dicer-like
DNA	Deoxyribonucleic acid
DOBA	Dropout base agar
DODA	DOPA 4,5-dioxygenase
dpi	Days postinoculation
dsRNA	Double stranded RNA

E	Glutamic acid
E. coli	*Escherichia coli*
eIF	Eukaryotic translation initiation factor
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethyl methanesulfonate
ETI	Effector-triggered immunity
G	Glycine
G	Guanine
Gal	Galactose
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
Glu	Glucose
GMO	Genetically modified organisms
GOI	Gene of interest
gRNA	Guide RNA
GTP	Guanosine triphosphate
GUS	β-glucuronidase
H	Histidine
HA	Influenza hemagglutinin
HC-pro	Helper component protease
het	Heterozygous
hom	Homozygous
HR	Hypersensitive response
inc	Inoculated
inf	Infected

K	Lysine
kb	Kilo base pairs
kDa	Kilodaltons
KO	Knockout
L	Leucine
lam	Lamin
L-DOPA	L-3,4-dihydroxyphenylalanine
LMV	Lettuce mosaic virus
LOV	Light, oxygen, or voltage
LRRs	Leucine-rich repeats
LZ	Leucine-zipper
m^7G	7-methylguanosine
MAMPs	Microbial-associated molecular patterns
MP	Movement protein
mRFP	Monomeric RFP
mRNA	Messenger RNA
MSNV	Melon necrotic spot virus
NBS	Nucleotide binding sequence
nCBP	Novel cap binding protein
NHR	Nonhost resistance
nt	Nucleotides
N-terminus	Amino-terminus
OD	Optical density
OD600	Optical density at 600 nm
ORF	Open reading frame

P1-pro	P1-protease
PABP	Poly-(A)-binding-protein
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PIC	Pre-initiation complex
PIPO	Pretty interesting potyvirus ORF
PLRV	Potato leaf roll virus
PPI	Protein-protein interaction
PPV	Plum pox virus
PRRs	Pathogen transmembrane pattern recognition receptors
P-site	Peptidyl site
PTI	PAMP-triggered immunity
PVMV	Pepper veinal mottle virus
PVY	Potato virus Y
QTL	Quantitative trait loci
R	Arginine
R genes	Resistance genes
Raf	Raffinose
RdRp	RNA-dependent RNA polymerase
RDV	Rice dwarf virus
RFP	Red fluorescent protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species

RTD	Read-trough domain
RTM	Restricted TEV movement
RT-PCR	Reverse-transcription PCR
RT-qPCR	Reverse transcriptase quantitative PCR
RYMV	Rice yellow mottle virus
SA	Salicylic acid
SAR	Systemic acquired resistance
SCAR	Sequence characterized amplified region
SD	Synthetic defined medium
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sGFP	Synthetic green fluorescent protein S65T
sgRNA	Subgenomic RNA
siRNA	Small interfering RNA
ssDNA	Single-stranded DNA
-ssRNA	Negative single-stranded RNA
+ssRNA	Positive single-stranded RNA
T	Threonine
T	Thymine
TAIR	The *Arabidopsis* Information Resource
TAS-ELISA	Triple antibody sandwich ELISA
T-DNA	Transfer DNA
TEV	Tobacco etch virus
Ti	Tumour-inducing
TILLING	Targeting induced local lesions in genomes

TIR	Toll- and interleukin 1 receptor
tRNAi	Initiator RNA
TRV	Tobacco rattle virus
TuMV	Turnip mosaic virus
TuYV	Turnip yellows virus
U	Uracil
VIGS	Virus-induced gene silencing
VPg	Viral protein genome linked
VY	Virus yellows
W	Tryptophan
wpi	Weeks posintoculation
w/v	Weight/volume
wt	Wild type
YTH	Yeast two-hybrid

1. Introduction

Until 2050 the world's population is expected to reach up to 9 billion people. With an increasing number of people, food availability appears to be one of the main factors to impede worldwide famine. Maximum food availability depends on extensive breeding and agricultural efforts, to gradually increase crop yields (Alexandratos and Bruinsma, 2012; Gu *et al.* 2021). However, food distribution and food waste have to be considered as well in regards of food availability (World Economic Forum, 2021) but will not be part of this thesis. Here, breeding strategies and techniques such as hybrid breeding or genome editing have to be mentioned as powerful tools to increase the genetic potential of plants (Breseghello and Coelho, 2013; Galvez *et al.* 2014). Abiotic and biotic stress factors are the two main causes negatively affecting the genetic potential of crops. Abiotic stress factors for plants are caused by limited or changed environmental conditions; this can be unavailability of nutrients or other effects mostly caused by the advancing climate change (Dresselhaus and Hückelhoven, 2018). Climate change favours the occurrence of more often, extreme weather events or successively events such as water availability or temperature, decreasing yields worldwide (Wiebe *et al.* 2019). While the consequences of the climate changes must be tackled by plant science research in the future (Atkinson and Urwin, 2012; Dresselhaus and Hückelhoven, 2018), biotic stress factors are an already existing threat for worlds food production.

Biotic stress is a result from various plant diseases caused by pathogenic fungi, oomycetes, nematodes, insects, bacteria, and viruses. These plant diseases are estimated to cause significant crop yield losses (Gull *et al.* 2019). Yield reduction induced by multiple biotic stress factors in total varies, but for some of the most important crops worldwide they have been reported to reach levels of up to e.g., 40.9 % in rice, 28.1 % in wheat or 41.1 % in maize even with chemical plant protection

available (Savary *et al.* 2019). Apart from reducing yields, plant diseases can reduce food quality and can cause a threat for consumer´s health, resulting all together in an economic risk for the farmer (Savary *et al.* 2019). Therefore, chemical plant protection was predominantly used to control these biotic stress factors directly, or in the case of viruses the insect vectors, to ensure high yields. However, chemical plant protection, in the public eye, is often seen as an environmental threat due to e.g., bee toxicity, causing different pesticides to lose their approval especially within Europe, resulting in the loss of active ingredients (Marchand, 2023). As a result of lost active ingredients, similar mechanisms of the plant diseases are repetitively targeted by the available pesticides. This high selection pressure within the pests or vector population, results in emerging resistances leaving the farmer without effective pest control. Furthermore, chemical plant protection is cost intensive, thus resistant cultivars are highly desirable especially for plant diseases which cannot be targeted directly such as these caused by plant viruses (Marchand, 2023). Viruses are among the most agriculturally important plant pathogens and were estimated to cause annually more than 30 billion dollars in economic losses (Jones and Naidu, 2019). They can infect most crops including sugar beet (*Beta vulgaris* subsp. *vulgaris* L.) where they cause an estimated yield and revenue loss of ~18 %, which can reach much higher rates at individual locations or during epidemic years (Rao and Reddy, 2020). Additionally viral diseases make up approximately half of all known emerging or re-emerging diseases in crops and lower food quality (Jones and Naidu, 2019).

Viruses are defined by the lack of a continuous membrane, absence of protein synthesizing machinery, a genome of either RNA or DNA, but not both, and the production of multiple components for their replication, which self-assemble the virion instead of binary fission. This makes plant viruses obligatory biotrophic parasites, which depend on their host for replication, recruiting the hosts cellular machinery for

their own replication (Hull, 2014). Unlike animal viruses, plant viruses are unable to perform endocytosis, because plant cells are enclosed by a cell wall, which must be overcome by mechanical damage. To spread from plant-to-plant external support is needed by e.g., parasitic plants, oomycetes, fungi, human activities and most often insect vectors. Even though their genomes are quite small and simple compared to the other plant pathogens, it is largely unknown how plant viruses interact with their host, resulting in the plethora of disease symptoms and yield losses (Hull, 2014; Li *et al.* 2020). Moreover, it is often unknown how plants resist these viral infections, for this reason, plant virologists frequently work on identification and characterization of resistance mechanisms and developed in addition different (molecular-) techniques and tools to facilitate these efforts. Therefore, this work should shed light on the development of molecular tools helping to identify plant virus resistance and furthermore if resistance against members of virus yellows (VY) disease can be implemented into sugar beets.

1.1 Virus yellows disease in sugar beets

Virus yellows disease in sugar beet (leaves) is an emerging problem in European sugar beet cultivation areas and is caused by multiple, aphid transmissible virus species. The ones present in Europe, have received their names from the leaf chlorosis caused by each of the viruses, respectively. These viruses are namely beet yellows virus (BYV; genus *Closterovirus*), beet mild yellowing virus (BMYV; genus *Polerovirus*) and beet chlorosis virus (BChV; genus *Polerovirus*) (Stevens *et al.* 2005b). Experimentally determined yield losses varied for the viruses and cultivars tested. Depending on time of inoculation and inoculation density, favouring early infection and high density, yield losses between inoculated to non-inoculated plots resulted in losses of approximately 11 - 47 % for BYV, 23 - 29 % for BMYV and 24 - 30 % for BChV (Hossain *et al.* 2021;

Stevens *et al.* 2004); R. Hossain personal communication). Besides that, beet mosaic virus (BtMV; genus *Potyvirus*) is an aphid transmissible virus, which is often associated with VY due to its transmission but generates mosaic symptoms instead of chlorosis in sugar beet. Still, BtMV is thought to exert synergistic effects in mixed infections with traditional members of the VY disease (Wintermantel, 2005). Thus, BtMV will be included as a member of VY in this work. Next to VY, beet curly top virus (BCTV) (Wintermantel and Kaffka, 2006) and beet necrotic yellow vein virus (BNYVV) (Liebe and Varrelmann, 2022) have to be mentioned as economically important viral diseases of sugar beet but will not be discussed any further here. However, VY is currently the fastest emerging threat in European sugar beet cultivation (Hossain *et al.* 2021). This emergence can be explained by the loss of neonicotinoid seed treatment due to an European Union wide ban in 2019 (European Commission, 2023). Neonicotinoids were effectively used in previous decades to control the aphid vector population, thus breeding efforts did not concentrate on VY, leaving the growers without an effective control measurement against the viral disease (Hauer *et al.* 2017). Monitoring activities carried out from 2017 to 2019 showed, especially in northern and western Europe, high abundance of BYV and the poleroviruses, while BtMV was not as widespread (Hossain *et al.* 2021). However, BtMV abundance could be underrepresented as plants with typical yellowing symptoms were sampled. Still, it is obvious that VY is a threat for sugar beet cultivation since 2019, hence breeding efforts now focus on generating VY-resistant plants.

1.2 Biological properties of the virus yellows disease

The genus *Polerovirus,* with its type-member potato leaf roll virus (PLRV), belongs to the family *Solemoviridae* (Sõmera *et al.* 2021). Out of this genus BMYV, BChV, beet leaf yellowing virus (BLYV) and beet western yellow virus (BWYV) were shown to infect

sugar beet, but the two latter have not been detected in Europe so far (Stevens *et al.* 2005a; Yoshida and Tamada, 2019). Likewise, within recent monitoring activities, the previously non-beet infecting polerovirus turnip yellows virus (TuYV) was detected in sugar beet, suggesting a potential host spill over which has not been investigated further (Filardo *et al.* 2021; Puthanveed *et al.* 2023). All poleroviruses are phloem-limited and exclusively aphid transmitted following a persistent circulative non-propagative mode. Multiple aphid species are able to transmit VY, here especially the green peach aphid *Myzus persicae* has to be mentioned as the most important vector for VY, and with less economic importance the black bean aphid *Aphis fabae* (Stevens *et al.* 2005a). The virions are non-enveloped, icosahedral particles with a diameter of approximately 26 nm consisting of a positive single stranded RNA (+ssRNA) genome of ~ 5.3 - 5.7 kb length with currently seven confirmed open reading frames (ORF; Figure 1a). In the following, a short overview on the genome organization of poleroviruses will be given, a more detailed description can be found in the recent review by Delfosse *et al.* (2021). The first three ORFs (ORF 0 - 2) at the 5'half of the genome are directly translated from the genomic RNA while the other four ORFs (ORF 3a - 5) downstream of a non-coding region of approximately 200 nucleotides are translated from subgenomic RNA (sgRNA) (Delfosse *et al.* 2021). Starting at the genome´s 5'end with a leader sequence, the translation initiation of ORF 0 starts at an AUG codon resulting in the P0 silencing suppressor (Kozlowska-Makulska *et al.* 2010). Ribosomes can skip the ORF0 through leaky scanning of its AUG codon, enabling them to initiate the translation at the ORF1 start codon (Hipper *et al.* 2013). The translation of ORF2 is a result of a ribosomal frameshift (-1) caused by a "shifty sequence" in ORF1 encoding the P1 + P2 fusion protein (Miller *et al.* 1995). Both ORF1 and ORF2 gene products were shown to play an essential role in viral replication (Prüfer *et al.* 1999; Stevens *et al.* 2005a). Thus, the P1 + P2 readthrough protein was

found to be a RNA-dependent RNA-polymerase (RdRp) (Mayo and Ziegler-Graff, 1996). However, the ORF1 gene product P1 displays multiple functions in the viral life cycle (Figure 1a). The ~ 70 kDa protein contains two transmembrane domains at its N-terminus, which are involved in formation of replication complexes (Hipper *et al.* 2013). P1 also represses jasmonic acid accumulation in plants, which is a defensive reaction towards the virus transmitting aphids, hence manipulating the vector-plant interaction (Patton *et al.* 2020). Furthermore, within the P1 the viral genome-linked protein (VPg) is encoded and released during maturation. Using the type-member PLRV, it was shown that P1 contains a central serine protease, which has a self-cleaving function (Li *et al.* 2007). Thus, releasing a protein from the P1 C-terminus with an apparent molecular weight of ~ 25 kDa. This 25 kDa protein shows RNA binding ability and is postulated to be a VPg precursor, which covalently binds to the 5'end of the virus genome (Figure 1a), and further maturates to the functional VPg (Prüfer *et al.* 1999). The mature VPg has a size of ~7 - 8 kDa (Mayo *et al.* 1982) and functions like a messenger RNA (mRNA) cap. Cap structures are needed to protect the mRNA from degradation or to recruit translation initiation factors, subsequently engaging the translation of mRNA, while for viruses, the VPg fulfils this task (Delfosse *et al.* 2021; Eskelin *et al.* 2011). Unfortunately, the exact VPg sequence of poleroviruses is currently unknown as the putative VPg encoding sequences are some of the most diverse sequences in poleroviral genomes and the protein is highly disordered (LaTourrette *et al.* 2021). Still, first investigations have been carried out as the N-terminal sequence for PLRV-VPg was determined experimentally (van der Wilk *et al.* 1997), while the C-terminus remains unknown. The ORF2 is followed by an intergenic non-coding region dividing the ORF clusters between the genomes 5'half and the 3'half. The ORFs from the genomic 3'half are translated from a sgRNA, which is synthesized by the RdRp. After synthesizing the sgRNA, the ORFs at the viruses 3'half

can be translated. Encoded near the 5'end of the sgRNA is P3a by the non-AUG initiated ORF3a, which is required for the viral long-distance transport throughout the plant (Smirnova *et al.* 2015) . Likewise, the gene products of the other sgRNA ORFs are involved in virus movement (Bruyère *et al.* 1997; Ziegler-Graff, 1996). Hence, ORF3 encodes the capsid protein (CP) which is needed for virion formation. Moreover, through a translational read-trough of ORF3's amber stop codon, P5 (P5 read-trough domain (RTD)) is produced (Mutterer *et al.* 1999). The resulting fusion Protein P3 + P5 is pivotal for the vector transmission (van den Heuvel *et al.* 1991) and is involved in virus accumulation, symptom induction, as well as systemic spread throughout the plant. As for ORF1 on the genomic mRNA, the translation of ORF4 encoding the movement protein (MP) is achieved via a leaky scanning of the respective ORF3 AUG start codon (Mayo and Ziegler-Graff, 1996). Further investigations on non-beet infecting viruses from the genus *Polerovirus* propose the existence of two additional sgRNAs coding for additional proteins of yet unknown function (Hwang *et al.* 2013). However, existence of these sgRNAs were not yet shown in beet infecting virus species. Due to highly conserved CP sequences, serological discrimination of the beet infecting poleroviruses BChV, BMYV and BWYV is difficult (Govier, 1985; Stevens *et al.* 2005a). On the other hand, they differ in host range and can be distinguished by species specific primers targeting the sequence diversity at the 5'ends of the viral genome (Stephan, 2005). BMYV host range includes for example parts of *Chenopodiaceae*, weed species including *Capsella bursa-pastoris* and *Senecio vulgaris*, as well as sugar beet and spinach (Stevens *et al.* 1994; Yoshida and Tamada, 2019). BWYV has a similar host range as BMYV but infects a wider range of weeds and additionally lettuce and broccoli (Duffus, 1975). BChV displays a narrower host spectrum compared to BMYV and BWYV as it does not infect *Montia perfoliata* and *Capsella bursa-pastoris* (Hauser *et al.* 2000; Hauser *et al.* 2002). The most recent

collection of experimental host ranges for beet infecting poleroviruses can be found in the review of Yoshida and Tamada (2019).

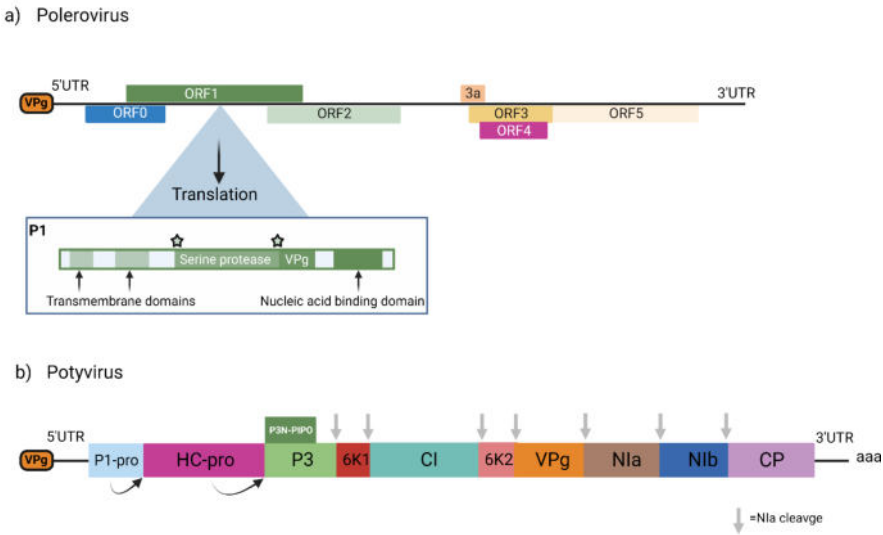

Figure 1: Schematic view of the genome organization for **a)** beet infecting poleroviruses. Poleroviral open reading frames (ORF) are shown with a detailed view on the domains of the translated P1-protein. The 5‘and 3‘untranslated regions (UTR) are indicated by straight lines. Viral protein genome linked (VPg) is shown at 5’UTR. Serine protease cleavage sites involved in VPg maturation identified by van der Wilk et al. (1997) and Li *et al.* (2007) are indicated by stars. **b)** Schematic view of BtMV genome organisation. VPg is shown at the 5’UTR and poly-(A)-tail is indicated at the 3’UTR. Genomic BtMV cistrons are shown as coloured boxes. Black arrows indicate autocatalytic cleavage sites of P1 and HC-pro respectively. NIa cleavage sites are shown as grey arrows. Figure created with BioRender.com.

BtMV belongs to the genus *Potyvirus*, which belongs to the family *Potyviridae*. The +ssRNA genome of ~ 10 kb is enveloped by its coat protein, forming ~ 730 nm long and ~ 13 nm wide flexuous filamentous virions (Fujisawa *et al.* 1983; Nemchinov *et al.* 2004). BtMV can be transmitted in a non-persistent manner by aphids as well as through infectious plant sap, which needs a mechanical damage of plant tissue for entry (Dusi and Peters, 1999). Similar to poleroviruses, viruses belonging to the genus *Potyvirus*, have a VPg at their 5’end, which has a comparable function to the poleroviral VPg. However, with a size of ~ 22 kDa they display neither structural nor sequence

resemblance (Figure 1b) (Eskelin *et al.* 2011). Indeed, potyviruses have a poly(A)-tail at their genomic 3'end, even more mimicking a cellular mRNA. During the translation of the potyviral genome, a single polyprotein is produced and subsequently cleaved by encoded proteases (Hull, 2014; Revers and García, 2015). Starting at the polyproteins N-terminus, the P1-protease (P1-pro) is encoded, which is a protease cleaving out itself and stimulating the helper component protease (HC-pro). HC-pro has multiple functions involved in transmission, as a silencing suppressor and as a protease. HC-pro autocatalytically cleaves itself from the polyprotein and is followed by P3, which is involved in symptom development and viral replication (Revers and García, 2015; Urcuqui-Inchima *et al.* 2001). The following proteins are the 6K1 protein influencing the jasmonic acid biosynthesis (Bera *et al.* 2022), the cylindrical inclusion (CI) protein, which is a helicase and forms inclusion bodies in the cytoplasm needed for replication, and the 6K2 protein, which is a potential transmembrane anchor for the potyviral replication complex. Next up is the VPg, followed by the NIa-protease, which cleaves the largest part of the polyprotein except the already mentioned P1-pro and HC-pro. The last two proteins within the polyprotein are the RdRp called NIb and the CP. A second ORF called pretty interesting potyvirus ORF (PIPO) is translated by a frameshift within P3, resulting in a fusion protein P3N-PIPO, which is involved in cell-to-cell movement (Hull, 2014; Revers and García, 2015; Urcuqui-Inchima *et al.* 2001). For BtMV, only a few experimental hosts have been identified mostly from the families of *Chenopodiaceae* and *Solanaceae*. Most notably infecting *Beta vulgaris and Spinacia oleracea,* however, a recent collection of host plants is not available (Grüntzig and Fuchs, 1979).

All in all, sugar beet is the most important agronomical host for viral pathogens of the VY disease complex in the past years. Due to similarities in their epidemiology and genome organisation, the two European beet infecting poleroviruses BChV and BMYV,

as well as the potyvirus BtMV, were investigated in this work with the aim to identify similar resistance mechanisms allowing to control these members of VY disease.

1.3 Resistance mechanisms against plant viruses

Multiple approaches to protect plants against viral diseases are known. They range from use of virus-free vegetative propagation plant material or seeds, cross protection with mild virus strains, increased hygiene standards during crop cultivation, control of virus vectors or even transgenic solutions (Jones, 2006). However, resistant cultivars are still considered to be the most ecological and economical effective approach to minimize agricultural losses if the resistance proves to be durable (Gómez *et al.* 2009; Jones, 2006). Still, especially RNA viruses have a high mutation rate due to their often error prone RdRp, which leads to an increased evolutionary adaptation, allowing to infect other hosts or overcome resistance (Duffy, 2018). In the past, different resistance genes (*R*-genes) have been identified and characterized against viruses, raising further research questions regarding host-pathogen interaction taking the environment into consideration (Ronde *et al.* 2014; Soosaar *et al.* 2005; Kang *et al.* 2005b). This research has a wide range, investigating on a molecular, cellular, or physiological level or even the co-evolution between virus and host. The resulting resistance mechanism are divided into two main categories, called host resistance and nonhost resistance, respectively (Baruah *et al.* 2020; Hull, 2014; Li *et al.* 2020).

Considering the amount of different virus species and plant hosts, the viral infection is rather an exception than a common outcome, as most plants are resistant to most viruses, which is called the nonhost resistance (NHR) (Baruah *et al.* 2020). This indicates that a defence mechanism in plants must exist, preventing an infection and by that conferring resistance (Li *et al.* 2020; Maule *et al.* 2007). Traditionally, NHR has been defined by non-host plants showing neither virus accumulation nor symptoms

upon inoculation, acting as a resistance on single cell level inhibiting viral replication in the initially infected cell as shown in isolated protoplasts (Baruah *et al.* 2020). All plants of one species displaying NHR confer hereby resistance against all isolates of one virus. Although the underlying mechanisms of NHR are not fully understood, they are most probable not encoded by a single *R*-gene as it is often the case for host resistance, which also could be polygenic (Mysore and Ryu, 2004). Interestingly, NHR can only act after the virus entered the cell through wounds or vectors during decapsidation. Thus, NHR is currently considered to be multi-layered and covers a broad spectrum of resistance mechanisms preventing these stages by impairing the virus replication for example by inhibiting translation of virus genomes through incompatibility of pathogen-host factors (Baruah *et al.* 2020). In brief, NHR does not necessarily need an active response of the host to confer resistance as it is rather an immunity or lack of susceptibility on the single cell level.

Host resistance on the other hand is defined as the ability of the host to hinder or arrest the spread or development of the pathogen (Hull, 2014). Still, the pathogen can infect and replicate within the initially infected cell of a host plant species. As a result of the resistance heritability, host resistance can be divided in two further subgroups the dominant and recessive resistance, which will be explained in more detail.

1.4 Dominant resistance

Plant-pathogen interaction in general can be divided into multiple layers, which according to the zig-zag model of Jones and Dangl (2006) are successive to each other if one fails. The first broader interaction between plants and pathogens is mediated by pathogen transmembrane pattern recognition receptors (PRRs) which sense rather unspecific microbial/pathogen-associated molecular patterns (MAMPS/ PAMPs) such as flagellin or chitin. The recognition of PAMPs results in PAMP-triggered immunity

(PTI) and can hamper further colonization of pathogens (Glazebrook, 2005). Where, local response reaction confines the pathogen infection to the primary infection site. However, viruses are unable to enter their host without external support, therefore a resistance reaction can only occur after entering the cell, therefore, this form of (traditional) PTI was not reported for viruses yet (Baruah *et al.* 2020). Nevertheless, if a wounding of plant tissue is needed for host plant colonization, remodelling of cell wall similar to PTI can be mediated by recognition of damage associated molecular patterns (DAMPs) during viral infections (Kozieł *et al.* 2021). Additionally, first studies occurred suggesting that the recognition of double stranded RNA (dsRNA) and the subsequent RNA interference (RNAi) reaction can also function over membranes and would therefore be an example for virus associated PTI (Amari and Niehl, 2020; Niehl and Heinlein, 2019). Compatible virus-host interactions are followed by an active signalling cascade mediating plant defence if the PTI was not sufficient to protect the plant (Ronde *et al.* 2014). Thus, following steps of plant defence are triggered. Often the first immune response is also mediated by RNAi, which is activated by dsRNA occurring during viral replication or in replicative structures and targets DNA and RNA viruses (Incarbone and Dunoyer, 2013). For this, dsRNA has to be sensed by Dicer-like (DCL) proteins, which are RNase type III-like enzymes. The dsRNA is fragmented into small interfering RNAs (siRNA) with a length of 21 – 24 nt (Jin *et al.* 2021). In the next step, siRNAs associate with the RNA-induced silencing complex (RISC) and function as the guide strands to specifically target the complementary virus RNA. Subsequently, viral RNA is cleaved and degraded by a member of the Argonaut protein family within the RISC. Secondary, siRNAs are produced by host RdRps to effectively induce local and systemic resistance (Jin *et al.* 2021; Sharma *et al.* 2013). However, this process can be time intensive and infected plants are often not fully cleared from the viral infection. Therefore, additional to RNAi on this layer of plant-pathogen interaction are dominant

resistance genes (Ronde *et al.* 2014). These genes act via intracellular host-pathogen interactions between *R*-genes/proteins and pathogenic avirulence effectors (*Avr*). This plant immune response is therefore called effector-triggered immunity (ETI). Technically, all virus-encoded proteins could be an *Avr*-factor involved in ETI (Hull, 2014; Kang *et al.* 2005b). Furthermore, pathogen colonization can result in a systemic cascade, which primes defence systems in other parts of the plant and is called systemic acquired resistance (SAR) (Vallad and Goodman, 2004). SAR is characterized by the interplay of salicylic acid (SA), jasmonic acid and ethylene as key defence hormones, which induce a broad defence response directed against various pathogens. SAR also involves expression of pathogenesis-related genes serving as reporters in SA-dependent defence mechanisms (Kunkel and Brooks, 2002) but underlying mechanisms are still under investigation and will not be described any further in this work as it would exceed the scope of this thesis.

About half of the known dominant resistances are monogenic and result most often in a hypersensitive response (HR), which follows an oxidative burst, a rapid production of reactive oxygen species (ROS), subsequently leading to a programmed cell-death of infected cells, limiting the pathogen's spread (Glazebrook, 2005; Kang *et al.* 2005b). In addition to that, few studies report other dominant resistance mechanisms such as the *RTM1*/*RTM2* in *A. thaliana* preventing the systemic virus spread of tobacco etch virus (TEV) without a HR (Chisholm *et al.* 2001). Models propose either a direct interaction of Avr- and R-protein or alternatively mediating a response in form of a "guardee" protein, which is modified due to its interaction with the pathogen and activates the R-protein reaction (Hull, 2014). Lastly, the decoy model hypothesizes that a host decoy protein renders the R protein non-functional (Głowacki *et al.* 2011). Strikingly, many dominant resistance genes share high similarity in their common features. Pathogen sensing is often mediated by proteins that contain a region of

leucine-rich repeats (LRRs) and a nucleotide binding sequence (NBS) (Głowacki *et al.* 2011). NBS-LRR proteins are the largest group of R-proteins. They further subdivide by N-terminal additions of coiled-coil (CC) sequences, or toll- and interleukin-1 receptor (TIR), respectively, to the NBS-LRR (Collier *et al.* 2011; Kang *et al.* 2005b). Other recurring features are leucine-zipper (LZ) and serine-threonine kinase domains. Thus, allowing to sense the infection and conferring resistance either by direct or indirect interaction of *R*-gene and *Avr*-gene products (Baker *et al.* 1997).

In sugar beet, some dominant resistances have been identified, which protect the plant from viral diseases. The most famous examples in practical sugar beet breeding are the *Rz1* and *Rz2* genes conferring resistance to BNYVV. While the genetic background of *Rz1* remains unknown, *Rz2* was shown to encode a CC-NBS-LRR protein (Capistrano-Gossmann *et al.* 2017). In contrast to that, no dominant resistance against VY members is currently identified or available, respectively, for sugar beet breeding. Still, three quantitative trait loci (QTL) directed against BYV were described by Grimmer *et al.* (2008) but were not sufficient to control the disease. Furthermore, the *Bm*-gene was described as an incomplete dominant resistance gene against BtMV (Lewellen, 1973). *Chenopodium amaranticolor* inoculated with infectious plant sap from sugar beets with a homozygous *Bm*-gene showed fewer local lesions indicating a reduced virus accumulation in *Bm*-plants compared to a susceptible control. Furthermore, symptom severity was decreased in homozygous *Bm*-plants, while the effects were intermediate in heterozygous progenies. However, the *Bm*-gene has not been investigated any further, except the development of a sequence characterized amplified region (SCAR) marker (Friesen *et al.* 2006), but is currently not used in commercial varieties.

1.5 Recessive resistance

Viruses have a limited genome size, making the use of host plant cell machinery pivotal for their translation, replication, and movement. If the initial host-virus interaction is perturbed, the plant is resistant without the need of an active signalling cascade and resistance response as it is the case for dominant resistance genes (Akhter *et al.* 2021; Mäkinen, 2020; Hashimoto *et al.* 2016; Sanfaçon, 2015; Truniger and Aranda, 2009). Resistance through incompatibility between virus and host factors was previously hypothesized to be one of the mechanisms behind NHR (Baruah *et al.* 2020). However, if the plant species contains dominant susceptibility factor towards a virus species in general, and the resistance is obtained by a loss of interaction mutation, it must be inherited in a recessive manner as the mutation must be present in all alleles (Hull, 2014; Sanfaçon, 2015).

In addition, the absence of factors that counteract resistance responses and result in an autoactivation of plant defence signalling if a negative regulator is missing, are a well-known alternative recessive resistance for fungal pathogens such as the *mlo*-gene in barley (Büschges *et al.* 1997), but have not been shown for viruses. In fact, identified recessive resistance have been described as loss of susceptibility by loss of interaction / null alleles of pivotal host proteins needed for virus accumulation. These so called positive regulators for viral infection can influence various stages of the pathogens multiplication such as transcription, translation, replication, cell-to-cell movement or long distance movement (Hashimoto *et al.* 2016). Examples for positive regulators of viral replication are the host integral membrane proteins TOM1, its homologs TOM3, and TOM2A. These proteins localize to the tonoplast, interact with each other and viral replication factors to facilitate the formation of tobamoviral replication complexes in *Arabidopsis.* Thus, making them necessary for efficient tobacco mosaic virus (TMV) replication in *Arabidopsis* protoplasts, conferring a virus

resistance in *tom1-1*, *tom2* or *tom3* mutants (Hagiwara *et al.* 2003; Hagiwara-Komoda *et al.* 2008). Another example is the recessive resistance allele *rim1-1* in rice, preventing rice dwarf virus (RDV) accumulation but not completely suppressing it. Resistant plants show a mutation in a gene encoding a novel NAC-domain transcription factor although the role of this protein in the virus infection cycle is unknown (Yoshii *et al.* 2010). However, it has to be noted that mutations conferring resistance by incompatibility must keep their cellular function otherwise, pleiotropic effects and by this a loss of fitness is expected.

Regardless of these identified recessive resistances and the underlying mechanism, it is known that no virus can autonomously perform protein synthesis, therefore they must recruit their host's ribosomes to obtain the viral gene products via translation (Sanfaçon, 2015). Thus, most recessive resistances fall into this category, conferring resistance by incompatibility between host factors and plant viruses trying to mimic messenger RNAs (mRNA) for their translation (Sanfaçon, 2015). This can be done either directly as shown for positive single stranded RNA (+ssRNA) viruses or through various intermediate steps as it is the case for DNA viruses. Indeed, this initiation of viral RNA translation is a central compatibility-interaction between host and virus, especially for +ssRNA viruses as this is the initial step during their replication (Diaz-Pendon *et al.* 2004). The underlying process of cellular mRNA translation initiation will be explained in more detail, to get a better understanding how translation initiation works and how viruses are able to use this mechanism for their own advantage before giving a detailed look on the resistance.

According to the central dogma of molecular biology, the mRNA is the mediator between DNA and proteins (Crick, 1970). The transcription process from DNA to RNA is described somewhere else and will not be explained as it would exceed the scope of this work (Franco-Zorrilla *et al.* 2014). However, after transcription in the nucleus,

mRNAs are capped at their 5'end by a methylated guanosine, called the 7-methylguanosine (m^7G)-cap and a stretch of adenine residues, the poly(A) tail, is added at their 3'end (Hunt, 2011). These additions have four main functions by regulating the nuclear export, preventing RNA degradation, promoting 5'proximal splicing and initiating the translation (Hunt, 2011; Mitchell *et al.* 2010). These processes follow similar strategies in all higher organisms with some unique specification for mammals, yeast, or plants (Hinnebusch, 2014). Still, plants have unique biological functions like photosynthesis, which led to different translational control mechanism (Browning and Bailey-Serres, 2015). Cytoplasmatic mRNA translation is divided into three phases starting with the initiation phase, elongation- and lastly termination phase, which need a reliable interplay of 10 or more different proteins in eukaryotic cells to ensure the correct decoding of mRNAs (Jackson *et al.* 2010). Especially the initiation phase has been studied for plants in the past as it is thought to be the main regulatory mechanism for translation and will be explained in the following (Browning and Bailey-Serres, 2015; Merchante *et al.* 2017). In plants, more than 16 different initiation factors are currently known with still increasing numbers, they are called eukaryotic translation initiation factor (eIF) 1 to 6. Moreover, each of the eIF subgroups can consist of single or multi-subunit complexes and they are often encoded by more than one functional gene (Browning and Bailey-Serres, 2015). As a result of that, multiple isoforms of the eIFs or their corresponding subunits are present in plants (Patrick *et al.* 2014). Interplay of the different isoforms with different quantities, spatial or temporal distribution, potentially have a regulatory function as well, but are not fully understood today (Merchante *et al.* 2017). In general, the translation initiation starts with an export of the mRNA from the nucleus into the cytosol, where its 5'end binds to the cap binding protein eIF4E or the plant specific eIF(iso)4E (Figure 2a), which form together with the scaffold proteins eIF4G/eIF(iso)4G

the eIF4F or eIF(iso)4F complex, respectively (Mayberry *et al.* 2011). The poly(A) tail at the mRNA 3'end is bound by the poly-(A)-binding-protein (PABP), which is also associated with the eIF4F/eIF(iso)4F complex. In the following, eIF4A, a DEAD box RNA helicase, and eIF4B, a RNA binding protein (Putnam and Jankowsky, 2013), are recruited to the eIF4F/eIF(iso)4F-mRNA complex and support the unwinding of secondary structures by ATP dephosphorylation before interacting with the 43S pre-initiation complex (PIC) and allow RNA circularization (Hinnebusch, 2014; Park *et al.* 2011). The 43S PIC is a multimeric complex on its own, consisting of the 40S ribosome subunit and the associated eIFs, namely eIF1, eIF1A, eIF3 and eIF5. To activate the 43S PIC, a ternary complex of eIF2 with the initiator RNA ($tRNA_i$) methionine-$tRNA_i$ and GTP is needed (Lorsch and Dever, 2010). The activated 43S PIC interacts with the mRNA and the aforementioned eIFs to form the 48S scanning complex in an open confirmation and scans the 5'UTR of the mRNA from 5' to 3'direction (Hinnebusch, 2014; Lorsch and Dever, 2010). If an AUG start codon in a favourable translation context is found (Kozak, 1986), the 48S complex changes its conformation into its closed form, binding the methionine-$tRNA_i$ in the ribosomal peptidyl site (P-site) and releases the eIF1 from the complex (Pisareva and Pisarev, 2014). It is not fully understood if the eIF4F/eIF(iso)4F complex dissociates at this stage from the 48S complex (Browning and Bailey-Serres, 2015). The joining of the 60S ribosomal subunit together with the 48S scanning complex is mediated by eIF5B+GTP binding to the 48S complex. During the conjunction of the subunits eIF2, eIF3, eIF5 and eIF6 are released from the 48S ribosome (Brina *et al.* 2011; Browning and Bailey-Serres, 2015; Pisareva and Pisarev, 2014). The ribosome assembly is completed when eIF5B+GTP is dephosphorylated and eIF5B+GDP together with eIF1A is released. As a result of this formation, the 80S ribosome is now able to start the following processes of elongation and termination to obtain a functional protein (Brina *et al.* 2011). During initiation

process, the function of eIF4F and eIF(iso)4F is especially intriguing, as the eIF(iso)4F complex and the eIFs of which it is made of, have been found exclusively in plants (Browning, 2004). This raises the questions if they are used for different biological purposes. The eIF4F complex displayed a higher affinity towards mRNAs with secondary structures than the eIF(iso)4F complex *in vitro,* indicating a distinct biological function (Gallie and Browning, 2001). Also, the optimal translation of intracellular mRNA appears to favour one of the isoforms even if both are able to promote the translation (Mayberry *et al.* 2009). However, to this day it is not known what advantages a multiplication of the eIF4F complex has for plants. Interestingly, the cap binding eIFs 4E and (iso)4E only share approximately 50% amino acid similarity and form exclusive complexes with their respective binding partner eIF4G and eIF(iso)4G. In absence of the correct binding partner, mixed complexes are formed, that allow translation *in vitro* (Mayberry *et al.* 2011). These finding have also been supported by numerous studies reporting that the cap binding proteins eIF4E/ eIF(iso)4E can be knocked out in plants without resulting in a pleiotropic effect indicating at least high functional redundancy for eIF4F and eIF(iso)4F (Lellis *et al.* 2002). Still, simultaneous null mutation of both *eIFiso4G* genes in *Arabidopsis thaliana* produce plants impaired in their growth, indicating the necessity of eIF4F-complexes, which potentially could be compensated by the formation of the aforementioned mixed complexes (Lellis *et al.* 2010). Interestingly, plants contain a third form of cap-binding protein, which is called novel cap binding protein (nCBP) or eIF4E-like. The nCBP shows cap binding affinity but appears not to play a role in the canonical translation, as it is rather involved in non-canonical translation (Patrick *et al.* 2014; Rhoads, 2009). As previously mentioned, viruses are unable to perform protein biosynthesis autonomously. Therefore, they must recruit their host's cell translational machinery and have to overcome the regulatory function of translation initiation. This viral translation

can be initiated through eIF-virus interaction with viral proteins covalently attached to their genome's 5'terminus resulting in initiation of a canonical translation complex together with the 3'terminus (Figure 2b) (Diaz-Pendon *et al.* 2004; Hwang *et al.* 2009). In contrast to that, some RNA viruses can recruit the eIF4F/ eIF(iso)4F complex by 3'cap-independent translation enhancers (3'CITES), mediated by secondary structures in the 3'UTR substituting the cap (Truniger *et al.* 2017). The 3'CITE uses RNA-RNA interaction to allow base pairing between 3'CITE and a 5'UTR loop, resulting in "kissing loop formation" a translation initiation process similar to the cap dependent canonical translation of mRNAs. Alternatively, some viral 3'UTRs imitate tRNAs by secondary structures and recruit the ribosomes directly (Simon and Miller, 2013; Truniger *et al.* 2017).

About half of all known ~200 virus resistance genes are of recessive nature (Kang *et al.* 2005b). Moreover, most studies mapping natural occurring recessive resistance genes against viruses, have been shown to confer resistance by mutations in specific members of the eIF4F/eIF(iso)4F complex, hindering the interaction between virus and host factors (Figure 2b) (Sanfaçon, 2015). These studies report most often a loss of susceptibility to different members of the family *Potyviridae* in inoculated leaves (Kang *et al.* 2005b; Truniger and Aranda, 2009). Well known examples of these resistance alleles have been identified in the past, like *lsp1,* which confers resistance against the potyviruses turnip mosaic virus (TuMV), plum pox virus (PPV) and lettuce mosaic virus (LMV) in *Arabidopsis thaliana* and was mapped to be a mutation within eIF(iso)4E resulting in a premature stop codon (Lellis *et al.* 2002). In contrast to that, resistance alleles *rym4-6* against barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) (both genus *Bymovirus*) were mapped to mutations in eIF4E (Kanyuka *et al.* 2005; Stein *et al.* 2005). Similar observations regarding resistance were made for the *pvr1* and *pvr2* alleles in pepper species resulting in a loss of susceptibility to

potato virus Y (PVY; *pvr1*), TEV (*pvr2*/*pvr1*) or pepper veinal mottle virus (PVMV; *pvr2*) (Ruffel *et al.* 2005; Ruffel *et al.* 2006). Today, only the recessive *mo1²* allele in lettuce has been reported to result in a reduced virus accumulation instead of a full resistance of whole plants depending also on the used virus isolate (Candresse *et al.* 2002). Interestingly, even though *pvr1* and *pvr2* have been mapped to eIF4E, the conferred resistance mechanisms appear to work differently, even though no virus accumulation was detected in inoculated leaves of plants carrying one of the alleles respectively. However, plants with *pvr1* displayed no virus accumulation in the protoplast, while *pvr2* carrying protoplasts showed no reduced virus accumulation. Therefore, *pvr2* resistance is thought to be conferred by an impaired cell-to-cell movement (Kang *et al.* 2005b; Ruffel *et al.* 2005; Ruffel *et al.* 2006). Hence, the underlying mechanisms of eIF-mediated resistance are not fully understood or have not been characterized in depth but appear to work differently for specific virus-host combinations (Jiang and Laliberté, 2011). Therefore, multiple theories for the underlying molecular mechanisms have been proposed (Truniger and Aranda, 2009). The first is that potyviruses resemble eukaryotic mRNA in their genome structure, however instead of a m^7G-cap they carry a viral genome-linked protein (VPg) covalently attached to their 5'terminus and a poly(A) tail at their 3'end, which were shown to directly interact most often with eIF4E (Figure 2b) (Jiang and Laliberté, 2011; Sanfaçon, 2015). The potyvirus resistant phenotype mediated by eIF4E is often associated with a single or few amino acid exchanges (Charron *et al.* 2008). Corresponding mutations often cluster near the cap-recognition pocket of eIF4E (Monzingo *et al.* 2007), which could be located either inside or on the outer surface of the pocket forming fingers (Truniger and Aranda, 2009). Although, these mutations are seldomly involved directly in the conserved cap binding amino acids, mutations of asparagine-96 in pepper (*pvr2*) and glutamate-109 in barley (*rym6*) were found to be directly involved in cap binding and confer resistance

(Truniger and Aranda, 2009). In contrast to eIF4E mutations conferring resistance by single amino acid substitutions, natural found eIF(iso)4E alleles are often corresponding to mutations resulting in knockouts (*lsp1*) or truncated proteins (*pvr6*) (Duprat *et al.* 2002; Lellis *et al.* 2002). Still, there is a high probability that also for eIF(iso)4E single amino acid exchanges are sufficient to disturb the interaction between VPg and eIF.

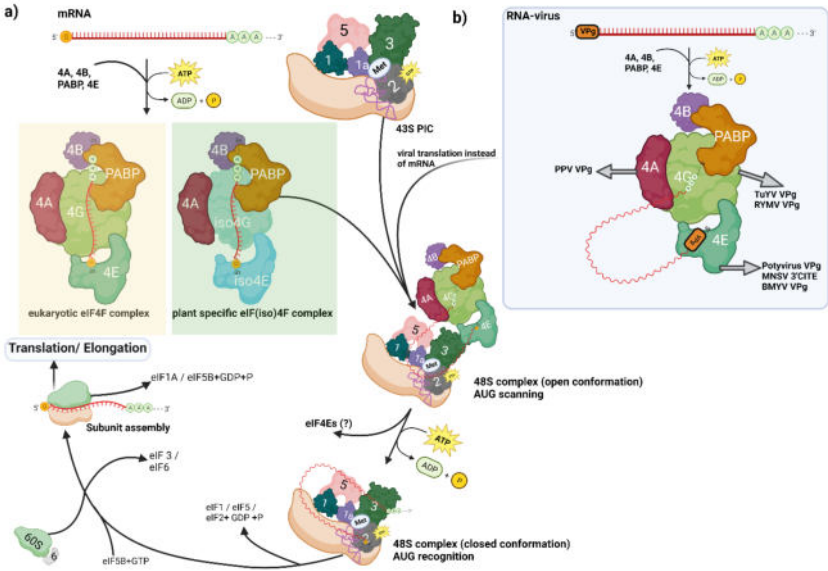

Figure 2 a) Schematic view of mRNA translation mediated by eukaryotic translation initiation factors (eIF) in plant cells. If not stated otherwise numbers refer to eIF name. mRNA cap is recruited by eIF4F complex or eIF(iso)4F complex and binds to the cap binding protein eIF4E/eIF(iso)4E while the poly(A) tail binds to poly-A-binding protein (PABP). The 43S pre-initiation complex (PIC) is formed by the 40S ribosome subunit and eIF1, eIF1A, eIF3 and eIF5. The 43S PIC is activated by a ternary complex of eIF2+methionine-tRNA$_i$+GTP. Both eIF4F and 43S PIC form the 48S complex in an open conformation, scanning for an AUG start codon in a favourable context. After the start codon is correctly identified, 48S complex changes into closed conformation and the methionine-tRNA$_i$ binds in the ribosomal P-site. Multiple eIFs (1,2,4,5) dissociated from the 48S complex allowing the ribosomal 60S subunit to join the 40S subunit mediated by eIF5B+GTP and eIF6, with the latter being responsible for 60S subunit recruitment. After the subunits assembled, translation elongation can be performed by the ribosomes. **b)** Simplified view on how RNA plant viruses (*Potyvirus*) interact with the eIF4F/eIF(iso)4F complex of their host and how viral translation is integrated into the cellular translation machinery. Individual identified interaction partners within the eIF4F/eIF(iso)4F complex for the viral protein genome linked (VPg) or 3'cap-independent translation enhancers (3'CITES) are shown for exemplary viruses. PPV= plum pox virus (*Potyvirus*); RYMV= rice yellow mottle virus (*Sobemovirus*); MSNV= melon necrotic spot virus (*Carmovirus*); TuYV= turnip yellows virus (*Polerovirus*); BMYV= beet mild yellowing virus (*Polerovirus*). Poleroviral interaction partners have been identified in *Arabidopsis thaliana* by Reinbold et al. (2013). Figure created with BioRender.com.

Noticeable, even though recessive resistance is often regarded as a more durable resistance compared to a dominant one (Hashimoto *et al.* 2016), single amino acid exchanges within the VPg can allow the virus to overcome these resistances. This can be achieved for resistance breaking isolates either by restoring the previous susceptibility reaction with the eIF or allows the virus to recruit different eIF isoforms (Saha and Mäkinen, 2020). With regard to that, mutational studies allow to investigate co-evolution involved in virus-host adaption and show that natural amino acid exchanges conferring resistance abolish the direct interaction between eIF4E and VPg. Underlining a direct involvement in the translation initiation for the viral RNA (Charron *et al.* 2008). However, for the potyvirus TuMV, VPg precursors have been found to interact with eIF4E and eIF(iso)4E *in planta.* These interactions were localized in subnuclear structures or cytoplasmic vesicles embedded in the endoplasmic reticulum (Beauchemin *et al.* 2007; Léonard *et al.* 2004), which were shown to be pivotal for the formation of the replication complex (Schaad *et al.* 1997). In addition to that, the circularization of viral RNA caused by the eIF4F complex is important for translation (Park *et al.* 2011). By RNA circularization the VPg is brought into proximity of the 3'end, where it potentially could perform its proposed function as a primer for complementary strand synthesis (Thivierge *et al.* 2008). Therefore, a direct involvement in the formation of replication complexes cannot be excluded either and indicate a coupling of potyviral translation and replication (Puustinen and Mäkinen, 2004). Another theory explaining the impairment of cell-to-cell movement e.g., in *pvr2* carrying plants, is that instead of the individual eIF4E/eIF(iso)4E rather the eIF4F/eIF(iso)4F complex is needed for effective virus spread as the eIF4F/eIF(iso)4F associated eIF4Gs display high binding affinity to the microtubules of the cytoskeleton and could influence viral spread (Bokros *et al.* 1995). In addition to that, the subnuclear localization of TuMV-VPg-precursor and eIF(iso)4E, could be important for viral

ribonucleoprotein formation (Beauchemin *et al.* 2007). This could be comparable to the findings on umbraviruses, where it was shown that nucleolar localization of the viral transport protein is important for the subsequent particle formation allowing the virion's long-distance movement and systemic infection (Kim *et al.* 2007). Each of the proposed models could be an explanation for the observed resistance phenotypes, as well as a combination of the respective models. However, most of these virus-host interactions are highly specific for each combination and indicate a direct involvement in translation. Hence, some viruses need different eIFs to complete their replication cycle in different hosts (Jiang and Laliberté, 2011). TEV and LMV rely on eIF(iso)4E in *A. thaliana* (Duprat *et al.* 2002; Lellis *et al.* 2002), while eIF4E is needed to colonize pepper, tomato, or lettuce (Kang *et al.* 2005b; Nicaise *et al.* 2003; Ruffel *et al.* 2005). Even multiple isoforms within one host can be recruited by plant viruses as for example eIF4E and eIF(iso)4E in pepper by PVMV (Ruffel *et al.* 2006).

Beside the numerous recessive resistances in plants against *Potyviridae* (Kang *et al.* 2005b; Kim *et al.* 2014; Ruffel *et al.* 2002), eIF-mediated resistance was also found for other virus families, which do not necessarily possess a VPg or poly(A)-tail at their UTRs respectively. Like the *rymv-1* allele in rice which is associated with the loss of interaction between eIF(iso)4G and the VPg of the *Sobemovirus* rice yellow mottle virus (RYMV) (Albar *et al.* 2003; Hébrard *et al.* 2006). Another well characterized eIF-mediated resistance is the *nsv* allele in melon conferring resistance against melon necrotic spot virus (MNSV; genus *Carmovirus*), conferred by a loss of interaction between MNSV 3'CITE and eIF4E (Nieto *et al.* 2006). Additionally, first reports focusing on poleroviruses, which do have a smaller VPg with no sequence homology compared to members of the *Potyviridae* and no poly-(A)tail were published (Bastet *et al.* 2018; Gallagher, 2013; Reinbold *et al.* 2013). Reinbold *et al.* (2013) used *A. thaliana* T-DNA mutants disrupted in their respective eIFs and yeast-two hybrid (YTH) assay, to identify

protein-protein interaction (PPI) between eIFs and poleroviral VPgs. Using this approach, the predicted VPgs of BWYV, BMYV and the non-beet infecting TuYV, were shown to directly interact with eIFs of their host (Reinbold *et al.* 2013). According to the authors, TuYV interacts preferably with eIF(iso)4G1 in *A. thaliana* and to minor parts with the redundant eIF(iso)4G2, if eIF(iso)4G1 was shut down. Knocking out the respective eIF(iso)4G made the mutants less susceptible to TuYV infection, however it should be noted that knocking out both eIF(iso)4G-forms resulted in a reduced growth phenotype, which could also explain the lower virus accumulation. Another study suggests that at least one additional TuYV-isolate is not depending on eIF(iso)4G, as disrupting the respective eIF(iso)4G genes appeared to have no influence on loss of susceptibility (Gallagher, 2013). On the other hand, the close relative poleroviruses BMYV and BWYV were shown to rely on eIF4E1, even though PPI was detected between the VPgs and eIF4E2 / eIF4E3 as well. Knocking out eIF4E2 / eIF4E3 appeared to have no influence on virus susceptibility, which can be explained by the low accumulation of eIF4E2 / eIF4E3 transcripts in *A. thaliana* (Patrick and Browning, 2012). Moreover, the work of Reinbold *et al.* (2013) is the first proof that an eIF-mediated recessive resistance can be implemented against poleroviruses even though no natural occurring allele has been identified so far. More recent studies on eIF-mediated resistance concentrate on genome editing techniques using e.g., CRISPR/Cas9 to implement a recessive resistance by inducing mutations knocking out eIFs, hindering host-viral PPI without causing pleiotropic effects (Chandrasekaran *et al.* 2016; Pyott *et al.* 2016). In addition to that, Kuroiwa *et al.* (2023) were able to perform genome editing on a base level, introducing non-synonymous mutation near the cap recognition site of tomato eIF4E1 resulting in a potyvirus resistance towards potato virus y, without influencing the cap binding ability. Therefore, leaving genes important for cellular translation unaffected, does not cause any loss of fitness as it

would be expected even if no obvious phenotype can be observed in eIF knockout plants. With regard to these knockout approaches, Zafirov *et al.* (2021) showed that a knockout of the *Arabidopsis eIF4E1* resulted in a resistance to clover yellow vein virus (ClYVV) relying on this factor for its accumulation, while increasing susceptibility towards TuMV, which relies on eIF(iso)4E, was observed. Indicating, that a knockout is not a feasible breeding approach. Moreover, a synthetic potyvirus resistance allele has been designed for *Arabidopsis* by genome editing (Bastet *et al.* 2018). For this, mutations conferring amino acid changes associated with potyvirus resistance in *Pisum sativum* were introduced into the eIF4E1 of *Arabidopsis.* Interestingly changing specific amino acids near the projected cap binding site of eIF4E1 is enough to confer a loss of susceptibility towards ClYVV, while the cellular mRNA translation is not perturbed. Combining this synthetic eIF with null alleles of the susceptibility factors towards TuMV and polerovirus BWYV allowed resistance gene pyramiding, conferring resistance respectively. Furthermore, such a pyramiding approach allows to design resistances towards viruses being able to recruit both eIF4E isoforms of their host at once (Bastet *et al.* 2017; Bastet *et al.* 2018). Overall, eIF-mediated resistance has been reported for poleroviruses in *Arabidopsis*, however no investigation in the genus species *Beta vulgaris* has been carried out yet. Due to the high specificity of eIF-VPg interaction for each virus-host combination, the previous findings cannot directly be transferred (Jiang and Laliberté, 2011) to the sugar beet host system but display a promising candidate gene approach for resistance breeding against the polero-/potyviral members of the VY disease.

1.7 Implementation and identification of virus resistances for practical use

With regard to the resistance mechanism described in the previous chapters, plant virologists have developed different techniques to obtain resistant plant material. The traditional way is performing large scale screens on heterogenous germplasm to evaluate symptom appearance by expert scoring and detecting virus accumulation in plants by serological or molecular biological techniques (Jones, 2006). Such screening methods do not target a specific mechanism, nor will it investigate the underlying resistance mechanisms. Indeed, the evaluation of infected material for virus accumulation and disease severity is one of the most laborious and therefore cost intensive process identifying new resistance traits. With increasing computing power, sensory based assessment in phytopathology is on the rise (Mahlein, 2016). Using non-invasive image processing of viral infections, disease severity and to some amount also virus accumulation can be identified already. In the upcoming future these techniques will widen the “bottleneck” resistance evaluation and allow automatization of these processes (Hossain *et al.* 2023; Mahlein, 2016).

However, especially for cross pollinating plants with high genetic diversity, mostly dominant resistance alleles or QTLs can be identified by such a descriptive approach (Jones, 2006; Kang *et al.* 2005b). To identify phenotypes displaying loss of susceptibility by incompatibility, mutations must be homozygous, which would increase the number of plants to be screened drastically (Nicaise, 2014). Therefore, a more sophisticated candidate gene approach is needed and techniques combining ethyl methanesulfonate (EMS) mutagenesis with targeting induced local lesions in genomes (TILLING) can be used to generate and identify new mutations e.g., near the cap recognition site in eIF4E, however to obtain a resistant phenotype an inbreeding step is necessary to produce homozygous plants (Nicaise, 2014). Therefore, TILLING

populations can help if already known resistance mechanisms are well established like eIF-mediated resistance, but no natural resistance could be found thus far. To identify these kind of resistance mechanisms, a better understanding of virus-host interaction is needed (McLeish *et al.* 2019). For this, plant virologists developed reverse genetic systems called infectious cDNA full length clones. These cDNA clones are powerful tools to identify interaction partners between viral proteins and host proteins and by this virulence and avirulence factors (Gilchrist and Haughn, 2010; Hull, 2014). This can be done by mutating the viral genome and investigate the resulting changes on virus accumulation or distribution (Gilchrist and Haughn, 2010). Moreover, these viruses or individual viral proteins can be labelled with different reporter genes, most often fluorescent proteins, to investigate (sub-)cellular distribution giving hints on genes/proteins which could be targeted to obtain a resistance (Baulcombe *et al.* 1995; Cruz *et al.* 1996; Dietrich and Maiss, 2003). Lastly, some labelled infectious cDNA clones are able to induce pigmentation in their host's leaves, allowing a visual virus tracking by the naked eye. For instance, the integration of the Rosea1 transcription factor into TEV induced the anthocyanin (Bedoya *et al.* 2012) biosynthesis in *Nicotiana tabacum* leaves, similar results were obtained by manipulating the carotenoid biosynthesis (Majer *et al.* 2017). Such tools simplify either expert screenings due to more distinguishable symptoms or they enable a high throughput screening by the aforementioned non-invasive image processing.

Another possibility to obtain virus resistant plants would be the use of biotechnology to produce genetically modified organisms (GMO). Using *Agrobacterium tumefaciens* meditated transformation, cis/transgene plants can be produced (Galvez *et al.* 2014; Jones, 2006). The resulting GMOs could contain a previously identified dominant resistance genes from other species, but more often (partial) sequences of viral genes are used. A commercially used example, is the resistance in papaya against papaya

ringspot virus (genus *Potyvirus*), which was obtained by overexpression of the coat protein, using, similar to cross protection (Fitch *et al.* 1992), the fact that potyviruses do not colonize cells already infected by a different virus isolate (Dietrich and Maiss, 2003). More recent research focuses on RNAi, integrating sequence complementary to viral genomes, producing double stranded RNA, which is subsequently degraded within its host resulting in a resistance (Galvez *et al.* 2014). Lastly, genome editing must be mentioned as a biotechnological solution to obtain virus resistance in plants (Jin *et al.* 2021; Tenllado *et al.* 2004). Genome editing techniques allow to specifically target host genes and modify these with no or less off target effects surpassing the accuracy of TILLING approaches, while also allowing to produce transgene free progenies (Bastet *et al.* 2017; Gu *et al.* 2021). Using CRISPR/Cas the straight forward approach to obtain virus resistance is by producing more frequent homozygous mutations knocking out susceptibility factors such as eIF4E as shown e.g., for cucumber where an eIF4E knockout resulted in resistance to one ipomovirus and two potyviruses (Chandrasekaran *et al.* 2016). Such approaches can get more sophisticated, as already mentioned in the previous chapter, where synthetic eIF alleles have been designed, carrying single amino acid exchanges inhibiting the interaction between virus and host, while remaining the cellular function without resulting in a potential loss of fitness (Bastet *et al.* 2018; Charron *et al.* 2008).

2. Research objectives

Since banning the neonicotinoids in the year 2019, sugar beet growers in the European Union are facing the threat of the subsequently emerging VY (including BtMV) disease. With no effective chemical or genetical control mechanism the growers are facing insecurity in their upcoming harvests. Especially, chemical plant protection is often criticized publicly and appears to be no feasible solution for the upcoming future.

Therefore, this work aims to support the already emerging breeding efforts to identify new recessive resistance mechanisms against members of the VY, especially focusing on the beet infecting poleroviruses and BtMV. Moreover, this work provides a molecular tool, which could make traditional resistance selection less labour intensive in the future.

Recessive resistance mediated by eIFs resulting in a loss of susceptibility was previously described for potyviruses and less often for other virus species including poleroviruses, although mostly in model species. However, it is known that these interactions are highly specific and cannot be transferred to other virus and plant species. Therefore, the main part of this thesis aimed to identify protein-protein interaction between the members and isoforms of *Beta vulgaris* eIF4F-complex and the VPgs of BMYV and BChV *in vitro* and *in planta*. Furthermore, the study had the goal to knockout the respective eIFs in *B. vulgaris* by CRISPR/Cas9. Subsequently the obtained T_0 mutant-lines were evaluated for resistance against BChV and BMYV. This study provides the first evidence that an eIF-mediated resistance against the polerovirus BChV is implementable in its agronomic important host sugar beet. Furthermore, different interaction patterns and interacting amino acids within the same eIF for BMYV compared to BChV indicate that a recessive resistance can be obtained against all beet infecting poleroviruses in the future (manuscript I).

With potential resistance mechanisms identified against two out of four VY members, an infectious cDNA clone of BtMV was produced to have a reverse genetic system available for further investigation on host-pathogen interaction. Thus, the clone was labelled with the monomeric red fluorescence protein (mRFP) to investigate its cellular localization. Furthermore, the *BvMYB1* transcription factor was integrated into the viral

genome. Heterologous expression of the aforementioned transcription factor as a result of viral replication produces red pigments in leaves allowing a virus tracking by the naked eye, making it the first reporter gene manipulating the betalain biosynthesis in a member of the order *Caryophyllales* to visualize a viral infection. This new tool could simplify either the evaluation of resistances during expert evaluation or alternatively allows a fast high throughput screen by non-invasive image processing (manuscript II).

With recessive resistance available for poleroviruses and an infectious cDNA clone for BtMV to investigate host-pathogen interaction, the last part of this thesis aims to identify further resistance mechanism against BtMV. While BtMV-VPg displayed interaction with *B. vulgaris* eIFs *in vitro* and *in planta*, a recessive resistance towards BtMV could not be obtained, even though recessive resistance has been mostly reported for potyviruses. Therefore, two sugar beet lines, differing in the presence of the *Bm*-gene, were evaluated for their resistance reaction and virus accumulation to characterize the *Bm*-gene further. Finally, the infectious cDNA clone of BtMV was labelled with the green fluorescence protein (sGFP) to investigate the virus distribution in whole plants by UV-handlamp or alternatively the cellular distribution starting from infection foci under the fluorescence microscope. This work provides new insights on how to obtain BtMV resistance and the feasibility of implementing the *Bm*-gene into breeding programs (manuscript III).

3. Manuscript I: Recessive resistance against beet chlorosis virus is conferred by the eukaryotic translation initiation factor (iso)4E in *Beta vulgaris*

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Recessive resistance against beet chlorosis virus is conferred by the eukaryotic translation initiation factor (iso)4E in *Beta vulgaris*

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Summary

Eukaryotic translation initiation factors (eIFs) are important for mRNA translation but also pivotal for plant-virus interaction. Most of these plant-virus interactions were found between plant eIFs and the viral protein genome-linked (VPg) of potyviruses. In case of lost interaction due to mutation or deletion of eIFs, the viral translation and subsequent replication within its host is negatively affected, resulting in a recessive resistance. Here we report the identification of the *Beta vulgaris Bv-eIF(iso)4E* as a susceptibility factor towards the VPg-carrying beet chlorosis virus (genus *Polerovirus*). Using yeast two-hybrid and bimolecular fluorescence complementation assays, the physical interaction between Bv-eIF(iso)4E and the putative BChV-VPg was detected, while the VPg of the closely related beet mild yellowing virus (BMYV) was found to interact with the two isoforms Bv-eIF4E and Bv-eIF(iso)4E. These VPg-eIF interactions within the polerovirus-beet pathosystem were demonstrated to be highly specific, as single mutations within the predicted cap-binding pocket of Bv-eIF(iso)4E resulted in a loss of interaction. To investigate the suitability of eIFs as a resistance resource against beet infecting poleroviruses, *B. vulgaris* plants were genome edited by CRISPR/Cas9 resulting in knockouts of different eIFs. A simultaneous knockout of the identified BMYV-interaction partners Bv-eIF4E and Bv-eIF(iso)4E was not achieved, but *Bv-eIF(iso)4E*^{KO} plants showed a significantly lowered BChV accumulation and decrease in infection rate from 100% to 28.86%, while no influence on BMYV accumulation was observed. Still, these observations support that eIFs are promising candidate genes for polerovirus resistance breeding in sugar beet.

Keywords: recessive resistance, polerovirus, eukaryotic translation initiation factor, *B. vulgaris*, CRISPR/Cas9.

Introduction

Plant viruses are important agricultural pathogens which cause a significant number of plant diseases resulting in several growth defects, reduced yield and plant quality (Gómez *et al.*, 2009). Since the ban of neonicotinoid seed treatment in the European Union in 2019 for sugar beet (*Beta vulgaris* subsp. *vulgaris* L.) production, the virus yellows disease complex (VY) is an increasing problem for European sugar beet growers resulting in sugar yield losses of up to 43% in mixed infections (Hossain *et al.*, 2021). VY is caused by a complex of different aphid transmissible viruses, namely beet yellows virus (BYV, genus *Closterovirus*), beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV) (both genus *Polerovirus*) (Stevens *et al.*, 2005b). At present, there are no effective methods or resistant cultivars to prevent the spread of VY. Therefore, breeding for natural plant resistance is essential to control the disease.

About half of all known virus resistance traits in plants are of recessive nature, which are most often described to act against potyviruses (Diaz-Pendon *et al.*, 2004; Gao *et al.*, 2004; Kang *et al.*, 2005; Ruffel *et al.*, 2002) but are also found to be effective against other positive single-stranded RNA viruses such as bymoviruses (Stein *et al.*, 2005), sobemoviruses (Hébrard *et al.*, 2010), carmoviruses (Nieto *et al.*, 2006) or cucumoviruses (Yoshii *et al.*, 2004). These recessive resistances have been found to be mostly associated with natural occurring polymorphisms in eukaryotic translation initiation factors (eIFs). eIFs are host cell factors, which are functional in the process of mRNA translation, forming the multimeric eIF4F-complex. The eIF4F-complex consists of the eIF4E, which binds the mRNA cap and eIF4G, which is a scaffold protein for further eIFs during translation initiation and subsequent ribosome assembly. Exclusively, plants possess a partly redundant isoform of this complex, the eIF(iso)4F complex, formed by eIF(iso)4E and eIF(iso)4G (Browning and Bailey-Serres, 2015). Like all positive-sense single-stranded RNA viruses, members of the *Potyviridae* mimic eukaryotic mRNA in their genome structure to initiate viral protein translation. Instead of an m^7G-cap, they carry a viral protein genome-linked (VPg) covalently attached to their 5′ terminus. Potyviral VPgs were shown in numerous studies to directly interact with different eIFs of their respective host plant, which were most often identified as eIF4E or eIF(iso)4E. When the specific VPg-eIF interaction is perturbed by knockout (KO), or natural as well as artificial mutations, a recessive resistance hampering the virus infection is achieved (Sanfaçon, 2015). Coding genes of the eIFs in plants belong to multigenic families resulting in multiple eIF isoforms

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and in addition to the different eIF4Es and eIF(iso)4Es, a non-canonical translation initiation factor called nCBP (novel cap binding protein) can be found in plants as well (Robaglia and Caranta, 2006). As such, members of the eIF4E or eIF4G gene family, and their respective isoforms, can be knocked out or mutated by genome editing to non-interacting eIFs without losing viability or displaying a phenotype (Bastet *et al.*, 2018; Chandrasekaran *et al.*, 2016; Pyott *et al.*, 2016).

The genus *Polerovirus* belongs to the family *Solemoviridae* with its *B. vulgaris* infecting members BMYV, beet chlorosis virus (BChV), beet leaf yellowing virus (BLYV) and beet western yellows virus (BWYV). They are exclusively aphid transmitted following a persistent circulative non-propagative mode. The main vector is the green peach aphid *Myzus persicae* (Stevens *et al.*, 2005a; Yoshida and Tamada, 2019). So far, only BMYV and BChV have been detected in Europe (Hossain *et al.*, 2021). *Polerovirus* virions are non-enveloped, icosahedral particles with a diameter of approximately 26 nm, consisting of a positive single-stranded RNA genome of ~5.3–5.7 kb, with up to seven confirmed open reading frames (ORF) and a VPg attached to the 5′ end of the genome (Delfosse *et al.*, 2021). The first three ORFs (ORF 0–2) at the 5′ half of the genome are directly translated from the genomic RNA, while the other four ORFs (ORF 3a-5) located downstream of a non-coding region (~200 nt), are translated from subgenomic RNA (Delfosse *et al.*, 2021; Stevens *et al.*, 2005a). Furthermore, it was shown that the VPg is encoded by the ORF1 and released after proteolytic processing of the protein precursor P1 (Prüfer *et al.*, 1999). van der Wilk *et al.* (1997) were able to identify the N-terminal amino acid sequence of the VPg from the P1 of potato leaf roll virus (PLRV), however the canonical polerovirus VPg sequence is still unknown and was only estimated to have a molecular weight of ~7 kDa via gel separation (Mayo *et al.*, 1982).

Reinbold *et al.* (2013) published the first report showing a recessive resistance towards poleroviruses. Using *Arabidopsis thaliana* eIF KO plants and protein–protein interaction (PPI) studies, poleroviral VPgs were shown to interact with different *A. thaliana* eIFs. Interestingly the non-beet infecting polerovirus turnip yellows virus (TuYV) was shown to interact preferably with eIF(iso)4G1 in *A. thaliana* and to a lesser extent with the redundant eIF(iso)4G2. While the *eIF(iso)4G1* × *eIF(iso)4G2* double KO mutant of *A. thaliana* showed lower virus accumulation during TuYV infection, simultaneously knocking out the *eIF(iso)4G* isoforms resulted in reduced plant growth, which might influence viral accumulation. On the other hand, the closely related beet infecting poleroviruses BMYV and BWYV were shown to interact with eIF4E1, which, when knocked out, resulted in significant lower viral accumulation in *A. thaliana* (Reinbold *et al.*, 2013). Furthermore, it was shown by Bastet *et al.* (2018) that resistance to the potyviruses clover yellow vein virus (ClYVV) can be obtained in *A. thaliana* by mutating eIF4E1 with mutations known to mediate potyvirus resistance in *Pisum sativum*. Besides resistance to potyviruses, the synthetic eIF4E1, when combined to mutated eIF4E1 and eIF(iso)4E, mediated complete resistance against BWYV while BMYV accumulation was not compromised (Bastet *et al.*, 2018). Interestingly, closely related virus species are able to use different eIFs for translation initiation. Moreover, the same virus can depend on varying eIFs in different host species (Jiang and Laliberté, 2011), making it impossible to transfer previous findings to sugar beet.

In this study, we investigated if an eIF-mediated recessive resistance against the poleroviruses BMYV and BChV can be generated in *Beta vulgaris*. Therefore, yeast two-hybrid (YTH) and bimolecular fluorescence complementation (BiFC) were used to identify interactions between eIFs and poleroviral VPgs. Furthermore, genome edited sugar beets with eIF knockouts were generated and challenged with BChV and BMYV, respectively. While no influence on viral accumulation was observed for BMYV, BChV accumulation was significantly decreased if *Bv-eIF(iso)4E* was knocked out. Our results demonstrate that different eIF4E isoforms are exploited for viral translation by closely related beet infecting poleroviruses in the sugar beet host. Moreover, it is the first report using eIF-mediated recessive resistance to viruses in sugar beet, making *Bv-eIF(iso)4E* a target for future resistance breeding in this crop species.

Results

Identification of interaction between poleroviral VPgs and *B. vulgaris* eIFs

Within the sugar beet genome, three loci with high sequence homology to the *A. thaliana*-eIF4Es were identified, which were thereafter named *Bv-eIF4E*, *Bv-eIF(iso)4E* and *Bv-nCBP*. Additionally, four loci with close relation to *A. thaliana*-eIF4G were identified and named *Bv-eIF4G1*, *Bv-eIF4G2*, *Bv-eIF(iso)4G1* and *Bv-eIF(iso)4G2* (Table S1). By yeast complementation assay, Bv-eIF4E and Bv-eIF(iso)4E could be verified as true eIFs, as they were able to complement translation in a yeast 4E deletion strain, while Bv-nCBP was not able to promote yeast growth in this background (Figure S1). The amplification of *Bv-eIF4G2* from sugar beet cDNA could not be achieved, but the remaining six eIF candidate genes were successfully cloned. To be used as bait in YTH, eIF candidate genes were fused to the CDS1 LexA DNA binding domain (BD).

Since the exact VPg sequence of any polerovirus is still unknown, two different VPg sequences were applied: The putative poleroviral VPg determined by the estimated molecular weight (VPg), and the VPg with the remaining C-terminus of P1 (VPg + C-term), which is postulated to be a VPg precursor (Prüfer *et al.*, 1999). Each VPg sequence was fused to the B42 transcription activation domain (AD) and used as prey in YTH.

Yeast super transformation with the plasmids for each eIF-VPg combination allowed the screen for potential PPI. To test for transcriptional autoactivation of the reporter gene *LEU2*, eIF-BD or VPg-AD containing plasmids were co-transformed with an empty AD- or BD-plasmid, respectively. In YTH, Bv-nCBP and Bv-eIF(iso)4E were identified as potential interaction partners for BMYV-VPg and BChV-VPg/ BChV-VPg + C-term, while no interaction could be detected for BMYV-VPg + C-term (Figure 1). The LexA based YTH did not display interaction between the poleroviral VPgs (BMYV and BChV) and the tested members of the eIF4G-group (Figure 1). Since Bv-eIF4E could not be tested for PPI in the LexA YTH due to autoactivation of the reporter gene (Figure 1), which even could not be abolished by reciprocal exchange of BD and AD (data not shown) Bv-eIF4E was subjected to a GAL4 based YTH. The fusion of BD-Bv-eIF4E resulted in autoactivation of the *HIS3*-reporter, but no autoactivation was observed for the *ADE2*-reporter. As such, a potential interaction between Bv-eIF4E and BMYV-VPg could be detected (Figure 2). In contrast, no interaction between Bv-eIF4E and BChV-VPg + C-term was detected in the GAL4 YTH for the *HIS3*-reporter, indicating a different eIF-interaction pattern for BMYV-compared to BChV-VPg (Figure 2).

We further investigated if a single amino acid change within the Bv-eIF(iso)4E could abolish the detected interactions. Multiple

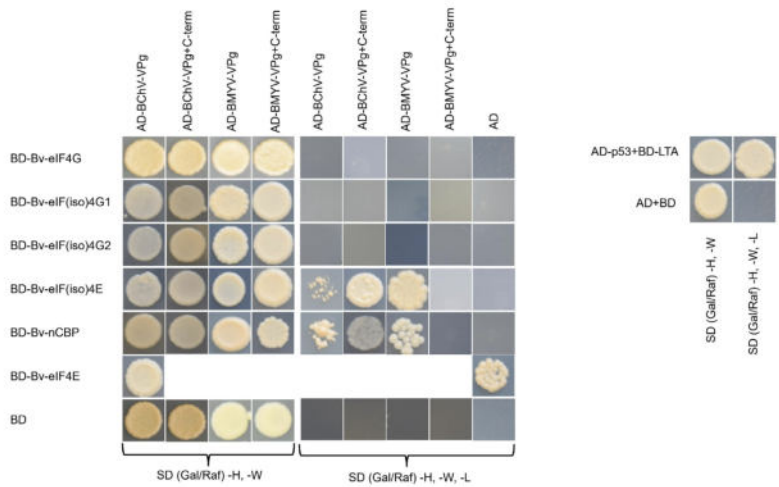

Figure 1 LexA-based YTH of direct physical interaction between poleroviral VPg/VPg + C-term and *Beta vulgaris* eIFs. Left panel: *Beta vulgaris* eIFs were fused to the binding domain (BD) and VPg/VPg + C-term to the activation domain (AD) for PPI evaluation. Yeast strain EGY48 was transformed with two plasmids containing AD and BD, respectively, positive transformants were selected on synthetic defined media (SD) lacking histidine (-H) and tryptophane (-W) using galactose and raffinose as a carbon source (Gal/Raf). To test for autoactivation, yeast cells were co-transformed with the plasmids encoding the proteins of interest and the corresponding AD or BD plasmids lacking a fusion protein. Single colonies were resuspended in water and diluted 1×10^{0}–1×10^{-3}. Then, 5 μL of each dilution was spotted on control medium SD [Gal/Raf (-H, -W)] and interaction medium lacking leucine (-L), and SD [Gal/Raf (-H, -W, -L)]. Spotted yeast was incubated at 30 °C for 3 days (control medium) or 5 days (selection medium), respectively. Only 1×10^{-2} dilution of spotted yeast is displayed. Right panel: Positive control AD-p53 with BD-LTA and the negative control AD (-empty) with BD (-empty). Expression of the fusion proteins was confirmed by Western blot analysis (Figure S2).

natural resistances against potyviruses were shown to be conferred by few non-synonymous mutations located in proximity to the cap-binding pocket of the respective eIF4E (Truniger and Aranda, 2009) but experimental data of German-Retana *et al.* (2008) showed that mutations conferring resistance to the potyvirus lettuce mosaic virus (LMV) in lettuce were unlinked to the cap-binding abilities of eIF4E. As poleroviral VPgs are smaller than the ones of potyviruses, we hypothesised that amino acids involved in cap-binding may also interact with poleroviral VPgs. Therefore, eight putative cap-binding amino acids (Monzingo *et al.*, 2007) of Bv-eIF(iso)4E were individually mutated to leucine by site directed mutagenesis (Figure 3). The Bv-eIF(iso)4E mutants were tested for interaction with BChV-VPg + C-term in YTH (Figure 3a). Mutations W47L, D81L, and W153L resulted in a complete loss of interaction between Bv-eIF(iso)4E and BChV-VPg + C-term, while W93L, E94L and R144L appeared to influence the interaction strength displaying reduced growth. On the other hand, mutations W64L and K149L did not affect the detected interaction. In addition, the interaction between Bv-eIF (iso)4E and BChV-VPg + C-term was restorable when W47L was mutated back into the wildtype sequence (L47W; Figure 3a). The mutational analysis for BMYV-VPg showed that, mutations E94L; R144L and K149L resulted in a loss of interaction, in addition to the previously identified amino acids also conferring the loss of interaction for BChV-VPg + C-term (Figure 3b), supporting the different interaction patterns for the polerovirus species analysed. The expression of the respective fusion proteins was confirmed by Western blot analysis for all YTH assays (Figures S2–S4). In summary, the LexA-YTH results showed an interaction between both poleroviral VPgs and Bv-eIF(iso)4E as well as Bv-nCBP. In addition to that, using a GAL4 based YTH approach, only for the BMYV-VPg an additional interaction with Bv-eIF4E was found. Furthermore, using the GAL4-system different interaction patterns in a mutational analysis were found for BChV-VPg + C-term or BMYV-VPg and Bv-eIF(iso)4E (Figure 3).

Due to differences in interaction strength between the eIFs and putative poleroviral VPgs, further PPI-experiments between members of the sugar beet eIF4E-group and poleroviral VPg were performed using BiFC as an *in planta* test system. BChV-VPg + C-term interacted only with Bv-eIF(iso)4E, while no interaction could be found for Bv-eIF4E and Bv-nCBP (Figure 4). For BMYV-VPg, there was no interaction found with Bv-nCBP, however an interaction between Bv-eIF(iso)4E, as well as Bv-eIF4E was found in the BiFC assay (Figure 4). The expression of all fusion proteins was confirmed by Western blot analysis (Figures S2–S5). Altogether, Bv-eIF(iso)4E is the only interaction partner identified

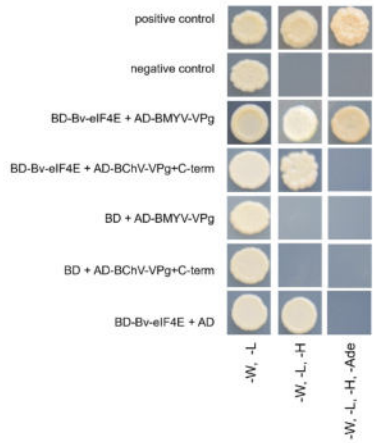

Figure 2 GAL4 based YTH experiments for interaction between poleroviral VPg/VPg + C-term and Bv-eIF4E. Bv-eIF4E was fused to the binding domain (BD) and VPg/VPg + C-term to the activation domain (AD) for PPI evaluation. Positive control: AD-p53 with BD-T; Negative control: AD-p53 with BD-Lam. Two plasmids containing AD and BD fusion proteins, respectively, were co-transformed into yeast. To test for autoactivation, yeast strain Y2HGold was co-transformed with the plasmids encoding the proteins of interest and the AD or BD plasmids lacking a fusion protein. Single colonies were resuspended in water and diluted 1×10^{0}–1×10^{-3}. Then, 5 µL of each dilution was spotted on control medium SD (-W, -L) and interaction media SD (-W, -L, -H) and SD (-W, -L, -H, -Ade). Spotted yeast was incubated at 30 °C for 3 days (control medium) or 5 days (selection medium), respectively. Only 1×10^{-2} dilution of spotted yeast is displayed. Expression of the fusion proteins was confirmed by Western blot analysis (Figure S3).

for BChV-VPg + C-term in both PPI systems (YTH and BiFC), while Bv-eIF(iso)4E and Bv-eIF4E were consistently identified as interaction partners for BMYV-VPg in both PPI systems. The putative interaction between poleroviral VPgs and Bv-nCBP was only found in YTH and not in BiFC.

Generation of eIF sugar beet knockout plants

According to the PPI studies presented in this work, Bv-eIF genes could be promising targets for engineering resistance against poleroviruses. As such, single and multiple KO lines were generated for the seven identified Bv-eIF genes in sugar beet using the CRISPR/Cas9 technology. A total of 12 GC-rich sequences with 20 bp in length, all located in Bv-eIF exons and upstream of a PAM (NGG), were selected as guide RNAs (gRNAs). Some gRNAs were gene-specific, while others targeted two closely related genes at once (Table S2). All gRNAs were introduced in a Cas9-expressing plasmid adapted for sugar beet transformation, harbouring 12 CRISPR/Cas9 KO constructs. Since the knockout of single genes from the Bv-eIF gene family might fail to produce viral-resistance due to redundancy, and since knocking out some of these genes may induce aberrant phenotypes or lethality, a multiplexing approach was adopted for generating single and multiple KO plants at once. The 12 CRISPR/Cas9 KO constructs were pooled at equimolar amounts and sugar beet protoplasts were transformed with the pooled DNA. The advantage of this strategy is that only viable Bv-eIF KO combinations are possible since lethal combinations will not result into regenerated plants. A total of 125 diploid T0 plants were obtained using the pooled strategy (Bv-O plants) and sequenced at the target loci to identify mutations for one or more of the Bv-eIF genes. A substantial amount of indels (mostly frameshift) were found in the 125 sequenced Bv-O lines: 25 lines were edited at a single gene, 42 lines contained two edited genes, 22 lines contained three edited genes, two lines carried four edited genes and one line displayed mutations in six genes (data not shown; the genotypes from all the Bv-O plants used in this study are listed in Table S2). Interestingly, the prevalence of mutations was not equally distributed among the different Bv-eIF genes: Many individual lines carrying homozygous mutations in *Bv-eIF(iso)4E*, *Bv-nCBP* and *Bv-eIF4G1* were detected. In contrast, no more than one line with a homozygous mutation within Bv-eIF4E was identified. Only heterozygous mutations were found for *Bv-eIF(iso)4G1*, *Bv-eIF(iso)4G2* and *Bv-eIF4G2*, suggesting that these genes might be essential for mRNA translation (with no redundancy). Unfortunately, a line with a simultaneous edit of *Bv-eIF(iso)4E* and *Bv-eIF4E* resulting in a double knockout was not obtained within our experiments (Tables 1 and 2; Table S2).

Additional sugar beet protoplast transformations were performed with each of the previously pooled plasmids one at a time, to obtain lines with single homozygous knockouts that were still missing for some of the Bv-eIF genes. This attempt succeeded only for Bv-eIF(iso)4E and Bv-nCBP. Two T0 lines regenerated from the single knockout strategy were included in the bioassay for virus resistance, namely Bv-D018 and Bv-G001 (Tables 1 and 2; Table S2).

Resistance screening of eIF sugar beet knockout plants to poleroviruses

The vegetatively propagated T0 plants were established from *in-vitro* culture into soil. After establishment these knockout lines were infected with BMYV (Table 1) and BChV (Table 2) using viruliferous aphids in two separate infection assays to investigate poleroviral accumulation within these plants. The regenerated plants from the edited lines obtained in this study did not display a phenotypic difference in comparison to *in-vitro* plants carrying the wildtype allele (Figure 5). Due to the use of a pooled genome editing approach, lines with multiple *eIF*^{KO} were produced and tested next to lines with a single *eIF*^{KO}. In particular, these lines were having different combinations of a triple or double knockout of *Bv-eIF(iso)4E*, *Bv-nCBP* and *Bv-eIF4G1* (Tables 1 and 2). In five independently performed BMYV bioassays all tested plants were susceptible towards BMYV, as shown in Table 1. None of the knockout lines displayed significantly altered OD_{405nm} values in the TAS-ELISA when compared with the wildtype. In addition, all inoculated plants were infected, resulting in an infection rate of 100% (Table 1). However, when knockout plants were challenged with BChV a different pattern was observed (Table 2). In detail, three independent bioassays were performed with multiple independent *Bv-eIF(iso)4E*^{KO} lines. In bioassay one, *Bv-eIF(iso)4E*^{KO} lines displayed a lower infection rate Bv-O054 (6/9; infected plants/inoculated plants), Bv-O055 (1/9) and Bv-O064 (6/11), as well as significantly lowered ELISA values

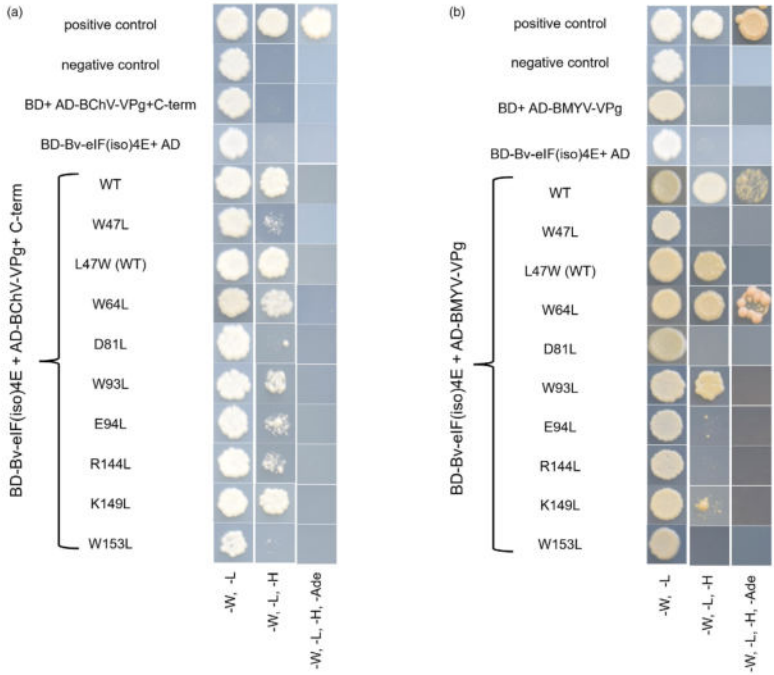

Figure 3 GAL4 based YTH experiments tested for interaction between (a) BMYV-VPg or (b) BChV-VPg + C-term and Bv-eIF(iso)4E mutated in its mRNA cap-binding amino acids. Mutations exchanging proteinogenic amino acid to leucine within *Bv-eIF(iso)4E* were introduced by site directed mutagenesis and are indicated with their position in the protein sequence. The *Bv-eIF(iso)4E* mutant was fused to the binding domain (BD) and VPg/VPg + C-term to the activation domain (AD) for PPI evaluation. Positive control: AD-p53 with BD-T; Negative control: AD-p53 with BD-Lam. Yeast was co-transformed with the two plasmids containing the AD and BD fusion proteins. To test for autoactivation, yeast cells were co-transformed with the plasmids encoding the proteins of interest and the AD or BD plasmids lacking a fusion protein. Single colonies were resuspended in water and diluted 1×10^{0}–1×10^{-3}. 5 µL of each dilution was spotted on control medium SD (-W, -L), interaction media SD (-W, -L, -H) and SD (-W, -L, -H, -Ade). Spotted yeast was incubated at 30 °C for 3 days (control medium) or 5 days (selection medium). WT = wildtype Bv-eIF(iso)4E. Only 1×10^{-2} dilution of spotted yeast is displayed. Expression of the fusion proteins was confirmed by Western blot analysis (Figure S4).

in comparison to *Bv-eIF(iso)4E*^{WT} plants. The lowest BChV infection rate of 7.14% for *Bv-eIF(iso)4E*^{KO} was found in bioassay two. In total 3 out of 42 plants were infected, thoroughly only line Bv-D018 (3/12) showed a significantly reduced ELISA value, while lines Bv-O025 (0/8), Bv-O054 (0/12) and Bv-O063 displayed no detectable infection. In bioassay three, detected ELISA values for *Bv-eIF(iso)4E*^{KO} lines were repeatedly significantly decreased, furthermore the infection rate for each line was decreased in lines Bv-O054 (3/10), Bv-O055 (9/24), Bv-O064 (3/23). A slight exception in comparison to other *Bv-eIF(iso)4E*^{KO} lines was found within the line Bv-O092, which is an isogenic line to Bv-O054, having a higher infection rate with 12 out of 21 and a significantly increased viral load. Still, the OD_{405nm} of TAS-ELISA in Bv-O092 was significantly lower compared to susceptible controls (Table 2). Summarising the BChV bioassay, a total of seven different *Bv-eIF(iso)4E*^{KO} lines from individual transformation events were challenged with BChV. Over the course of three independent experiments, 173 out of 173 plants containing the wildtype allele for *Bv-eIF(iso)4E* were infected, resulting in an infection rate of 100%. When *Bv-eIF(iso)4E* was knocked out, 43 out of 149 plants were infected, with an overall infection rate of 28.86%. It must be noted that some of these lines contained multiple homozygous edits of *Bv-nCBP*^{KO} and/or *Bv-eIF4G1*^{KO} in addition to the *Bv-eIF(iso)4E*^{KO}, such as Bv-O064. By challenging Bv-O051 with

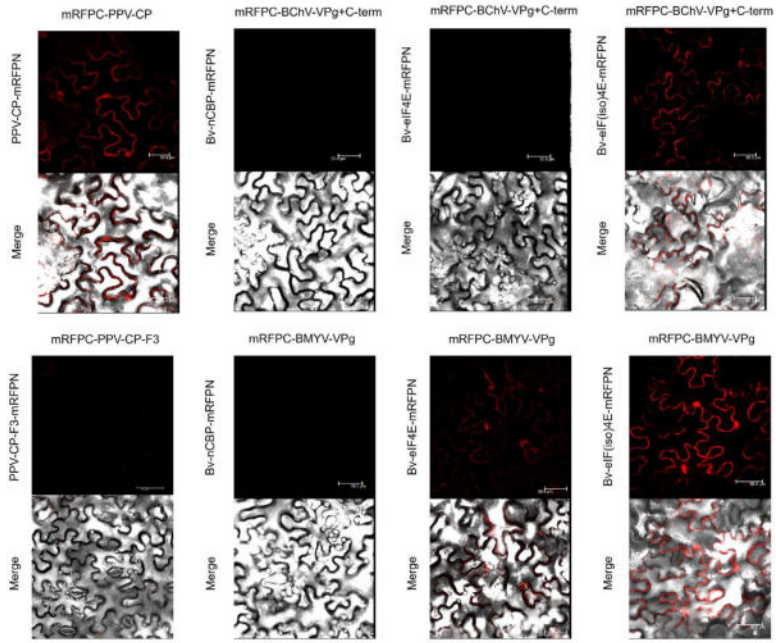

Figure 4 BiFC assay using *N. benthamiana* leaves co-infiltrated with *Agrobacterium tumefaciens* C58C1 cells harbouring pCB:Bv-Bv-nCBP-mRFPN/pCB:Bv-eIF4E-mRFPN/pCB:Bv-eIF(iso)4E-mRFPN and pCB:mRFPC-BMYV-VPg/pCB:mRFPC-BChV-VPg + C-term. Co-expression of CB:PPV-CP-mRFPN + pCB:mRFPC-PPV-CP was used as positive control, while pCB:PPV-CP-F3-mRFPN + pCB:mRFPC-PPV-CP-F was used as a negative control. CLSM images were taken at 4 dpi. Scale bars = 50 μm. Expression of the fusion proteins was confirmed by Western blot analysis (Figure S5).

BChV, it was shown that homozygous mutations of *Bv-nCBP*^{KO} and *Bv-eIF4G1*^{KO} did not influence the viral accumulation significantly in comparison to the susceptible control. Furthermore, using line Bv-O054 in all three BChV bioassays, allowed to reproduce the significantly lowered viral accumulation and lowered infection rate in independent experiments. Testing BChV-infected Bv-O054 showed in some of the tested plants slight amplification by RT-PCR using BChV specific primers, while clear amplification in *Bv-eIF(iso)4E*^{WT} plants was observed (Figure S6). Six weeks after inoculation, *Bv-eIF(iso)4E*^{WT} plants of the third experiment displayed yellowing symptoms in their oldest leaves, while lines with *Bv-eIF(iso)4E*^{KO} did not display symptoms (Figure 5), however as symptom development of poleroviral infection under greenhouse conditions is inconsistent, no further scoring was performed.

Discussion

Recessive resistance in the form of non-interacting eIF alleles is frequently used in breeding programs to obtain virus resistance usually against the VPg-carrying potyviruses (Sanfaçon, 2015). In this study, we aimed to investigate if an eIF-based recessive resistance is implementable into the sugar beet host system. Therefore, we identified and functionally characterised six *B. vulgaris* eIF isoforms of 4E and 4G. We showed that knocking out *Bv-eIF(iso)4E* in sugar beet resulted in a significantly lowered *in planta* viral accumulation of BChV. In comparison to *Bv-eIF(iso)4E*^{WT} plants, the infection rate within *Bv-eIF(iso)4E*^{KO} plants dropped from 100% to 28.86%, showing for the first time that an eIF-based recessive resistance against poleroviruses can be generated in an agronomical important host. On the other hand, challenging the Bv-eIF knockout lines with BMYV infection, had no influence on viral accumulation as part as experiments carried out here under potentially high virus pressure from the ten used viruliferous aphids. The involvement of Bv-eIF(iso)4E in the initiation of the BChV infection process is supported by the direct interaction between eIF and predicted BChV-VPg/ VPg + C-term observed in two independent PPI assays. In contrast, BMYV-VPg showed physical interaction with both functionally redundant Bv-eIF(iso)4E and Bv-eIF4E. Mutational analysis shows that a single

Table 1 Summary of BMYV bioassay challenging different eIF-knockout plants

Experiment	(T0-)line	*Bv-eIF4E*	*Bv-eIF(iso)4E*	*Bv-nCBP*	*Bv-eIF(iso)4G1*	*Bv-eIF(iso)4G2*	*Bv-eIF4G1*	*Bv-eIF4G2*	OD_{405nm}	±SD	inf/inoc (n)	Tukey test ($P \leq 0.05$)
1	Bv-WT								1.36	0.39	8/8	a
1	Bv-O050						hom		1.49	0.19	8/8	a
1	Bv-O054		hom						1.30	0.23	8/8	a
1	Bv-O055		hom	hom					1.39	0.29	8/8	a
1	Bv-O068			hom	het		hom		1.59	0.32	8/8	a
1	Bv-O097								0.88	0.56	8/8	a
1	Bv-O110	het		hom					1.64	0.39	4/4	a
2	Bv-WT								1.46	0.36	4/4	a
2	Bv-O015		hom	hom			hom		1.35	0.23	8/8	a
2	Bv-O064		hom	hom			hom		1.47	0.33	11/11	a
2	Bv-O067			hom			hom		1.25	0.14	4/4	a
2	Bv-O076			hom	het		hom		1.42	0.28	7/7	a
2	Bv-O092		hom						1.81	0.39	5/5	a
2	Bv-O096						hom		1.18	0.22	4/4	a
2	Bv-O097								1.58	0.40	6/6	a
2	Bv-O123		hom	hom					1.46	0.26	3/3	a
3	Bv-WT								0.42	0.13	8/8	a
3	Bv-G001			hom					0.34	0.12	7/7	a
3	Bv-O025	het	hom				bi		0.51	0.08	5/5	a
3	Bv-O065	het	hom	bi		het	hom	het	0.25	0.08	3/3	a
3	Bv-O097								0.40	0.12	8/8	a
3	Bv-O135	hom							0.42	0.10	5/5	a
4	Bv-WT								0.98	0.52	10/10	a
4	Bv-G001			hom					0.74	0.24	3/3	a
4	Bv-O050						hom		1.00	0.22	12/12	a
4	Bv-O051			hom			hom		1.09	0.20	10/10	a
4	Bv-O063		hom				hom		1.17	0.37	9/9	a
4	Bv-O096						hom		0.74	0.18	7/7	a
4	Bv-O097								0.75	0.28	10/10	a
4	Bv-O135	hom							0.66	0.24	10/10	a
5	Bv-WT								1.52	0.30	4/4	a
5	Bv-O097								1.70	0.71	6/6	a
5	Bv-O107						hom	het	0.97	0.37	7/7	a

Shown are the used *Beta vulgaris* lines having a homozygous (hom) or biallelic (bi) mutation in different *eIFs* resulting in a knockout of the respective eIF. Heterozygous (het) mutations are indicated as well, if not stated otherwise, the *eIF* sequence was indistinguishable from the wildtype allele. The obtained T0-lines were challenged with BMYV infection and evaluated 4 wpi by means of TAS-ELISA. Mean ELISA absorption (OD_{405nm}) standard deviation (±SD) and number of infected plants to inoculated plants (inf/inoc) are shown for BMYV infection. Same letters (a) indicate no significantly different virus contents within each experiment identified by ANOVA with a *post-hoc* Tukey test ($P < 0.05$).

amino acid change of the cap-binding amino acids within Bv-eIF(iso)4E is sufficient to disturb the VPg-interaction. These results indicate that poleroviral VPgs may interact differently with their host eIFs in comparison to the potyvirus VPg interactors as the findings of German-Retana *et al.* (2008) show that the infection of the potyvirus LMV in lettuce is unlinked to the cap-binding amino acids of eIF4E. However, only specific mutations in Bv-eIF(iso)4E resulted in loss of interaction, underlining the high specificity of the detected interaction, which also varied between the closely related BChV and BMYV (Figure 3). The amino acids evaluated are involved in mRNA cap-binding and therefore are expected to be critical for cellular mRNA translation, which is highly conserved throughout the plant and animal kingdom (Marcotrigiano *et al.*, 1997). Still, it can be speculated that at least parts of the poleroviral VPg bind to the cap-binding pocket. This could also explain the limited effects on polerovirus resistance observed by Bastet *et al.* (2018), as they introduced mutations into *A. thaliana eIF4E1*, which were previously found in potyvirus resistance alleles and therefore not directly part of the cap-binding pocket. Naturally occurring eIF-mutations in resistance alleles to potyviruses cluster in close proximity to cap-binding amino acids (Truniger and Aranda, 2009), therefore a systematic approach by performing mutational studies to fine map VPg-interacting domains within Bv-eIF(iso)4E and eIF4E is an important objective for future research on recessive resistance against poleroviruses.

It has to be noted that the putative VPg encoding region of poleroviruses is highly diverse and disordered in both species (LaTourrette *et al.*, 2021), which complicates VPg predictions. Thus, two potential VPg sequences (VPg/VPg + C-term) were used to reduce artefacts in the YTH experiments. Here, the putative BChV-VPg displayed a weak interaction with Bv-eIF(iso)

Table 2 Summary of the BChV bioassay challenging different eIF-knockout plants

Experiment	(T0-)line	*Bv-eIF4E*	*Bv-eIF(iso)4E*	*Bv-nCBP*	*Bv-eIF(iso)4G1*	*Bv-eIF(iso)4G2*	*Bv-eIF4G1*	*Bv-eIF4G2*	OD_{405nm}	±SD	Infected/inoculated (n)	Dunn test ($P \leq 0.05$)
1	Bv-WT								1.53	0.15	9/9	a
1	Bv-O054		hom						0.12	0.15	6/9	b
1	Bv-O055		hom	hom					0.02	0.05	1/9	b
1	Bv-O064		hom	hom			hom		0.01	0.03	6/11	b
1	Bv-O068			hom	het		hom		1.27	0.40	9/9	a
1	Bv-O097								1.29	0.27	10/10	a
1	Bv-O110	het		hom					1.22	0.26	9/9	a
2	Bv-WT								0.66	0.34	10/10	a
2	Bv-D018		hom						0.11	0.25	3/12	b
2	Bv-O025	het	hom				bi		0.00	0.00	0/8	b
2	Bv-O050						hom		0.96	0.66	18/18	a
2	Bv-O051			hom			hom		0.55	0.38	22/22	a
2	Bv-O054		hom						0.00	0.00	0/12	b
2	Bv-O063		hom				hom		0.00	0.00	0/10	b
2	Bv-O097								1.03	0.60	17/17	a
2	Bv-O107						hom	het	0.84	0.70	14/14	a
2	Bv-O135	hom							0.99	0.57	22/22	a
3	Bv-WT								1.73	0.34	9/9	a
3	Bv-G001			hom					1.16	0.59	12/12	a
3	Bv-O054		hom						0.07	0.14	3/10	c
3	Bv-O055		hom	hom					0.02	0.06	9/24	c
3	Bv-O064		hom	hom			hom		0.00	0.01	3/23	c
3	Bv-O092		hom						0.12	0.15	12/21	b
3	Bv-O097								1.38	0.30	12/12	a

Shown are the used *Beta vulgaris* lines having a homozygous (hom) or biallelic (bi) mutation in different *eIFs* resulting in a knockout of the respective eIF. Heterozygous (het) mutations are indicated as well, if not stated otherwise, the *eIF* sequence was indistinguishable to the wildtype allele. The obtained T0-lines were challenged with BChV infection and evaluated 4 wpi by TAS-ELISA. Mean ELISA absorption (OD_{405nm}) standard deviation (±SD) and number of infected plants to inoculated plants (inf/inoc) are shown for BChV infection. Different letters indicate significantly different virus contents within each experiment identified by Kruskal–Wallis test on ranks with a *post-hoc* Dunn's test ($P < 0.05$). Lines significantly differing from the susceptible genotypes (a; high virus accumulation) are highlighted in blue (b/c; low virus accumulation) and green (b; intermediate virus accumulation).

4E in YTH. In contrast, BChV-VPg + C-term, which in case of PLRV was shown to possess genome binding capacity and postulated a VPg precursor (Prüfer *et al.*, 1999), showed a stronger interaction with Bv-eIF(iso)4E in YTH. These findings are similar to the observations of Reinbold *et al.* (2013), who discovered a stronger interaction between eIF(iso)4G1 of *A. thaliana* and TuYV-VPg + C-term than for the initially used TuYV-VPg prediction. An involvement of the potential VPg precursor in initiating and coupling between translation and viral replication cannot be excluded. However, we suspect that the low precision of the VPg size estimation might have influenced the PPI experiments. Therefore, the VPg + C-term was regarded as a more suitable approximation for the BChV-VPg and was used for further BiFC evaluation, confirming the previously identified interaction with Bv-eIF(iso)4E. While the molecular mechanisms of the interaction are not yet fully understood, we assume that BChV predominantly (or even exclusively) exploit Bv-eIF(iso)4E for translation initiation and not both 4E isoforms as it appears to be the case for BMYV. On the other hand, only the putative BMYV-VPg displayed interactions with the sugar beet eIFs, while the BMYV-VPg + C-term was not showing any interaction. These different results for the putative BMYV- and BChV-VPgs indicate the importance of obtaining the exact poleroviral VPg sequences in future research. Using YTH and BiFC, the functional redundant Bv-eIF(iso)4E and Bv-eIF4E were identified as BMYV-VPg interaction partners. While Bv-eIF(iso)4E and Bv-eIF4E are able to complement each other when individually knocked out *in planta*, we were not able to knockout both genes simultaneously. An involvement of Bv-nCBP in poleroviral translation seems rather unlikely since the potential interaction seen in the YTH could not be confirmed in BiFC. Moreover, the knockout of Bv-nCBP did not lead to a reduced polerovirus titre in *Bv-nCBP*^{KO} plants.

Interestingly, even in case of BChV, a low level of virus was still detectable by ELISA in single plants. Low BChV amplification was observed in RT-PCR, indicating that the virus is still able to replicate and spread systemically even if the virus titre remains low. An explanation for this might be the functional redundancy of eIFs within plants, leading to additional VPg-eIF interaction. Such a decrease of virus accumulation instead of a full resistance was also observed for TuYV in *A. thaliana* eIF(iso)4G-knockouts (Reinbold *et al.*, 2013) and for some potyvirus isolates of the LMV in lettuce with the *mo1*-allele corresponding to mutations in eIF4E (Candresse *et al.*, 2002). However, a recent report by Miras *et al.* (2022) shows that the polerovirus cucurbit aphid-borne yellows virus (CABYV) contains mRNA cap-independent translation enhancers (3′-CITE) in the 3′ untranslated regions, which allows the recruitment of the eIF4F complex without a cap (Simon and Miller, 2013). Studies of Miras *et al.* (2022) suggest that 3′-

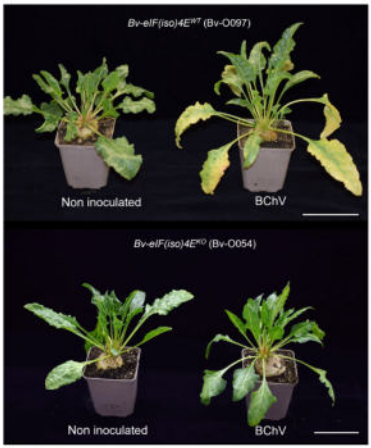

Figure 5 Wild type (*Bv-eIF(iso)4E*^{WT} line Bv-O097) and *Bv-eIF(iso)4E*^{KO} (Bv-O054) sugar beet plants after a 6-week greenhouse cultivation period. Symptoms caused by BChV 6 weeks post inoculation in line Bv-O097 and Bv-O054, respectively, are shown; white bar = 10 cm.

CITE could be present in other poleroviruses too. The presence of 3′CITE in beet-infecting poleroviruses could be another explanation why no full infection resistance was observed in all tested sugar beet knockout lines. Independent of the resistance type, dominant or recessive, resistance breaking virus strains might occur with yet unpredictable frequency (Hashimoto *et al.*, 2016; Li *et al.*, 2016). In addition, the emergence of resistance breaking isolates of BChV, due to increased selection pressure in *Bv-eIF(iso)4E*^{KO}, cannot be fully excluded, and should be addressed in future research like the re-infection experiments of Pechar *et al.* (2022).

Still, recessive resistance is widely regarded as the most durable approach, which might confer resistance towards multiple viruses at once, due to the VPg-eIF interaction that is often conserved (Sanfaçon, 2015). As we could show, an eIF mediated resistance towards members of the VY could be implemented in the agronomically important host sugar beet. However, for breeding purpose a knockout of an eIF is not feasible as it can increase susceptibility towards other diseases. This phenomenon was already described in *A. thaliana* where a knockout of eIF4E-1 resulted in resistance towards the potyvirus CIYVV, while simultaneously increasing susceptibility towards another member of the genus, turnip mosaic virus (TuMV) (Zafirov *et al.*, 2021). Therefore, there is no alternative to decipher the specific VPg-interacting domains within the sugar beet eIFs, to modify these domains into non-interacting domains for further investigation. It remains to be demonstrated if the here identified mutations in the cap-binding pocket are the only ones to mediate a loss of interaction to the poleroviral VPg, or if other mutations outside the cap-binding pocket are a resistance resource as well. In addition, the influence on cap-binding ability for these respective eIFs needs to be investigated in more detail for example, by the performed yeast complementation assay to exclude that such a non-synonymous mutation, is effectively a KO of the respective eIF losing all cellular function. If mutations not compromising the mRNA translation can be identified, a pyramiding approach of multiple non-interacting eIFs could be used to investigate whether an eIF-mediated resistance in sugar beet against BMYV can be implemented. An eIF-mediated control of BMYV appears to be a possibility, as the here performed experiments suggest an involvement of Bv-eIF(iso)4E and Bv-eIF4E in viral translation through their direct interaction with the VPg and by this opens the door for future applications screening for mutation in beet germplasm using high throughout sequencing techniques.

Experimental procedures

Identification of *Beta vulgaris* eIF homologues

The *Beta vulgaris* genome RefBeet 1.2 (Dohm *et al.*, 2014) was screened for all the available eIF4E and eIF4G homologues (mRNA sequences) by reciprocal best hit analysis using the *Arabidopsis thaliana* eIFs from TAIR (Berardini *et al.*, 2015) as bait.

Complementation assay in yeast

The different *B. vulgaris* eIF4E isoforms were subjected to a yeast complementation assay as described by Kuroiwa *et al.* (2023). Therefore, Bv-eIF4E candidates were subcloned from amplified cDNA into p424-GPD (Mumberg *et al.*, 1995) by Gibson isothermal assembly (Gibson *et al.*, 2009). *Saccharomyces cerevisiae* yeast strain J055, lacks the native Sc-eIF4E and galactose inducible expresses the human eIF4E (H-eIF4E) to complement its translation machinery. J055 was transformed with the *B. vulgaris* eIF-containing plasmids. The transformed yeast cells were selected on synthetic defined medium (SD) with galactose as carbon source lacking uracil and tryptophan (-U, -W). To investigate whether the Bv-eIFs complement the lack of the native Sc-eIF4E, transformants were serial diluted and 5 μL of the yeast cell suspension was spotted on SD with glucose as carbon source lacking tryptophan (-W). The complementation assay was evaluated after incubation for 3 days at 30 °C.

Yeast-two hybrid assay

To evaluate potential protein–protein-interaction (PPI) between poleroviral VPg and sugar beet eIFs, YTH was used (Fields and Song, 1989). Therefore, a LexA based YTH (MoBiTec; Göttingen) was performed as described in Muellender *et al.* (2021). Total RNA was extracted from sugar beets infected with BMYV or BChV using the Nucleospin RNA plant mini kit (Macherey-Nagel; Düren). Using RevertAid H Minus reverse transcriptase (Thermo Fisher Scientific; Waltham), cDNA synthesis was performed with random hexamer primers, according to the manufacturer's instruction. All cloning steps were performed by standard restriction enzyme cloning (Thermo Fisher Scientific; Waltham), resulting in in-frame fusions of activation/binding domain and protein of interest, respectively. Each obtained plasmid was confirmed by restriction enzyme digest and Sanger sequencing of the complete insert. The sugar beet eIF-genes (Table S1) were cloned into pEG202 to be translationally fused to CDS1 LexA DNA binding domain, while the poleroviral VPg sequences were cloned into pJG4-5 containing the C-terminal B42 transcription activation domain- hemagglutinin (HA-) epitope. Positive controls with translational fusion to the activation domain (AD) AD-p53 and to the DNA binding domain (BD) BD-LTA, as well as the negative control AD (−empty) with BD (−empty) were supplied by

MoBiTec. To identify and clone the putative VPg for beet infecting poleroviruses, P1 amino acid alignment was performed using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004). Different VPg sequences were estimated for BChV and BMYV based on the work of van der Wilk *et al.* (1997) and Mayo *et al.* (1982) using the N-terminal sequence of the PLRV VPg and its molecular weight, respectively. For these estimations, nucleotide 1374–1589 of BMYV isolate BMYV-IPP (acc-no. DQ132996) and nucleotide 1381–1620 of BChV isolate BChV-2a (acc-no. MH271171.1), including a stop codon at the 3′ end, were RT-PCR amplified and cloned. The full amino acid sequences can be found in Supplemental S1. Furthermore, to exclude artefacts of VPg estimation, the putative VPg was additionally cloned with the remaining poleroviral P1 C-terminus of BMYV (1374–2126 nt) and BChV (1381–2133 nt) (VPg + C-term) respectively. The GAL4 based Matchmaker Gold YTH (Takara Bio Europe; Saint-Germain-en-Laye) was used according to the manufacturer's instructions using the reporter genes *HIS3* and *ADE2* to detect PPI. AD-p53 and BD fused with the large T-antigen (BD-T) were used as positive controls, while negative controls consisted of AD-p53 and BD fused with lamin (BD-lam). Using standard restriction enzyme cloning, the poleroviral VPg sequences were cloned into pGADT7 encoding the activation domain, while *Bv-eIF4E* was cloned into pGBKT7 encoding the DNA-binding domain. *S. cerevisae* cells EGY48 (LexA) / Y2HGold-strain (GAL4) were super transformed by lithium acetate transformation of the plasmids (Gietz and Schiestl, 2007).Trans-formants were resuspended in water and serial diluted (10^0 - 1×10^{-4}). 5 µL of each dilution was spotted on petri dishes with culture media and incubated at 30 °C for 3 days (control medium) or 5 days (selection medium).

Site directed mutagenesis of *Bv-eIF(iso)4E*

To investigate the specificity of the Bv-eIF(iso)4E-VPg interaction, amino acids most likely involved in mRNA cap-binding (Monzingo *et al.*, 2007) were mutated to leucine. Plasmid pGBKT7 encoding *Bv-eIF(iso)4E*^{WT} was mutated by site directed mutagenesis using overlapping PCR primers (Zheng, 2004) and Phusion™ high-fidelity DNA polymerase (Thermo Fisher Scientific; Waltham). *S. cerevisiae* Y2HGold cells were co-transformed with pGADT7-BChV-VPg + C-term and the mutated eIF-gene containing plasmids.

Bimolecular fluorescence complementation

BiFC was performed according to Zilian and Maiss (2011) using the self-interaction of plum pox virus (PPV) coat protein as a positive control. The shortened PPV coat protein CP3, unable to self-interact, was used as negative control. To test the proteins of interest for PPI, Bv-eIF4E, Bv-eIF(iso)4E and Bv-nCBP were translationally fused at the C-terminus to the N-terminal part of mRFP (mRFPN). The C-terminal part of mRFP (mRFPC) was fused to the N-terminus of the putative BMYV-VPg and BChV-VPg + C-term. Cloning was performed by Gibson isothermal assembly. Each plasmid assembly was confirmed by restriction enzyme digest and Sanger sequencing of the insert. *Agrobacterium tumefaciens* C58C1 cells were transformed with the plasmid constructs using electroporation at 1440 V. The *A. tumefaciens* strain C58C1 containing the different plasmids was grown overnight at 28 °C. OD_{600} was adjusted to 0.5 in inoculation buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 µM acetosyringone). After 3 h of incubation at room temperature, 4-week old *Nicotiana benthamiana* leaves were co-infiltrated with *Agrobacterium* containing the respective eIF-VPg combination and the tomato bushy stunt virus P19 silencing suppressor (Voinnet *et al.*, 2003). The infiltration patches were assessed for mRFP fluorescence 3 to 5 days post inoculation (dpi). The mRFP fluorescence was visualised with the TCS-SP5 confocal laser-scanning microscope (CLSM) (Leica Microsystems, Wetzlar). Excitation/emission wavelengths for mRFP were 584/607 nm. All confocal images were processed with the LAS-AF software version 2.6.3.8173 (Leica Microsystems; Wetzlar).

Western blot analysis

Yeast containing both AD/BD constructs of pJG4-5:VPg + pEG202:eIF (LexA) or pGBKT7:eIF + pGADT7:VPg (GAL4) were grown over night in liquid media depleted of tryptophane and histidine or tryptophane and leucine, respectively. Total protein from yeast was extracted according to Zhang *et al.* (2011) using sodium dodecyl sulfate (SDS). Total protein from 100 mg of infiltrated *N. benthamiana* leaf patches was extracted according to van Loon and van Kammen (1968). Protein extracts were electrophoresed on 12% (7.5% eIF-4Gs) SDS-polyacrylamide gels and blotted onto PVDF-membrane (Roche Life Science; Basel). Rat hemagglutinin A monoclonal antibodies (Roche Life Science) and mouse c-Myc antibodies (Sigma-Aldrich; St. Louis) were used in dilution of 1/1000 and 1/500 respectively (GAL4-YTH/BiFC). Rabbit anti LexA antibodies (Sigma Aldrich) were used at a dilution of 1/4000 (pEG-202; LexA-YTH). Secondary antibodies (mouse—Jackson Immuno Research, Cambridgeshire; rat and rabbit—Thermo Fisher Scientific) with alkaline phosphatase were used at a dilution of 1/10 000 and developed using ready to use NBT/BCIP substrate (Thermo Fisher Scientific).

Generation of eIF knockouts in sugar beets by gene editing

Each gRNA listed in Table S2 was ligated into pChimera, a vector with an AtU6-26 promoter and sgRNA backbone (Fauser *et al.*, 2014). The AtU6-26 promoter and sgRNA were introduced in a transformation vector for sugar beet containing Cas9 driven from parsley PcUbi4-2 and the Arabidopsis AtAHAS gene (AT3G48560) as selection marker, which enables the selection of transformed events on imidazolinone. Protoplasts from sugar beet leaves were isolated, transformed and regenerated into *in vitro* plantlets via the formation of friable calli as described earlier in Hall *et al.* (1996) using a single plasmid or a plasmid pool containing the 12 CRISPR/Cas9 KO constructs at equimolar amounts. Only the diploid T0 plants were Sanger sequenced at all their Bv-eIF target sites. A selection of eIF knockout lines together with non-mutated lines originating from the same regeneration process, were maintained *in vitro* by micropropagation and subjected to bioassays (Table S3).

BMYV and BChV infection assay of knockout plants

In-vitro grown sugar beet plantlets with specific single or multiple *eIF* knockouts were transferred to soil for further cultivation under controlled greenhouse conditions (24 °C/14 h light, 18 °C/10 h dark photoperiod). In addition to the edited *B. vulgaris* lines, a wild type (Bv-WT) and an unmutated line (Bv-0097), which was obtained during the regeneration process of the eIF knockouts were used as susceptible controls in each bioassay (Table 1). After the emergence of the first true leaves, plants were challenged with BMYV or BChV. To avoid co-infection with both poleroviruses, bioassays were performed individually. Non-viruliferous aphids (*Myzus persicae* Sulz) were placed for 2 days on plants infected with the respective polerovirus

for virus acquisition. For the infection tests, plants were set up randomly in insect tents (Bugdorm, Taiwan). Ten viruliferous aphids were placed on each plant using a brush for a feeding period of 48 h and subsequently killed with the systemic insecticide Tepekki (Belchim Crop Protection Deutschland GmbH, Burgdorf). Four weeks post inoculation (wpi), a total of 200 mg fresh leaf tissue was harvested from 4 to 5 different leaves of each plant using a cork drill ensuring homogeneous sampling. Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) was performed with two technical replicates according to the protocol provided by the antiserum manufacturer (DSMZ, Braunschweig). Plants were considered infected when OD_{405nm} of inoculated plants exceeded the mean value of sample buffer and control samples of non-inoculated plants plus three times standard deviation (D'arcy and Hewings, 1986).

Virus detection by RT-PCR

Total RNA was extracted from sugar beet plants challenged with BChV 4 weeks post infection using GeneJET Plant RNA purification Kit (Thermo Fisher Scientific; Waltham). cDNA was synthesised with RevertAid H Minus reverse transcriptase (Thermo Fisher Scientific; Waltham) and random hexamer primers, followed by a Phusion™ high-fidelity PCR (Thermo Fisher Scientific; Waltham). BChV was detected by using primers BChVP0s (5′-GTGACGA GCGAAAGACACTTGC-3′) and BChVP0as (5′-CGCTTAAGGCCA TCAATGAGG-3′) that specifically amplify a 580 bp DNA fragment when infected (Stephan, 2005).

Statistical analysis

Statistical analysis was performed with SigmaPlot 14.5 (SigmaPlot 14.5, Systat Software Inc.). ELISA OD_{405nm} mean values of technical replicates were first tested by the Shapiro–Wilk test for normal distribution ($P \leq 0.05$), followed by a Brown-Forsythe test to evaluate the equal variance distribution. Data with a normal distribution were tested for differences by an ANOVA with a subsequent *post-hoc* Tukey test ($P \leq 0.05$). If the test for normal distribution failed, a Kruskal–Wallis test with a subsequent *post-hoc* Dunn's test ($P \leq 0.05$) was performed.

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Conflict of interest

The authors declare that the research was conducted in the absence of commercial or financial relationships that could be construed as a potential conflict of interest.

Author contribution

MV, NW, RH, GW, HVH and LR contributed to the conception and design of the study. HVH selected the sgRNAs, prepared the CRISPR/Cas9 plasmids, transformed, regenerated and sequenced the KO plants. LR performed cloning, YTH, BiFC, the yeast complementation assay and the greenhouse bioassay and is the principal author of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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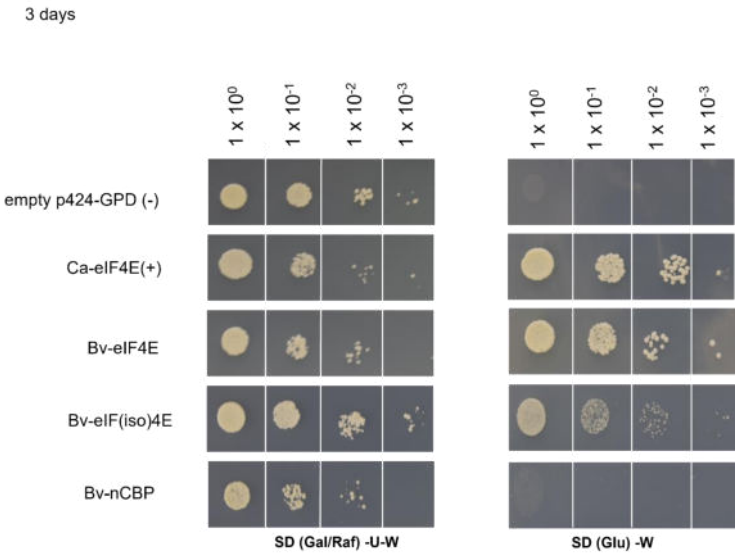
Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

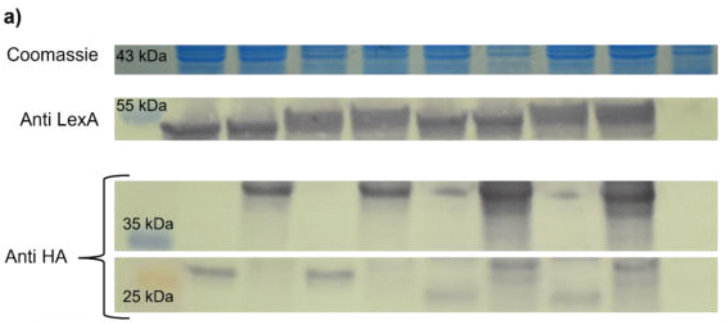
Figure S1 eIF4E-complementation assay using a Sc-eIF4E deletion strain (JO55).
Figure S2 Western blot analysis of LexA-YTH.
Figure S3 Western blot analysis of GAL4-YTH.
Figure S4 Western blot analysis of GAL4-YTH eIF(iso)4E mutants.

Figure S5 Detection of the fusion-proteins expressed in the BiFC assay by Western blot analysis.
Figure S6 Gel electrophoresis of RT-PCR products for detection of BChV infection in different gene edited sugar beets.
Table S1 *Arabidopsis thaliana* eIF genes and corresponding eIF genes within the *Beta vulgaris* genome.
Table S2 gRNAs used to generate Bv-eIF knockout sugar beet plants.
Table S3 Indels found in the *Bv-eIF* genes of the T0 sugar beet knockout plants used in this study.
Data S1 Protein Sequences of eIFs and VPgs used in this study.

Supporting Information

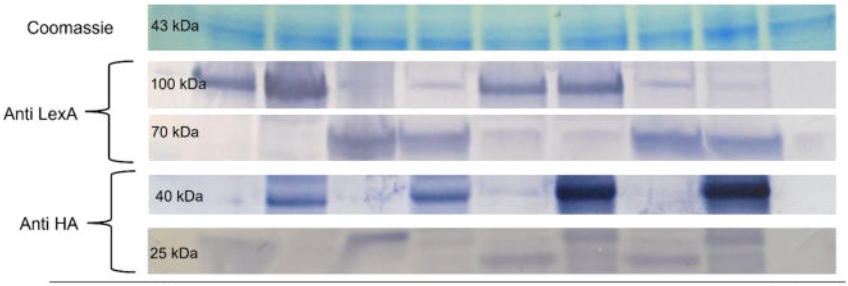


Supplementary Figure S1: eIF4E-complementation assay using a Sc-eIF4E deletion strain (J055) in serial dilution of 1x 10^0 -1 x10^{-3}. SD (Gal/Raf; -U, -W) is used as control medium, allowing growth for all transformed yeast strains. SD (Glu; -W) is used as selective medium for the eIF4E functionality assay. Growth of yeast strain J055 on SD (Glu; -W) indicates that Bv-eIF(iso)4E and Bv-eIF4E can retain the function of translation initiation in yeast. J055 transformed with pGDP-424 empty vector is shown as a negative control. *Capsicum annuum* eIF4E (Ca-eIF4E) is used as positive control.

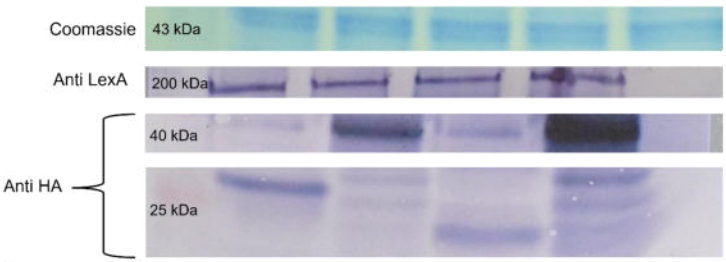


	Marker									Negativ control empty EGY
eIF (LexA)	-	(iso)4E	(iso)4E BChV+C-term	nCBP	nCBP BChV+C-term	(iso)4E	(iso)4E BMYV+C-term	nCBP	nCBP BMYV+C-term	
VPg (HA)	-	BChV		BChV		BMYV		BMYV		-
LexA (kDa)	-	45.2	45.2	49.3	49.3	45.2	45.2	49.3	49.3	-
HA (kDa)	-	26.6	38.9	26.6	38.9	22.7	38.8	22.7	38.8	-

b)

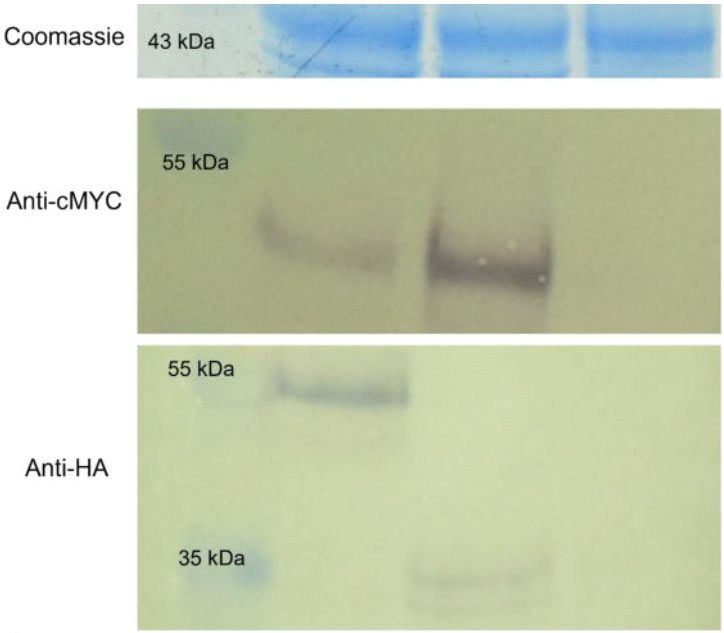


	Marker									Negativ control
eIF (LexA)	-	(iso)4G1	(iso)4G1	(iso)4G2	(iso)4G2	(iso)4G1	(iso)4G1	(iso)4G2	(iso)4G2	empty EGY
VPg (HA)	-	BChV	BChV+C-term	BChV	BChV+C-term	BMYV	BMYV+C-term	BMYV	BMYV+C-term	-
LexA	-	109.3	109.3	64.5	64.5	109.3	109.3	64.5	64.5	-
HA	-	26.6	38.9	26.6	38.9	22.7	38.8	22.7	38.8	-



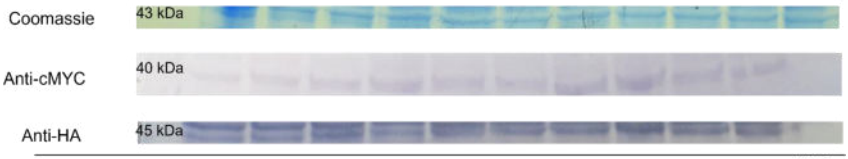
	Marker					Negativ control
eIF (LexA)	-	4G1	4G1	4G1	4G1	empty EGY
VPg (HA)	-	BChV	BChV+C-term	BMYV	BMYV+C-term	-
LexA	-	212.6 kDa	212.6	212.6	212.6	-
HA	-	26.6	38.9	22.7	38.8	-

Supplementary Figure S2: LexA-YTH: a) Expression of eIF4Es/VPg fusion proteins in yeast were detected using a HA tag (VPg-AD) or LexA tag (eIF-BD). β-actin (~ 43 kDa) is shown as loading control in Coomassie stained 12 % SDS polyacrylamide (PA) gels. b) Expression of eIF4Gs/VPg fusion proteins in yeast were detected using a HA tag (VPg-AD) or LexA tag (eIF-BD). β-actin (~ 43 kDa) is shown as loading control in Coomassie stained 7.5 % SDS PA gels. The molecular weight of each eIF/VPg fusion protein is indicated in the table as well as the approximate molecular weight indicated by PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific). The yeast strain EGY48 without any plasmid served as negative control.



Order:	Marker			Negativ control
eIF (cMYC)	-	4E	4E	empty Gold
VPg (HA)	-	BChV+C-term	BMYV	-
cMYC (kDa)	-	46.9	46.9	-
HA (kDa)	-	45.6	26.4	-

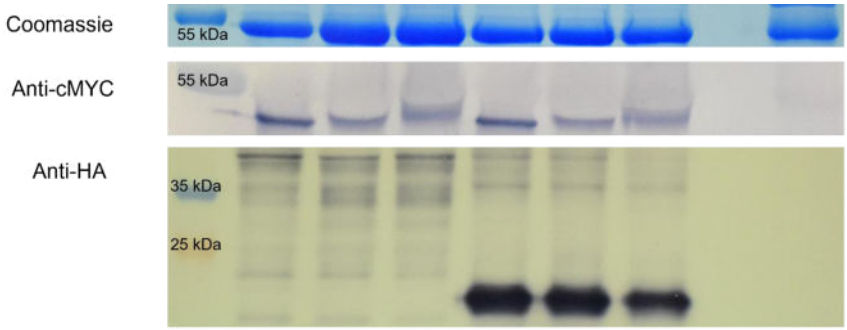
Supplementary Figure S3: GAL4-YTH: Expression of eIF4E/VPg fusion proteins in yeast were detected using a HA tag (VPg-AD) or cMYC tag (eIF-BD). β-actin (~ 43 kDa) is shown as loading control in Coomassie stained 12 % SDS PA gels. The molecular weight of each eIF/VPg fusion protein is indicated in the table as well as the approximate molecular weight indicated by PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific). The yeast strain Matchmaker Gold without any plasmid served as negative control.



	Marker										Negativ control
eIF(iso)4E (cMYC)	-	WT BChV+C-term	W47L BChV+C-term	L47W BChV+C-term	W64L BChV+C-term	D81L BChV+C-term	W93L BChV+C-term	E94L BChV+C-term	R144L BChV+C-term	K149L BChV+C-term	W153L BChV+C-term	empty Gold
VPg (HA)	-											-
cMYC (kDa)	-	40.8	40.8	40.8	40.8	40.8	40.8	40.8	40.8	40.8	40.8	-
HA (kDa)	-	45.6	45.6	45.6	45.6	45.6	45.6	45.6	45.6	45.6	45.6	-

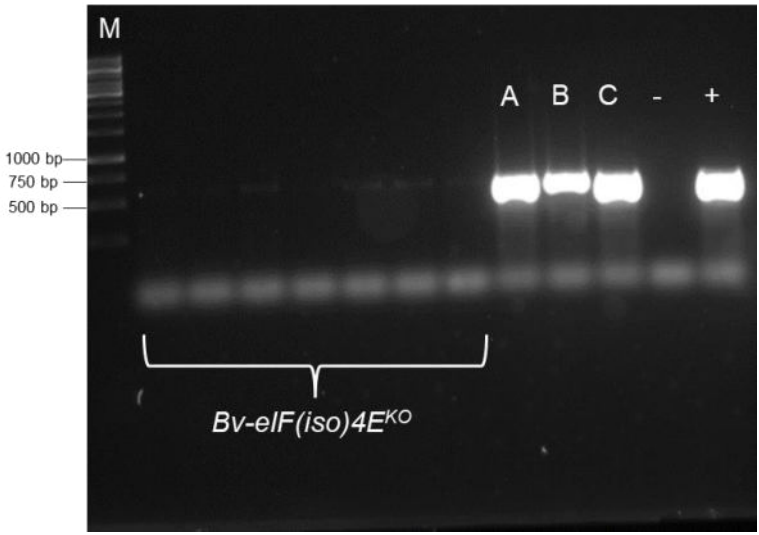
Order:	Marker										Negativ control
eIF(iso)4E (cMYC)	-	WT	W47L	L47W	W64L	D81L	W93L	E94L	R144L	K149L	W153L	empty Gold
VPg (HA)	-	BMYV	BMYV	BMYV	BMYV	BMYV	BMYV	BMYV	BMYV	BMYV	BMYV	-
cMYC (kDa)	-	40.8	40.8	40.8	40.8	40.8	40.8	40.8	40.8	40.8	40.8	-
HA (kDa)	-	26.4	26.4	26.4	26.4	26.4	26.4	26.4	26.4	26.4	26.4	-

Supplementary Figure S4: GAL4-YTH: Expression of eIF(iso)4E-mutants/VPg fusion proteins in yeast were detected using a HA tag (VPg-AD) or cMYC tag (eIF-BD). β-actin (~ 43 kDa) is shown as loading control in Coomassie stained 12 % SDS PA gels. The molecular weight of each eIF/VPg fusion protein is indicated in the table as well as the approximate molecular weight indicated by PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific). The yeast strain Matchmaker Gold without any plasmid served as negative control.



	Marker							empty	healthy plant
eIF (cMYC)	-	(iso)4E	nCBP	4E	(iso)4E	nCBP	4E		
VPg (HA)	-	BChV+C-term	BChV+C-term	BChV+C-term	BMYV	BMYV	BMYV	-	-
cMYC (kDa)	-	43.4	47.1	47.5	43.4	47.1	47.5	-	-
HA (kDa)	-	35.7	35.7	35.7	16.5	16.5	16.5	-	-

Supplementary Figure S5: Detection of the fusion-proteins expressed in the BiFC assay by western blot analysis. The estimated molecular weight for each fusion protein is given in the table. RuBisCo (~ 55 kDa) is shown as loading control in Coomassie stained 12% SDS PA gels. The western blots show the immunoblot for detection of cMYC-tagged fusion-proteins (eIFs) and HA tagged proteins (VPg) in *N. benthamiana* leaves. A protein sample from non-inoculated, healthy *N. benthamiana* leaves served as negative control to exclude unspecific binding of the antibodies separated by an empty lane where only sample buffer was loaded.



Supplementary Figure S6: Gel electrophoresis of RT-PCR products (580 bp) for detection of BChV infection in different gene edited sugar beets. M= GeneRuler 1 kb (Thermo Fisher), fragment sizes are indicated. *Bv-eIF(iso)4E*^{KO}= plants from line Bv-O054 challenged with BChV infection. A= line Bv-O051; B= line Bv-O097; C= Bv-WT; - = H_2O control; += BChV infectious cDNA full length as template.

Supplementary Table S1: *Arabidopsis thaliana* eIF genes and corresponding eIF genes within the *Beta vulgaris* genome identified and characterized in this work. N/A= not available.

Arabidopsis thaliana	**Gene ID**	***Beta vulgaris***	**Gene ID**
At-eIF4G	825194	*Bv-eIF4G1*	104905238
		Bv-eIF4G2	104900826
At-eIF(iso)4G1	835897	*Bv-eIF(iso)4G1*	104901664
			104901717
		Bv-eIF(iso)4G2	
At-eIF(iso)4G2	816939	N/A	N/A
At-eIF4E1	827529	*Bv-eIF4E*	104882920
At-eIF4E2	839832	N/A	N/A
At-eIF4E3	839836	N/A	N/A
At-eIF(iso)4E	833534	*Bv-eIF(iso)4E*	104887879
At-nCBP	831929	*Bv-nCBP*	104906715

Supplementary Table S2: gRNAs used to generate Bv-eIF knockout sugar beet plants.

gRNAs	Target gene(s)	Exon(s)
CCACTGTTGAAGAATTCTGG	*Bv-eIF4E*	1
ACCCAAACAAGGCGCTGCTT	*Bv-eIF(iso)4E*	1
TCTGTGCTAATGGAGGCAAG	*Bv-eIF4E, Bv-eIF(iso)4E*	2,2
AATGTGCAAATGGAGGCAAG	*Bv-eIF4E, Bv-eIF(iso)4E*	2,2
TGTTTGTGTTCTCACACCGG	*Bv-nCBP*	2
GGGAATTCGCCCATTATGGG	*Bv-nCBP*	3
CAGAAGCAATTCGCTGACAC	*Bv-eIF(iso)4G1*	2
TCAACTGCTGCAACTCAGAG	*Bv-eIF(iso)4G2*	2
GACGTTCAGCTTCTTGCTCA	*Bv-eIF(iso)4G1, Bv-eIF(iso)4G2*	5,7
TCAATTCCTCGTGCGTCTCT	*Bv-eIF4G1*	7
TGTCAGGAGGAATTTGAGAG	*Bv-eIF4G1, Bv-eIF4G2*	9,7
CACAAAGGTGGGGAGGAACC	*Bv-eIF4G1, Bv-eIF4G2*	11,9

Supplementary Table S3: Indels found in the *Bv-eIF* genes of the T0 sugar beet knockout plants used in this study. Wt and 0 = wild type allele(s).

T0 line	Bv-eIF4E exon1	Bv-eIF4E exon2	Bv-eIF(iso)4E exon1	Bv-eIF(iso)4E exon2	Bv-nCBP exon2	Bv-nCBP exon3	Bv-eIF(iso)4G1 exon2	Bv-eIF(iso)4G1 exon5	Bv-eIF(iso)4G2 exon2	Bv-eIF(iso)4G2 exon7	Bv-eIF4G1 exon7	Bv-eIF4G1 exon9	Bv-eIF4G1 exon11	Bv-eIF4G2 exon7	Bv-eIF4G2 exon9
Bv-O015	Wt	Wt	+C/+C	Wt	+A/+A	0/+T	Wt	Wt	Wt	Wt	0/+1	Wt	+A/+A	Wt	Wt
Bv-O025	Wt	+1/0	+A/+A	-4/0	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	+A/+T	Wt	Wt
Bv-O050	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	+A/+A	Wt	Wt
Bv-O051	Wt	Wt	Wt	Wt	+T/+T	Wt	Wt	Wt	Wt	Wt	Wt	Wt	+A/+A	Wt	Wt
Bv-O054	Wt	Wt	+G/+G	-4/-4	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-O055	Wt	Wt	Wt	+A/+A	+G/+G	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-O063	Wt	Wt	-1/-1	+1/-6	Wt	Wt	Wt	Wt	Wt	Wt	+T/+T	Wt	+A/+A	Wt	Wt
Bv-O064	Wt	Wt	+C/+C	Wt	+A/+A	0/+1	Wt	Wt	Wt	Wt	0/+1	Wt	+A/+A	Wt	Wt
Bv-O065	Wt	0/+1	+T/+T	Wt	+A/+A(+T)	0/+1	Wt	Wt	Wt	0/+1	+T/+T	0/-4/-4	Wt	+1/0	Wt
Bv-O067	Wt	Wt	Wt	Wt	Wt	+T/+T	Wt	Wt	Wt	Wt	Wt	-2/-2	+A/+A	Wt	Wt
Bv-O068	Wt	Wt	Wt	Wt	+A/+T	+T/+T	+1/0	Wt	Wt	Wt	Wt	0/-5/-5/-5	+C/+C	Wt	Wt
Bv-O076	Wt	Wt	Wt	Wt	+A/+T	+T/+T	+1/0	Wt	Wt	Wt	Wt	0/-5/-5/-5	+C/+C	Wt	Wt
Bv-O092	Wt	Wt	+G/+G	-4/-4	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-O096	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	+A/+A	Wt	Wt
Bv-O097	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-O107	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	-2/+1	+T/+T	0/-5	Wt
Bv-O110	0/+1	0/+1	Wt	Wt	+T/+T	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-O123	Wt	Wt	+T/+T	Wt	Wt	+T/+T	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-O135	+A/+A	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-D018	Wt	Wt	Wt	+53	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-G001	Wt	Wt	Wt	Wt	Wt	-4/-4	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt
Bv-WT	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt

4. Manuscript II: Beet mosaic virus expression of a betalain transcription factor allows visual virus tracking in *Beta vulgaris*

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TECHNICAL ADVANCE

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Beet mosaic virus expression of a betalain transcription factor allows visual virus tracking in *Beta vulgaris*

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Abstract

In the field of plant virology, the usage of reverse genetic systems has been reported for multiple purposes. One is understanding virus–host interaction by labelling viral cDNA clones with fluorescent protein genes to allow visual virus tracking throughout a plant, albeit this visualization depends on technical devices. Here we report the first construction of an infectious cDNA full-length clone of beet mosaic virus (BtMV) that can be efficiently used for *Agrobacterium*-mediated leaf inoculation with high infection rate in *Beta vulgaris*, being indistinguishable from the natural virus isolate regarding symptom development and vector transmission. Furthermore, the BtMV clone was tagged with the genes for the monomeric red fluorescent protein or the *Beta vulgaris BvMYB1* transcription factor, which activates the betalain biosynthesis pathway. The heterologous expression of *BvMYB1* results in activation of betalain biosynthesis genes in planta, allowing visualization of the systemic BtMV spread with the naked eye as red pigmentation emerging throughout beet leaves. In the case of BtMV, the *BvMYB1* marker system is stable over multiple mechanical host passages, allows qualitative as well as quantitative virus detection and offers an excellent opportunity to label viruses in plants of the order *Caryophyllales*, allowing an in-depth investigation of virus–host interactions on the whole plant level.

KEYWORDS
beet mosaic virus, betalain biosynthesis, *BvMYB1*, *Caryophyllales*, infectious cDNA clone, virus tracking

1 | INTRODUCTION

The infection of sugar beet with aphid-transmissible yellowing viruses consisting of beet yellows virus (BYV, genus *Closterovirus*), beet mild yellowing virus (BMYV, genus *Polerovirus*), beet chlorosis virus (BChV, genus *Polerovirus*) and a mosaic disease caused by beet mosaic virus (BtMV, genus *Potyvirus*) is an increasing problem for European sugar beet growers. Today, aphid-transmissible viruses have re-emerged in Europe with widespread distribution in all sugar beet-growing areas due to the ban of neonicotinoid seed coating in the European Union, resulting in a strong need for natural plant resistance and the development of biotests for fast and reliable identification and phenotypic characterization (Hauer et al., 2017; Hossain et al., 2021). The BtMV genome sequence with 9591 nucleotides (nt)

in length was first published by Nemchinov et al. (2004), displaying a typical potyvirus genome organization with a single polyprotein translated and cleaved into 10 mature and one fusion protein by a virus-encoded protease or autocatalytic processing (Nigam et al., 2019). BtMV causes mosaic-like patterns on leaves and a stunted growth with an estimated yield loss of approximately 10%; however, it is highly probable that BtMV in mixed infection with other viruses of the yellowing virus complex increases disease severity in sugar beet and is potentially under-represented in field monitoring activities due to the light mosaic deviating from the conspicuous yellowing caused by the other species (Wintermantel, 2005).

Virus-induced symptoms in plants as well as yield losses do not always correlate with virus replication level and systemic tissue colonization, which is frequently inhomogeneous over time (Crespo et al., 2020; Kaweesi et al., 2014). This often requires extensive work for reliable and detailed evaluation of potential resistance/tolerance in breeding material relying on specialized personnel and cost-intensive molecular analysis.

There was limited work done on virus–host interaction for the yellowing viruses in the sugar beet host, as only one reverse genetic system for BMYV has been reported being fully infectious (Klein et al., 2014). Other previously published cDNA clones of yellowing virus displayed low (Stephan & Maiss, 2006; Wetzel et al., 2018) or no infectivity (Hasan, 2004; Peremyslov et al., 1998) in beet using *Agrobacterium*-mediated inoculation. The field of plant virology has benefitted from reporter genes integrated into the viral genome, enabling virus tracking throughout the plant for a better understanding of the viral life-cycle in plants (Oparka et al., 1996). Most of the tagged recombinant viruses rely on the use of green or red fluorescent proteins (Baulcombe et al., 1995; Cruz et al., 1996; Dietrich & Maiss, 2003) allowing virus tracking throughout the whole plant. Alternative plant-derived fluorescent proteins made from light, oxygene, or voltage (LOV) domains have been described, such as the flavoprotein improved LOV (Chapman et al., 2008). All these reporter systems rely on technical equipment such as a UV light handlamp or fluorescence microscopes, which often cannot be used directly in a greenhouse. In addition to this, the need for technical equipment and suitable facilities limits the feasability of a high-throughput resistance screen, and fluorescence microscopy does not allow the visualization on the whole plant level but only a rather small leaf section or tissue. A reporter system allowing visual virus tracking without the need for any technical devices was first reported by Bedoya et al. (2012). It was shown that the integration of the MYB-related Rosea1 transcription factor gene derived from *Antirrhinum majus* into the genome of tobacco etch virus, tobacco mosaic virus and potato virus X resulted in an increased anthocyanin biosynthesis and subsequently clearly visible red pigmentation of *Nicotiana tabacum* or *Nicotiana benthamiana* leaves during viral replication and systemic colonization (Bedoya et al., 2012). Potato virus Y expressing Rosea1 initiates anthocyanin production in other plant species such as tomato and potato as well (Cordero et al., 2017). An alternative system used phytoene synthase (*crtB*) to initiate carotenoid biosynthesis and visualize virus spread (Majer et al., 2017). Still, every virus–host combination has to be tested for the suitability of this reporter system (Bedoya et al., 2012).

However, members of the order *Caryophyllales* such as beet, spinach, prickly pear or quinoa produce a different class of red pigments, the betalains (Polturak & Aharoni, 2018). Furthermore, no plant family is known to produce both betalains and anthocyanins in the same species (Stafford, 1994). Betalains possess pH stability in a wider range, which is thought to be beneficial mostly in the context of acidification for vacuoles of CAM and C4 plants (Jain & Gould, 2015). Betalains are mostly redundant in their cellular functions as an antioxidant compared to anthocyanins but it is hypothesized that the ability to synthesize betalains might have an evolutionary advantage under specific environmental conditions like high salinity (Jain & Gould, 2015; Timoneda et al., 2019).

Starting with tyrosine as a precursor from the shikimate pathway, L-3,4-dihydroxyphenylalanine (L-DOPA) is formed by tyrosine hydroxylase activity of the cytochrome P450 CYP76AD1 and other CYP76AD-family members (Polturak et al., 2016; Sunnadeniya et al., 2016). It was then shown that the DOPA 4,5-dioxygenase (DODA) and CYP76AD1 are the catalysts for betalain biosynthesis resulting in the red/violet betalacyanins and yellow betaxanthins as a result of multiple spontaneous reactions (Hatlestad et al., 2012). Betalain synthesis is regulated differently compared to the anthocyanin biosynthesis. The beet *Y* locus, responsible for red-fleshed beet and red leaf colour, encodes the *BvMYB1* transcription factor responsible for the bottleneck for betalain pigmentation in beets. *BvMYB1* is a plant R2R3-MYB that contains R2 and R3 MYB domains and a C-terminal activation domain; however, *BvMYB1* does not interact with the basic helix–loop–helix (bHLH) of the heterologous MYB-bHLH-WD40 activation complex for anthocyanin biosynthesis, leaving *BvMYB1* without an influence on pigmentation for anthocyanin-producing plants (Hatlestad et al., 2015).

Here we report the construction of the first beet-infecting BtMV cDNA full-length clone and assess its biological properties, such as symptom development and vector transmission. Furthermore, the obtained BtMV clone was labelled by integration of monomeric red fluorescent protein (mRFP) or the *BvMYB1* gene. Using the *BvMYB1* as a reporter system in *Beta vulgaris* enables virus tracking with the naked eye in whole plants as red pigmentation was induced in *B. vulgaris* plants wherever the virus replicated. This makes it a useful molecular tool to study the virus biology, estimate viral loads and assess information about the disease severity within a leaf without the need for technical devices.

2 | RESULTS

2.1 | Generation of a BtMV cDNA clone for agroinoculation and infectivity testing in different host plant species

We obtained three different cDNA clones of BtMV from the cloning experiments (Figure 1). The first of the three clones (pDIVA-BtMV1228) is identical to sequence of the BtMV wild-type

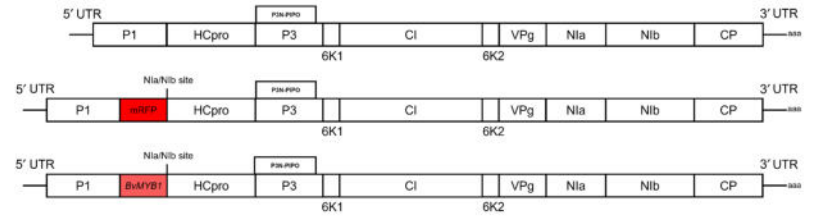

FIGURE 1 Schematic view of the BtMV genome organization for the constructed cDNA clones pDIVA-BtMV1228, pDIVA-BtMV-mRFP and pDIVA-BtMV-MYB. The 5′ and 3′ untranslated regions (UTR) are indicated by straight lines. The poly(A) tail is indicated at the 3′ UTR. Genomic BtMV cistrons are shown as white boxes. Inserted *mRFP* and *BvMYB1* cistrons are shown as red boxes; the additional NIa/NIb-protease cleavage site is also indicated.

isolate (DSMZ PV-1228) and was confirmed by Sanger sequencing. After successful cloning, different host plants from the order of *Caryophyllales* (*B. vulgaris*, $n=24$) and *Solanales* (*N. benthamiana*, $n=20$) were agroinoculated with the recombinant cDNA clone. The resulting recombinant virus was named BtMV1228. Each of the tested virus–host combinations resulted in 100% symptomatic plants at 7 days postinoculation (dpi) (Figures 2 and 3, and Table S1). First symptoms of BtMV1228 were observed starting at 5 dpi in emerging leaves of *B. vulgaris* plants and developed over the observation period of 4 weeks (Figure 2a–f). The start of symptom expression was comparable to the mechanically inoculated wild-type BtMV isolate. Symptom severity at later time points did not show any discrimination between recombinant and wild-type virus (Figure 2b–f and Table S1).

2.2 | Labelling of recombinant BtMV with mRFP or *BvMYB1* and effect on infectivity

After BtMV1228 infectivity in *B. vulgaris* and *N. benthamiana* was shown, the reporter genes *mRFP* and *BvMYB1* were integrated into the cDNA clone of pDIVA-BtMV1228 without a stop codon, allowing translation of the viral polyprotein, resulting in the cDNA clones pDIVA-BtMV-mRFP and pDIVA-BtMV-MYB (Figure 1). The recombinant viruses were named BtMV-mRFP and BtMV-MYB, respectively. Here we have chosen an insertion between the P1 proteinase (P1) and the helper component proteinase (HC-Pro), which has been used in previous works for potyvirus labelling (Beauchemin et al., 2005; Bedoya et al., 2012; Kelloniemi et al., 2008). By using the already existing autoproteolytic P1 cleavage site (RTMHY/SS) at the 5′ end of the insert and via a duplication of the NIa/NIb (EVVEQ/G) at the insert's 3′ end, proper translation and release of the heterologous proteins was ensured. Again, different host plants from the order of *Caryophyllales* (*B. vulgaris*, $n=24$) and *Solanales* (*N. benthamiana*, $n=20$) were agroinoculated with the recombinant cDNA clones pDIVA-BtMV-mRFP and pDIVA-BtMV-MYB. Independent of the tested host plant, the plants displayed less severe symptoms when infected by BtMV-MYB and BtMV-mRFP compared to BtMV1228 (Figures 2a–f and 3). The anthocyanin-producing plant *N. benthamiana* did not accumulate red pigments at any time point after inoculation with BtMV-MYB although virus symptoms were clearly visible (Figure 3). When *B. vulgaris* was infected by one of the recombinant labelled viruses, the plants displayed milder BtMV symptoms compared to a BtMV1228 infection; however, red pigments extensively accumulated over time in BtMV-MYB infected plants (Figures 2 and 5) while BtMV-mRFP infected plants displayed a mRFP fluorescence signal (Figure 4). Symptom development appeared to be slower and less severe in *B. vulgaris* plants inoculated with the labelled viruses as symptoms at 6 dpi were not as distinguishable as for the wild-type viruses and fewer plants infected with BtMV-MYB showed symptoms compared to early time points of BtMV1228 infection (Figure 2a and Table S1). However, at 7–8 dpi symptoms caused by each labelled clone increased, showing symptoms in all of the tested plants, being comparable in disease severity for BtMV-MYB and BtMV-mRFP (Figure 2b). In the case of BtMV-mRFP a clear signal (607 nm) could be observed using a confocal laser-scanning microscope correlating with the beginning of mosaic symptoms at 5 dpi (Figure 2a). Furthermore, BtMV-MYB-infected *B. vulgaris* plants displayed an accumulation of red pigments in oldest leaves first, which was particularly visible on the lower leaf surface by the naked eye starting at 8–10 dpi (Figure 5a,b) and could be confirmed photometrically to be caused by betalains, as the red pigments had a specific absorption peak at c.536 nm after chloroform extraction, which is typical for betalains (Khan & Giridhar, 2015). Old leaves displayed pigmentation that appeared to follow the veins and spread out from there into the surrounding tissue (Figure 5c); pigmentation in leaves that emerged after infection did not exclusively follow the veins as most leaves already displayed full viral symptoms and subsequently produced red pigments in a mosaic-like pattern during emergence (Figure 5d,e). The beet root itself only displayed minor pigmentation except occasional red spots (Figure S1) and an inhomogeneous virus distribution throughout the plant was observed as well (Figures 2g and S2). To test for the

FIGURE 2 BtMV symptom development in *Beta vulgaris* infected with the different recombinant viruses (BtMV1228, BtMV-mRFP, BTMV-MYB) compared to healthy plants, mock-inoculated plants and mechanically BtMV DSMZ PV-1228 (BtMV-WT) infected plants. (a) 5 days postinoculation (dpi), (b) 7 dpi, (c) 10 dpi, (d) 14 dpi, (e) 21 dpi, (f) 28 dpi. (g) Side view of *B. vulgaris* plants 4 weeks postinoculation with the recombinant viruses in comparison to a healthy/mock-inoculated plant. (a–f) Bar = 1 cm, (g) bar = 10 cm.

stability of the inserted reporter genes into the genome of BtMV, five consecutive rounds of mechanical transmission of the recombinant viruses BtMV-mRFP and BtMV-MYB were carried out in *B. vulgaris*. Over the course of five passages, mRFP signal as well as red pigmentation was observed without a loss in signal intensity. Furthermore, all of the mentioned viruses were aphid transmissible, and the symptoms caused were indistinguishable to mechanical/agroinoculation (data not shown).

2.3 | Quantification of viral load and betalain gene expression by reverse transcription-quantitative PCR

The chloroform betalain extraction is a destructive method that does not allow betalain content and viral load from the same sample to be measured, therefore reverse transcription-quantitative PCR (RT-qPCR) was performed to analyse the influence of BtMV-MYB on the mRNA expression of betalain biosynthesis genes *BvMYB1*,

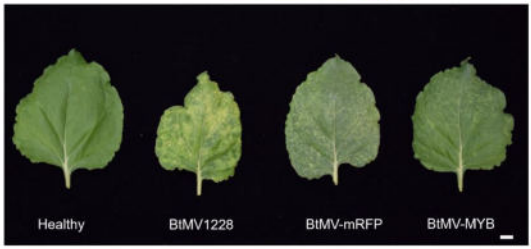

FIGURE 3 Leaf symptoms in *Nicotiana benthamiana* after inoculation with BtMV1228, BtMV-mRFP and BtMV-MYB recombinant viruses at 21 days postinoculation compared to a healthy plant. White bar = 1 cm.

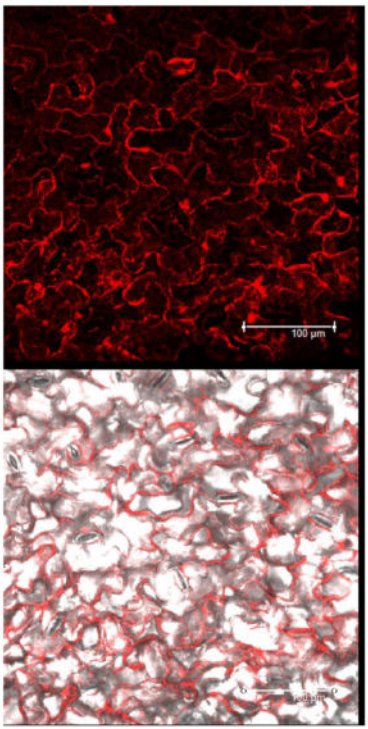

FIGURE 4 Red fluorescence recorded by confocal laser-scanning microscopy in white beet epidermal cells from systemically BtMV-mRFP-infected leaf tissue displaying mRFP signal at 6 days postinoculation. White bar = 100 µm.

BvDODA and *BvCYP76AD1* in comparison to BtMV1228 replication. *Agrobacterium*-mediated inoculation was performed to allow better comparability of inoculum among treatments. White beets cv. Albina Vereduna were leaf infiltrated with *Agrobacterium* C58C1 harbouring no additional plasmid (mock control), pDIVA-BtMV-1228 or pDIVA-BtMV-MYB. Starting from 6 dpi, whole systemically infected leaves of the same age were sampled, homogenized and used for total RNA extraction on a weekly basis. BtMV was detectable by RT-qPCR in all infected plants independent of the tested construct, while no amplification was observed in mock-inoculated plants (data not shown). However, copy numbers of the viral genome at 6 dpi was 875 times higher in BtMV1228 than in BtMV-MYB-infected plants (Figure 6). Over the test period of 4 weeks, the difference in viral load between BtMV1228 and BtMV-MYB continuously decreased significantly, resulting in a 22-fold higher viral load for BtMV1228 compared to BtMV-MYB at the end of the test period. This mainly resulted from a rising BtMV content in BtMV-MYB-infected leaves, and both viruses' accumulation increased significantly over time (Figure 6). The gene expression level of the betalain biosynthesis genes continuously increased during the experiment in plants infected with BtMV-MYB compared to the steady gene expression in BtMV1228-infected plants (Figure 7). After only 6 days the detected level of *BvMYB1* mRNA was significantly up-regulated in BtMV-MYB- compared to BtMV1228-infected plants, even though no symptoms were observed at this time point in BtMV-MYB-infected plants (Figure 7a). However, the two betalain biosynthesis genes *BvDODA* and *BvCYP76AD1* only displayed a slightly increased expression in BtMV-MYB-infected plants at 6 dpi, which was not significantly increased compared to BtMV1228 (Figure 7a,b). Apart from that, *BvDODA* and *BvCYP76AD1* expression significantly increased over time in BtMV-MYB-infected beets (Figure 7a,b), subsequently leading to an accumulation of red pigments being visible with the naked eye (Figure 5). With this, absolute viral content and relative betalain biosynthesis gene expression in BtMV-MYB-infected plants compared to BtMV1228-infected plants were determined. To evaluate whether an increasing BtMV content in BtMV-MYB-infected plants correlates with an increased gene expression of *BvMYB1*, *BvDODA* and *BvCYP76AD1*, a linear regression model was used to prove a highly significant correlation ($r^2 \geq 0.95$,

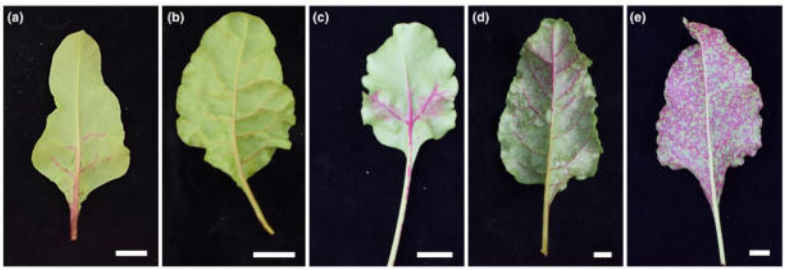

FIGURE 5 Representative pictures of increasing betalain pigmentation as a result of BtMV-MYB infection on the lower leaf side of *Beta vulgaris* 'Albina Vereduna' (a–e) in systemically infected tissue in comparison to a mock-inoculated leaf (f). (a) First observable betalain pigmentation at 8 days postinoculation (dpi). Development of betalain pigmentation at 10 dpi (b), 14 dpi (c), 21 dpi (d) and 28 dpi (e). White bars = 1 cm.

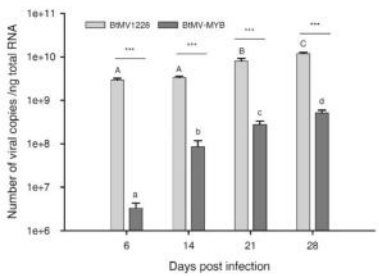

FIGURE 6 Quantification of wild-type (BtMV1228) compared to recombinant (BtMV-MYB) BtMV accumulation in systemically infected leaves of white beet cv. Albina Vereduna tested by reverse transcription-quantitative PCR. Bars show means with standard deviation of number of copies per nanogram total RNA from five biological replicates. Different upper/lower case letters indicate significantly different virus contents between time points for each virus identified by two-way analysis of variance with a post hoc Tukey test ($p < 0.05$, $n = 5$). Differences in virus accumulation between the tested viruses for each time point are indicated with asterisks (***$p \leq 0.001$; $n = 5$).

$p \leq 0.001$) between increasing BtMV content and increased gene expression for the betalain biosynthesis genes (Figure 8). The highest correlation ($r^2 = 0.97$, $p \leq 0.001$) between BtMV content and gene expression was found for *BvMYB1*.

3 | DISCUSSION

This study describes the successful construction and modification of the first recombinant BtMV full-length clone with 100% infection rate in beets. No differences were observed regarding viral load, symptom development or transmissibility for BtMV1228 compared to a mechanically inoculated wild-type virus isolate. Furthermore, we were able to effectively label the recombinant virus to allow virus tracking with the naked eye in systemically infected tissue and could show that viral load and betalain gene expression in beets were highly correlated after infection with the labelled recombinant BtMV-MYB.

Introducing the *BvMYB1* transcription factor into the genome of BtMV resulted in an activation of the beet endogenous betalain biosynthesis pathway. Due to its nuclear functional localization, it is anticipated that the transcription factor *BvMYB1* is not only properly and functionally released from the polyprotein during viral translation and subsequent replication but also transported into the nucleus. Throughout the viral infection cycle, heterologous expression of a protein with a size of 25.4 kDa (mRFP) or 26.3 kDa (BvMYB1) was possible while retaining their function in planta. However, the integration of additional 675 nt (*BvMYB1*) into the genome of BtMV had a negative influence on viral replication and speed of virus spread throughout the plant, especially during early infection compared to BtMV1228, similar to what was observed by Bedoya et al. (2012). For BtMV-MYB infection, similarities in the estimated delay of approximately 2 days between first mild symptoms and subsequent pigmentation were observed and verified by the gene expression experiments performed, limiting the application for such a cDNA clone in specific experiments especially investigating viral cell-to-cell movement. At 6 dpi *BvMYB1* expression was already significantly up-regulated compared to BtMV1228, while *BvDODA* and *BvCYP76AD1* expression just started to increase (Figure 7). This indicates that even though the virus is present and replicating, betalain biosynthesis needs additional time after infection in planta, as the BvMYB1 must be released from the polyprotein, localized to the nucleus, the transcription of the other biosynthesis genes started, and pigments must accumulate in such a high amount that they are visible by naked eye without the need for technical devices. The

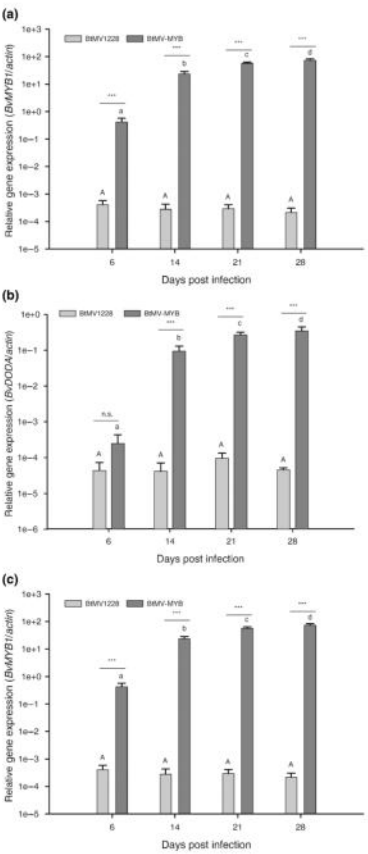

FIGURE 7 Relative gene expression of (a) *BvMYB1*, (b) *BvCYP76AD1* and (c) *BvDODA* in *Beta vulgaris* 'Albina Vereduna' infected with BtMV-MYB compared to BtMV1228 over time. Bars show means with standard deviation of relative gene expression compared to the internal control gene *actin* from five biological replicates. Different upper/lower case letters indicate significantly different gene expression over time for each virus identified by two-way analysis of variance with a post hoc Tukey test ($p < 0.05$, $n = 5$). Differences in gene expression between the tested viruses for each time point are indicated with asterisks (***$p \leq 0.001$; n.s., not significant; $n = 5$).

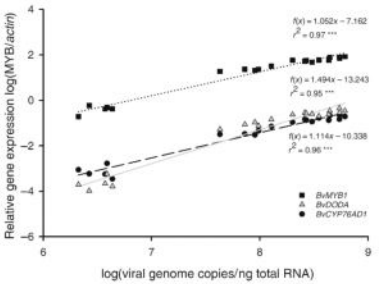

FIGURE 8 Correlation of log(relative gene expression compared to an internal control gene) for *BvMYB1*/*BvDODA*/*BvCYP76AD1* in white beet cv. Albina Vereduna in relation to the BtMV-MYB content shown as log(viral genome copies per ng total RNA) of 20 independent biological replicates. Linear regressions were performed, significant differences are indicated by ***$p \leq 0.001$ ($n = 20$).

2-day delay in pigmentation also displays one of the disadvantages of the BtMV-MYB clone compared to fluorescence-labelled ones. While fluorescently labelled clones, depending on the fluorophore, supply a real-time picture, a *BvMYB1*-labelled clone is only indirectly labelled, as the virus is already present in green tissue before betalains accumulate to a visually detectable amount. On the other hand, the reporter can be used without any artificial irradiation and offers a potential way to investigate the virus spread on the whole plant level, especially in non-model plants such as *B. vulgaris*. As betalains are stored in the vacuole (Jain & Gould, 2015), movement tracking through cells without or with small vacuoles, such as vascular tissue, can be limited. Besides the limitation inside the plant, the viral genome organization is an additional factor that has to be considered, as the genome size of the recombinant virus negatively influences viral replication and efficacy of fluorescence labels (Chapman et al., 2008). Additionally, partial RNA silencing due to the presence of the *BvMYB1* cistron cannot be excluded either, as small quantities of intracellular *BvMYB1* transcript can be found in white beet without being infected with BtMV-MYB. Similar to virus-induced gene silencing (VIGS), this could lead to a lack of pigmentation in some cells (Lu et al., 2003). Nonetheless, besides these limitations BtMV-MYB showed similar properties comparable to the wild-type virus regarding in planta localization, symptom development, host range and transmission. Strikingly the virus distribution was not homogenous throughout the plant, which has implications for representative sampling and foreshadowing the advantage of such a cDNA clone to get a better understanding of virus–host interactions. In addition to that, betalain biosynthesis genes showing such a strong correlation to the BtMV content is an indicator for the suitability of this clone to be used in noninvasive high-throughput screens, correlating an image-determined betalain content to the viral load. Studies determining the anthocyanin content in plant material from pictures (e.g., apples) have already been published (Grimm et al., 2022). Such a quantification algorithm combined with a fast-phenotyping apparatus through sensor

technology could be used to reduce the need for experts to evaluate, for example, disease severity, making the selection procedure of tolerant plant material less error prone (Mahlein, 2016). Even without the use of such technical equipment, the more distinguishable symptoms caused by BtMV-MYB would facilitate the evaluation of disease severity for an expert. Still, a decrease or loss of betalain pigmentation for a tested genotype does not directly imply a resistance, as the *R*-locus, which is responsible for red hypocotyl colour, mapped to be *BvCYP76AD1* and is a prerequisite to express betalains (Hatlestad et al., 2012). With these limitations, the use of BtMV-MYB for a breeding programme does need additional future research; however, it is the first proof of principle that the betalain biosynthesis pathway in general can be manipulated by a plant virus, as already shown for anthocyanins (Bedoya et al., 2012) and carotenoids (Majer et al., 2017). We expect that every virus–host combination needs careful evaluation for the suitability of such a reporter gene before being used in scaled-up experiments.

4 | EXPERIMENTAL PROCEDURES

4.1 | Virus source and cloning strategy for BtMV cDNA full-length clone construction

Total RNA was extracted from *B. vulgaris* systemically infected with BtMV (DSMZ PV-1228, GenBank accession no. MT815987) using the NucleoSpin RNA plant mini kit (Machery-Nagel). Four different cDNAs were synthesized with RevertAid H minus reverse transcriptase (Themo Fisher Scientific) covering the virus genome, using the primers C1 for cDNA1, C2 for cDNA2, C3 for cDNA3 and C4 for cDNA4. All primers used for cloning purposes are listed in Table S1. Using C4, a poly-A-tail of 22 nt was added to the genomic BtMV sequence, increasing the genome size up to 9613 nt. These cDNAs were processed by Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) to a total of six overlapping PCR products covering the entire BtMV genome. The PCR products were separated by gel electrophoresis and purified by NucleoSpin gel and PCR clean-up kit (Macherey-Nagel). All subsequent cloning steps were performed by introducing PCR products into a small binary vector pDIVA (accession no. KX665539) downstream of the 2×35S promoter from cauliflower mosaic virus and upstream of a hepatitis delta virus ribozyme and the nopaline synthase terminator using the standard protocol for Gibson isothermal assembly (Gibson et al., 2009). Using PCR primers, appropriate overlaps to the PCR fragments were introduced, resulting in five different subclones that were used as templates to assemble the full-length cDNA clone. Chemically competent *Escherichia coli* NM522 cells were transformed with the plasmids (Inoue et al., 1990). After plasmid purification (Birnboim & Doly, 1979), positive clones were identified by restriction enzyme digest and complete sequencing of the viral insert. Plasmid preparations of six to eight positive clones were mixed for population-based cloning. For each subclone assembly the pDIVA vector backbone was amplified in two overlapping PCR products using primers PDIVA1 + PDIVA2 and PDIVA3 + PDIVA4, which were subsequently combined with partial BtMV genome PCR fragments. Starting with BtMV cDNA1, pDIVA-BtMVsub1 was produced using a PCR product corresponding to BtMV nt 1–2370 with the primers M1 and M2. pDIVA-BtMVsub2 using cDNA2 consisted of two BtMV PCR products amplified by M3 + C1 and M4 + M5, respectively. These PCR products correspond to the nt 2344–3077 and 3058–4704, respectively. Using primers M6 + C3 and cDNA3, pDIVA-BtMVsub3 was generated corresponding to the nt 4679–6762 of the BtMV genome. The last subclone pDIVA-BtMVsub4 consisted again of two BtMV PCR products amplified by primers M7 + M8 and M9 + C4 using cDNA4, which amplified the genomic BtMV nt 6736–8708 and 8684–9614, respectively. The correct sequence of all subclones was confirmed by Sanger sequencing. Amplifying not only PCR products from the BtMV cDNAs, but additional vector sequences, the pDIVA-BtMVsub34 was generated from pDIVA-BtMVsub3 using primers V1 + PDIVA4 and pDIVA-BtMVsub4 using primers V2 + PDIVA3. After the subcloning steps were performed, and three final PCR products including genomic BtMV sequence and pDIVA sequence were generated from the subclones pDIVA-BtMVsub1 pDIVA-BtMVsub2 pDIVA-BtMVsub34 using primers M1 + M10, M11 + C3 and M12 + pDIVA1, respectively, to assemble the final clone pDIVA-BtMV1228. Correct assembly was confirmed by complete Sanger sequencing of the final product.

4.2 | Integration of reporter genes into pDIVA-BtMV1228

The pDIVA-BtMV1228 clone was PCR-amplified in two overlapping fragments using the primers PDIVA4 + M14 and M15 + PDIVA6. With the primer M15, a duplication of the NIa/NIb-cleavage site (EVVEQ/G) was introduced 5′ to the HC-Pro coding sequence. The coding sequence without the stop codon of *B. vulgaris BvMYB1* (accession no. JF432080.1) was RT-PCR amplified from total RNA extracts of red beet cv. Bulls Blood (Pieterpikzonen B.V.), which was extracted by NucleoSpin RNA plant mini kit (Machery-Nagel). The cDNA was synthesized with RevertAid H minus reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers followed by a Phusion High-Fidelity PCR (Thermo Fisher Scientific) with primers BMYB1 + BMYB2. The monomeric red fluorescent protein (mRFP) coding sequence was amplified from plasmid pDIVA-BNYVV-P2-mRFP (Laufer, Mohammad, Christ, et al., 2018) using primers BRFP1 + BRFP2. Both primer pairs added appropriate overlaps to the inserts, allowing an integration of the reporter between P1 and HC-Pro coding sequence in pDIVA-BtMV1228 using Gibson assembly (Table S2).

4.3 | Plant inoculation with pDIVA-BtMV variants

White beet *B. vulgaris* var. *conditiva* 'Albina Vereduna' (Pieterpikzonen B.V.), and *N. benthamiana* were grown under controlled greenhouse

conditions (24°C/14 h light, 18°C/10 h dark photoperiod). *Agrobacterium tumefaciens* C58C1 cells were transformed with the plasmid constructs using electroporation at 1440 V. *A. tumefaciens* C58C1 containing the different plasmids pDIVA-BtMV1228, pDIVA-BtMV-MYB or pDIVA-BtMV-mRFP were grown overnight at 28°C. OD_{600} was adjusted to 0.5 (*N. benthamiana*) or 1.5 (*B. vulgaris*), respectively, in inoculation buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 150 μM acetosyringone). After 3 h of incubation at room temperature, beet cotyledons were inoculated with a needleless syringe (agroinoculation) 14 days after emergence, while first true leaves of *N. benthamiana* were inoculated 21 days after emergence (Laufer, Mohammad, Maiss, et al., 2018). Virus symptom development was evaluated daily over 6 weeks.

4.4 | Betalain extraction

To verify that produced red pigments in BtMV-MYB-infected plants were betalains, 100 mg of red pigmented leaf tissue was harvested and homogenized in liquid nitrogen. Using a chloroform–methanol–water mixture (2:1:1) red pigments were separated from green leaf tissue after centrifugation for 10 min at 21,000 *g*. Red pigments were taken from the aqueous phase and extinction at 476 nm (betaxanthins) and 536 nm (betacyanins) was measured photometrically and compared to a red beet extract (Chang et al., 2021).

4.5 | Fluorescence microscopy

To investigate the recombinant BtMV-mRFP, infiltration patches, as well as systemically infected leaves of beet and *N. benthamiana*, were evaluated for a distinguishable signal at 600–610 nm. The mRFP fluorescence was visualized with the TCS-SP5 confocal laser-scanning microscope (Leica Microsystems). Excitation/emission wavelengths for mRFP were 584/607 nm. All confocal images were processed with LAS-AF software v. 2.6.3.8173 (Leica Microsystems).

4.6 | BtMV transmission procedures

Leaves of infected and symptomatic plants were ground 1:10 (wt/vol) in 10 mM phosphate buffer (pH 7.3). Infectious plant sap was mechanically inoculated together with Celite 535 on 14-day-old seedlings of beet and evaluated for symptoms and presence of reporter.

For analysis of BtMV aphid transmissibility, nonviruliferous aphids (*Myzus persicae*) were placed for 24 h on beet plants infected with each recombinant BtMV variant for virus acquisition. Ten aphids were then brush transferred to healthy plants and left for an additional day on these plants before being treated with a systemic insecticide (Teppeki; Belchim Crop Protection). Plants were evaluated for BtMV symptoms over a period of 4 weeks and the presence of reporter gene signal, if applicable.

4.7 | Virus and plant mRNA quantification with RT-qPCR

Total RNA was extracted from virus-infected white beet cv. Albina Vereduna on a weekly basis with the GeneJET Plant RNA purification kit (Thermo Fisher Scientific). All subsequent RT-qPCRs were carried out using the CFX96 Real time system (Bio-Rad). cDNA synthesis was performed with 1 μg of total RNA and 5 μM oligo$(dT)_{18}$ primer. All primers used for gene expression analysis can be found in Table S3.

The RT-qPCR for quantification of *BvMYB1*, *BvDODA* and *BvCYP76AD1* transcripts was performed with specific primers and iTaq Universal SYBR Green Supermix (Bio-Rad) as described in Hatlestad et al. (2015) using *actin* as a housekeeping gene.

Viral load in BtMV-infected plants was quantified by one-step RT-qPCR using 100 ng of total RNA. For cDNA synthesis, Superscript IV reverse transcriptase (Thermo Fisher Scientific) was used. In addition to that 2× Maxima Probe qPCR mix (Thermo Fisher Scientific) with 400 nM primers BtMVqs1, BtMVqas1 and the Fam-BHQ1-labelled probe BtMVq-p1 were added to the reaction mix. A calibration curve to determine absolute viral genome copies for each recombinant virus was produced using serial dilution of the corresponding purified full-length clone plasmid of known concentration. *Beet cytochrome oxidase* was used as a housekeeping gene to confirm integrity of samples with 400 nM primers COX-F, COX-R and the Fam-BHQ1-labelled probe COX-P-FAM as described (Mahillon et al., 2022). Cycler conditions were 3 min at 95°C, 10 min at 52°C and 10 min at 95°C for cDNA synthesis, followed by 40 cycles 15 s at 95°C and 60 s at 60°C. Gene expression data was analysed using the ΔC_t method (Schmittgen & Livak, 2008).

4.8 | Statistical analysis

Relative gene expression was calculated by the ΔC_t method, values have been log-transformed before being used for statistical analysis using SigmaPlot v. 14.5 (Systat Software). Mean values are displayed with standard deviation (*SD*). Mean values of expression data between different viruses and time points were tested for differences by a two-way analysis of variance with a subsequent post hoc Tukey test ($p \leq 0.05$). Linear regressions were performed, and significant differences were determined and indicated by $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supporting information

Figure S1: [redacted]

Figure S2: [redacted]

Table S1: Symptom development of BtMV infection in *B. vulgaris* after mechanical inoculation of BtMV DSMZ PV-1228 (BtMV-WT) or agroinoculation of the recombinant viruses BtMV-1228/MYB/-mRFP until all plants displayed clear systemic BtMV symptoms. Shown are numbers of *B. vulgaris* plants (n= 24) displaying BtMV symptoms from 4 to 8 days postinoculation. Mock inoculated plants (n=12) were infiltrated with *Agrobacterium tumefaciens* C58C1 harbouring no additional plasmid, while healthy plants (n =12) were untreated.

	days postinoculation				
Virus (n)	**4**	**5**	**6**	**7**	**8**
BtMV-WT (24)	0	8	23	24	24
BtMV-1228 (24)	0	8	23	24	24
BtMV-MYB (24)	0	0	4*	24*	24
BtMV-mRFP (24)	0	7	17	24	24
Mock (12)	0	0	0	0	0
Healthy (12)	0	0	0	0	0

* Slight symptom occurrence but no clear pigmentation in systemically infected leaves.

Table S2: Primers used for generation of pDIVA-BtMV1228, pDIVA-BtMV-mRFP and pDIVA-BtMV-MYB. To distinguish between vector and viral sequence, the vector sequences are italicized.

Primer	**Sequence 5' → 3'**
BMYB1	GAACCATGCACTACAGTAGTATGTACCAGCAGAATAGTGAAACTGG
BMYB2	TCTCCCTGCTCGACTACTTCTGCCCACAAGTTCACAACATCAAAATCC
BRFP1	GAACCATGCACTACAGTAGTATGGCCTCCTCCGAGGACG
BRFP2	TCTCCCTGCTCGACTACTTCGCCGGTGGAGTGGCGGCCCTCGGC
C1	TGGCGTCGATATCTTCTGAC
C2	ATTGACACTTTCTTGTAGCGCATGC
C3	*GAGATGCCATGCCGACCC*CACAATCACCTTCCTTTGTTGAGACC
C4	*GAGATGCCATGCCGACCC*TTTTTTTTTTTTTTTTTTTTTTT
M1	*AGGAAGTTCATTTCATTTGGAGAGG*AAAATTAAAACATCTCAATACAACAC
M10	CCAAATGAATCAATGACATGCATTG
M11	ACAATGCATGTCATTGATTCATTTGG
M12	TGCATGCGCTACAAGAAAGTGTC
M14	ACTACTGTAGTGCATGGTTCTACCAAC
M15	GAAGTAGTCGAGCAGGGAGAGGAAAGATTCTTTGCTGG
M2	*GAGATGCCATGCCGACCC*CAAATGAATCAATGACATGCATTG
M3	*GTTCATTTCATTTGGAGAG*GACAATGCATGTCATTGATTCATTTGG
M4	GTCAGAAGATATCGACGCCA
M5	*GAGATGCCATGCCGACCC*ATTGACACTTTCTTGTAGCGCATGC
M6	*GTTCATTTCATTTGGAGAG*GTGCATGCGCTACAAGAAAGTGTC

M7	*GTTCATTTCATTTGGAGAG*GGGTCTCAACAAAGGAAGGTGATTGTGG
M8	GCTCGATTGTCCCACATCTTTGC
M9	AAGGACAAGCAGAGTGTCATCAAGC
PDIVA1	*CCTCTCCAAATGAAATGAACTTCCTTATATAG*
PDIVA2	*GCTAAGCGGCTGTCTAATGAATTCGTATAGGGACAATCCG*
PDIVA3	*GGGTCGGCATGGCATCTCCACCTCCTC*
PDIVA4	*CGGATTGTCCCTATACGAATTCATTAGACAGCCGCTTAGC*
PDIVA6	*GCTAAGCGGCTGTCTAATGAATTCGTATAGGGACAATCCG*
V1	CCACAATCACCTTCCTTTGTTGAGACC
V2	GGTCTCAACAAAGGAAGGTGATTGTGG

Table S3: Reverse transcription-quantitative PCR primers used in this study to confirm and quantify BtMV accumulation and evaluate gene expression of the betalain biosynthesis pathway in *Beta vulgaris*.

Primer Name	**Sequence 5‘ → 3‘**
BtMV-detec1	AGGGAAGAGGCGTCAGATGC
BtMV-detec2	CTACTCTTCCCTGACAACCTCTG
COX-F	CGTCGCATTCCCGATTATCCA
COX-R	CAACTACAGAGATATAAGAGC
COX-P-FAM	[FAM]TGCTTACGCAGGATGGAATGCCCT[BHQ1]
BtMVqs1	GATGCAGCAGAAGCGTATATTGA
BtMVqas1	TGTCTCTCAGATTTCTCTGAGC
BtMVq-p1	[FAM]CAACAGAGAAAGGCCATACATGCC[BHQ1]
RTPCRBvACTF	TCTATCCTTGCATCTCTCAG
RTPCRBvACTR	ATCATACTCGCCCTTGGAGA
RTPCRMYB1F	GCCGACGATTCTGGCC
RTPCRMYB1R	GATGGTCTTTGATAGCAGC
RTPCRDODA1F	CATTGGTTCAGGAAGTGCAA
RTPCRDODA1R	ACGAAGCCATGAATCAAAGG
RTPCR76AD1F	CTTTTCAGTGGAATTAGCCCACC
RTPCR76AD1R	CCCAATATCTTCCATAATGTTCCA

5. Manuscript III: Characterization and evaluation of beet mosaic virus resistance in sugar beet

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Manuscript in preparation for publication.

Abstract

The aphid transmissible beet mosaic virus (BtMV; genus *Potyvirus*) is often associated with the (re-)emerging virus yellows (VY) disease in sugar beet, displaying synergistic effects with VY members during co-infection. In this study we aimed to investigate possible future control strategies for BtMV in *Beta vulgaris*. First, we tried to identify and generate a recessive resistance via incompatibility between the BtMV viral protein genome linked (VPg) and corresponding eukaryotic translation initiation factors (eIF) from the host. To achieve this, yeast two hybrid (YTH) assay and bimolecular fluorescence complementation (BiFC) were used to identify eIFs interacting with BtMV VPg. However, even though BtMV-VPg + Bv-eIF(iso)4E interaction was detected in yeast and *in-planta,* genome edited sugar beets carrying a single Bv-eIF(iso)4E knockout displayed no resistant phenotype. Hypothesized double interaction between the VPg with Bv-eIF(iso)4E and Bv-eIF4E could not be tested in knockout plants due to lethality. Bibliographic analysis identified a previously described dominant resistance gene *Bm* that was lacking detailed phenotypic characterisation to BtMV infection. Virus inoculation resulted in clearly delayed symptom development in a *Bm*-carrying *B. vulgaris* breeding line compared to a susceptible *bm*-carrying line. To facilitate the study of delayed systemic colonisation, a BtMV full-length clone was labelled with a GFP variant that can be visualized with a hand-held UV lamp. Comparison of colonisation between resistant *Bm*-plants and susceptible *bm*-plants showed a decreased virus spread and delayed host colonialization in *Bm*-carrying plants. Even though not conferring a complete BtMV resistance, the *Bm*-gene significantly lowers BtMV accumulation measured by RT-qPCR. Hence, the *Bm*-gene appears to be an effective way to reduce yield losses by BtMV and even more allows pyramiding of multiple resistances such as an eIF-mediate resistance against VY and *Bm* against the associated BtMV. For this a proper marker development should be performed in the

future, which would support the breeding process and allows to understand the underlying mechanism of the *Bm*-resistance.

Introduction

Plant viruses make up about half of the (re-)emerging plant diseases in agricultural crops (Jones and Naidu, 2019). Thus, they cause a significant number of plant diseases resulting in various growth defects, reduced yield and plant quality (Gómez *et al.* 2009). The aphid transmissible potyvirus beet mosaic virus (BtMV) is a prevalent virus in beet growing areas causing estimated yield losses of 10 % in single infections (Shepard *et al.* 1964; Wintermantel, 2005). Furthermore, co-infection of multiple viruses within one host is more common than expected; these infections often display synergistic or antagonistic effects regarding e.g., disease severity and reduce yields to a higher extent (Syller, 2012). BtMV displays these synergistic effects with members of the virus yellows (VY) disease complex (Wintermantel, 2005). VY is an increasing problem in European sugar beet cultivation and consists of different aphid transmissible viruses, namely beet yellows virus (BYV, genus *Closterovirus*), beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV; both genus *Polerovirus*) (Stevens *et al.* 2005a). A co-infection of multiple viruses including the VY associated BtMV can result in sugar yield losses of up to 43 % (Hossain *et al.* 2021; Shepard *et al.* 1964). Recent VY monitoring activities only detected BtMV in few plants, however VY monitoring only focused on plants with typical chlorosis, therefore BtMV could be underrepresented (Hossain *et al.* 2021). Older monitoring activities suggest a high prevalence of BtMV in sugar beet growing areas (Mali *et al.* 2000; Russell, 1971; Shepherd, 1970). Currently no effective methods or resistant cultivars to prevent the spread of VY and BtMV throughout European growth areas are available. Therefore, natural plant resistance is essential to control VY and the associated BtMV.

There are different resistance mechanisms against plant viruses such as RNA meditated interference (Amari and Niehl, 2020) or systemic acquired resistance, however, resistances against plant viruses are often divided in two main categories based on their heritability. These resistances are called dominant or recessive resistance making up approximately half of the known resistances respectively (Gómez *et al.* 2009; Ronde *et al.* 2014; Truniger and Aranda, 2009). The latter named recessive resistance is based on incompatibility between host and virus (Mäkinen, 2020). The recessive resistance was most often described to act against potyviruses (Diaz-Pendon *et al.* 2004; Gao *et al.* 2004; Kang *et al.* 2005a; Ruffel *et al.* 2002) but also for other positive single-stranded RNA viruses such as sobemoviruses (Hébrard *et al.* 2010), cucumoviruses (Yoshii *et al.* 2004), bymoviruses (Stein *et al.* 2005) or carmoviruses (Nieto *et al.* 2006). In our previous work we were able to implement a recessive resistance against BChV in sugar beets, being the first report of recessive resistance in sugar beets and against a member of the VY (Rollwage *et al.* 2024). When mapped, most of these recessive resistances were found to represent natural occurring polymorphism(s) in eukaryotic translation initiation factors (eIF). eIFs are host cell factors, forming the multimeric eIF4F-complex, which is important for the cellular mRNA translation. To initiate their genome translation, members of the *Potyviridae* mimic eukaryotic mRNA. However, they carry a viral protein genome linked (VPg) covalently attached to their 5´terminus instead of the mRNAs m^7G-cap (Urcuqui-Inchima *et al.* 2001). In numerous studies VPgs of potyviruses have been shown to interact with different eIFs of their host, which was most often the cap binding proteins eIF4E or eIF(iso)4E. Perturbing these VPg-eIF interactions impairing the viral replication cycle sufficient to result in a resistance (Mäkinen, 2020; Sanfaçon, 2015). The main theories behind the underlying molecular mechanisms propose either an involvement of the eIFs in the formation of potyviral replication-complexes, translation

(-initiation) of the potyviral genome or involvement in viral cell-to-cell movement (Truniger and Aranda, 2009).

The second category of virus resistance is the dominant resistance. Here, a signalling cascade is activated by a resistance gene (*R*-gene) recognizing the avirulence effector (*Avr*) of the pathogen. Resistance is conferred by a programmed cell death mediated by reactive oxygen species called hypersensitive response (HR) confining the virus to its initial infection site (Ronde *et al.* 2014). Most often *R*-genes encode proteins that contain a region of a nucleotide binding sequence (NBS) and leucine-rich repeats (LRRs) (Głowacki *et al.* 2011). N-terminal additions of coiled-coil (CC) sequences, or toll- and interleukin 1 receptor (TIR) to the NBS-LRR, respectively, allow further subdividing of the proteins (Collier *et al.* 2011; Kang *et al.* 2005b). Thus, allowing to sense *Avr*-gene products during infection, conferring resistance either by direct or indirect interaction with the R-protein (Baker *et al.* 1997). Non NBS-LRR dominant resistance genes have been reported as well e.g. conferring broad potexvirus resistance or preventing systemic virus movement (Ronde *et al.* 2014). Only few dominant resistances are used in commercially available sugar beet varieties. The most famous dominant resistance genes in sugar beet are *Rz1* and *Rz2* allowing an effective control of rhizomania diseases. *Rz2* was shown to encode a CC-NBS-LRR protein while the *Rz1* encoded R-protein is still unknown (Capistrano-Gossmann *et al.* 2017). Still, no comparable resistance towards the traditional VY members has been identified so far in sugar beet. However, Lewellen (1973) reported, a dominant resistance gene mediating incomplete resistance against BtMV subsequently called *Bm*. The *Bm*-gene was deemed incomplete as it showed less local lesion on *Chenopodium amaranticolor* indicating a lowered viral concentration, instead of a full resistance, with heterozygous F_1 progenies having a higher viral load than the homozygous *Bm*-parent line. Additionally, the *Bm*-gene conferred only resistance

towards BtMV but had no effect on the other VY (Lewellen, 1973). However, besides determining the heritability of *Bm*-gene, no further characterization of the *Bm*-resistance was done. More than 30 years later a sequence characterized amplified region (SCAR) marker was developed by using bulked segregant analysis. Localizing the *Bm*-resistance on chromosome 1 in linkage group III of the sugar beet reference genome (Friesen *et al.* 2006). However, in Friesen *et al.* (2006) work no genomic interval is mentioned, therefore based on the available data it is impossible to determine the coding interval as only the SCAR marker is individually available .

In this study we aimed to investigate how BtMV can be controlled in *Beta vulgaris* by different approaches: generation of eIF-based recessive resistance and by the use of *Bm*-resistance gene. To investigate the first hypothesis, yeast two hybrid (YTH) assay and bimolecular fluorescence complementation (BiFC) were used to identify interacting eIFs subsequently trying to implement a recessive resistance in genome edited sugar beets by eIF knockout. For the second hypothesis, the previously described dominant resistance gene *Bm* has been characterized in detail using wildtype BtMV isolates, and a fluorescence labelled cDNA clone of BtMV. Besides the visual analysis of GFP distribution throughout the plant, molecular virus quantification using RT-qPCR was performed. Our results demonstrate that a recessive resistance is currently not implementable. However, the *Bm*-gene, which was discovered 50 years ago, significantly lowers virus accumulation of a current BtMV isolate but does not confer a complete BtMV resistance. Therefore, the *Bm*-gene appears to be an effective way to control the VY associated BtMV. Hence, a proper marker development should be performed to make the *Bm*-gene available for breeding purposes, which has not been done to our knowledge.

Results

A direct interaction between VPg and eIF is needed for potyviral infection, therefore by using YTH and BiFC assays protein-protein-interaction (PPI) between BtMV-VPg and *B. vulgaris* eIFs were investigated. Using a LexA based YTH assay, the Bv-eIF(iso)4E and Bv-eIF4-like were identified as potential interaction partners for BtMV-VPg (Figure 1), while the Bv-eIF4Gs and Bv-eIF(iso)4G displayed no interaction. As described in our previous work (Rollwage *et al.* 2024) the Bv-eIF4E showed an autoactivation in the LexA based YTH. However, switching to a GAL4 based YTH system did not allow to investigate the BtMV-VPg and Bv-eIF4E into more detail, as no signal exceeding the autoactivation was obtained (data not shown). To confirm the PPI identified via YTH, a BiFC assay was performed testing the potential interaction partners of the different Bv-eIF4E isoforms. However, only the interaction between Bv-eIF(iso)4E and BtMV-VPg could be confirmed (Figure 2).

The previously detected interaction between BtMV-VPg and Bv-eIF4E-like by means of YTH was not reproducible nor was an interaction with Bv-eIF4E detected. Even though an interaction between Bv-eIF(iso)4E and BtMV-VPg was detected in two independent PPI systems, none of the Bv-eIF knockout (KO) plants produced in Rollwage *et al*. (2024) displayed a fully resistant phenotype nor reduced symptom severity (Table 1). As a result of the missing resistance phenotype, we concluded that with our current knowledge an eIF-mediated recessive resistance against BtMV cannot be obtained in *Beta vulgaris* using single eIF-KO plants, while a potential double KO of *Bv-eIF(iso)4E* and *Bv-eIF4E*, which potentially could confer resistance was lethal

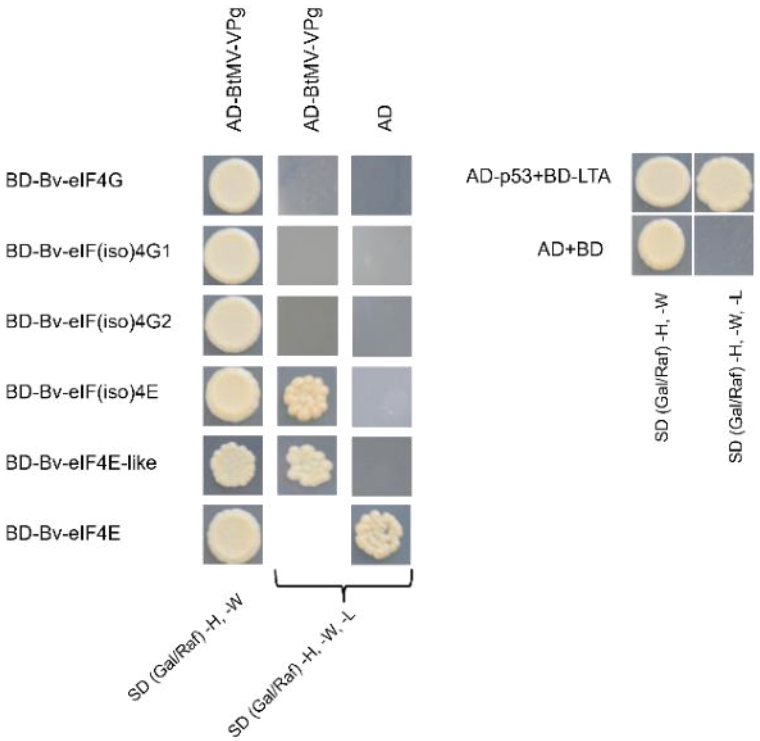

Figure 1: 1 x 10^{-2} dilution of spotted yeast from LexA based YTH experiments tested for interaction between BtMV-VPg and eIFs of *Beta vulgaris*. *Beta vulgaris* eIFs were fused to the binding domain (BD) and BtMV-VPg to the activation domain (AD) for PPI evaluation. Positive control AD-p53 with BD-LTA and the negative control AD (- empty) with BD (-empty) were supplied by MoBiTec. Yeast strain EGY48 was transformed with two plasmids containing AD and BD respectively. Positive transformants were selected on synthetic defined media (SD) lacking histidine (-H) and tryptophane (-W) using galactose and raffinose as a carbon source (Gal/Raf). To test for autoactivation, yeast cells were co-transformed with the plasmids encoding the proteins of interest and the AD or BD plasmids lacking a fusion protein. Single colonies were resuspended in water and diluted 1 x 10^{0} – 1 x10^{-3}. 5 µL of each dilution was spotted on control medium SD [Gal/Raf (-H, -W)] and interaction medium lacking leucine (-L), and SD [Gal/Raf (-H, -W, -L)]. Spotted yeast was incubated at 30 °C for 3 days (control medium) or 5 days (selection medium), respectively.

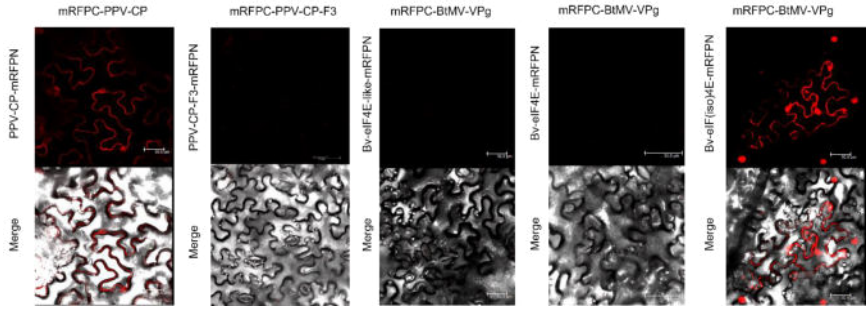

Figure 2: CLSM images of *N. benthamiana* leaf parenchymatic tissue during the BiFC assay co-infiltrated with *Agrobacterium tumefaciens* C58C1 cells harbouring pCB:Bv-eIF4E-like-mRFPN/ pCB:Bv-eIF4E-mRFPN/ pCB:Bv-eIF(iso)4E-mRFPN and pCB:mRFPC-BtMV-VPg, respectively, to confirm the interactions between BtMV-VPg and sugar beet eIF isoforms previously detected by YTH. Co-expression of CB:PPV-CP-mRFPN + pCB:mRFPC-PPV-CP was used as positive control, while pCB:PPV-CP-F3-mRFPN + pCB:mRFPC-PPV-CP-F3 was used as a negative control. Images were taken at 4 dpi. White bars = 50 µm.

Subsequently we decided to investigate the effect of the *Bm*-resistance gene on virus colonialization in more detail as it was not yet described properly. To investigate virus spread on a single cell level, we used our previously published BtMV cDNA full length clone (Rollwage *et al.* 2023) and integrated a synthetic green fluorescent protein S65T (sGFP) gene into the virus genome between the P1 and HC-pro ORFs (Figure 3a). After the obtained pDIVA-BtMV-sGFP plasmid was verified by Sanger sequencing, it was tested by *Agrobacterium tumefaciens* mediated leaf inoculation (agroinoculation) for infectivity in *Nicotiana benthamiana* (Figure 3b).

The resulting recombinant virus was subsequently named BtMV-sGFP and allows to visualize the virus spread under illumination of a handheld UV-lamp as green fluorescence, while the uninfected leaves themselves appear red due to the chlorophyll autofluorescence (Figure 3b).

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Table 1: Summary of *Beta vulgaris* lines having a homozygous (hom) or biallelic (bi) mutation in different *eIFs* resulting in a knockout of the respective *eIF* obtained in *Rollwage et al.* (2024). Heterozygous (het) mutations are indicated as well, if not stated otherwise, the *eIF* sequence was indistinguishable from the wildtype allele. The obtained T_0-lines were challenged with BtMV infection and scored for symptom expression over the course of four weeks.

Experiment	(T_0-) line	*Bv-eIF4E*	*Bv-eIF(iso)4E*	*Bv-eIF4E-like*	*Bv-eIF(iso)4G1*	*Bv-eIF(iso)4G2*	*Bv-eIF4G1*	*Bv-eIF4G2*	inf/inoc (n)	Symptoms after 4 weeks
1	Bv-Control								5/5	yes
1	Bv-O050						hom		8/8	yes
1	Bv-O054		hom						6/6	yes
1	Bv-O055		hom	hom					5/5	yes
1	Bv-O068			hom	het		hom		5/5	yes
1	Bv-O097								4/4	yes
1	Bv-O110	het		hom					4/4	yes
2	Bv-Control								4/4	yes
2	Bv-O015		hom	hom			hom		4/4	yes
2	Bv-O064		hom	hom			hom		4/4	yes
2	Bv-O067			hom			hom		4/4	yes
2	Bv-O076			hom	het		hom		5/5	yes
2	Bv-O092		hom						4/4	yes
2	Bv-O096						hom		4/4	yes
2	Bv-O097								4/4	yes
2	Bv-O123		hom	hom					3/3	yes
3	Bv-Control								3/3	yes
3	Bv-G001			hom					4/4	yes
3	Bv-O025	het	hom				bi		5/5	yes
3	Bv-O065	het	hom	bi		het	hom	het	3/3	yes
3	Bv-O097								5/5	yes
3	Bv-O135	hom							3/3	yes

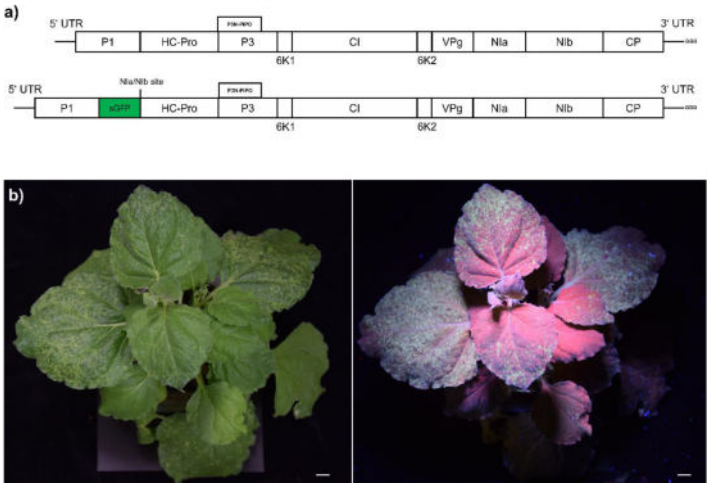

Figure 3: a) Schematic view of the wildtype BtMV genome organization compared to the modified sGFP labelled cDNA clone pDIVA-BtMV-sGFP. The 5'and 3'untranslated regions (UTR) are indicated by straight lines. The poly-A-tail is indicated at the 3' UTR. Genomic BtMV cistrons are shown as white boxes. The sGFP cistron is shown as green box; the additional NIa/NIb-protease cleavage site is indicated as well. **b)** Systemic symptoms and GFP fluorescence caused by BtMV-sGFP in *Nicotiana benthamiana* 21 days postinoculation under white light (left) or UV-light (right). White bars= 1cm.

After systemic infectivity in *N. benthamiana* was confirmed, resistant *Bm*-plants were compared to susceptible *bm*-plants evaluating the symptoms caused by BtMV-WT and BtMV-sGFP (Figure 4). Plants with the *bm*-gene showed first BtMV symptoms after 5 - 7 days postinoculation (dpi), while plants with the *Bm*-gene displayed first faint symptoms not earlier than 14 dpi only visible as small patches with slightly lower colour intensity (Figure 4a). Over a period of four weeks virus symptom severity in *Bm*-plants increased, however each plant still showed individual circular patches on their leaves (Figure 4a) and appeared to recover from the viral disease approximately two months after infection (not shown). In contrast, symptom severity increased over the course of 28 days in plants carrying the *bm*-gene with all patches fusing, resulting in a fully symptomatic leaf surface with typical mosaic (Figure 4a). Similar observations regarding symptom development could be made after inoculation with the recombinant

BtMV-sGFP virus even though, symptoms under transmitted white light appeared to be less severe compared to the BtMV wildtype infection (Figure 4b/c). Intriguingly, plants carrying the *Bm*-allele infected with the BtMV-sGFP, displayed no detectable GFP signal under UV-illumination at 28 dpi (Figure 4b/c) and neither using the epifluorescence microscope (data not shown). To investigate how the *Bm*-resistance works on a single cell level, a leaf-prick inoculation with a needle dipped into an agar plate culture of agrobacterium containing pDIVA-BtMV-sGFP was performed (Figure 5). The cell damage caused by the needle at the infection foci already resulted in a false positive fluorescence signal. However, it was visible that BtMV-sGFP started to spread from the infection foci displaying a distinguishable fluorescence signal in adjacent cells. However, the timepoint of first signals caused by the spreading BtMV-sGFP and subsequently the signal intensity varied for *Bm*- and *bm*-plants. *Bm*-plants showed first virus spread from the infection foci at 10 dpi, in comparison to that the spread of BtMV-sGFP in *bm*-plants started already at 5 dpi. Hence, at 10 dpi the BtMV-sGFP signal in *bm*-plants covered a larger surface than in *Bm*-plants, additionally systemically infected leaves of *bm*-plants showed a strong GFP fluorescence, while no comparable signal could be found in *Bm*-plants (Figure 5). Furthermore, using RT-qPCR the influence of the *Bm/ bm*-allele on wildtype BtMV accumulation was investigated. For this the relative BtMV content in comparison to an internal housekeeping gene (cytochrome oxidase) control was determined in systemically infected leaves (Figure 6).

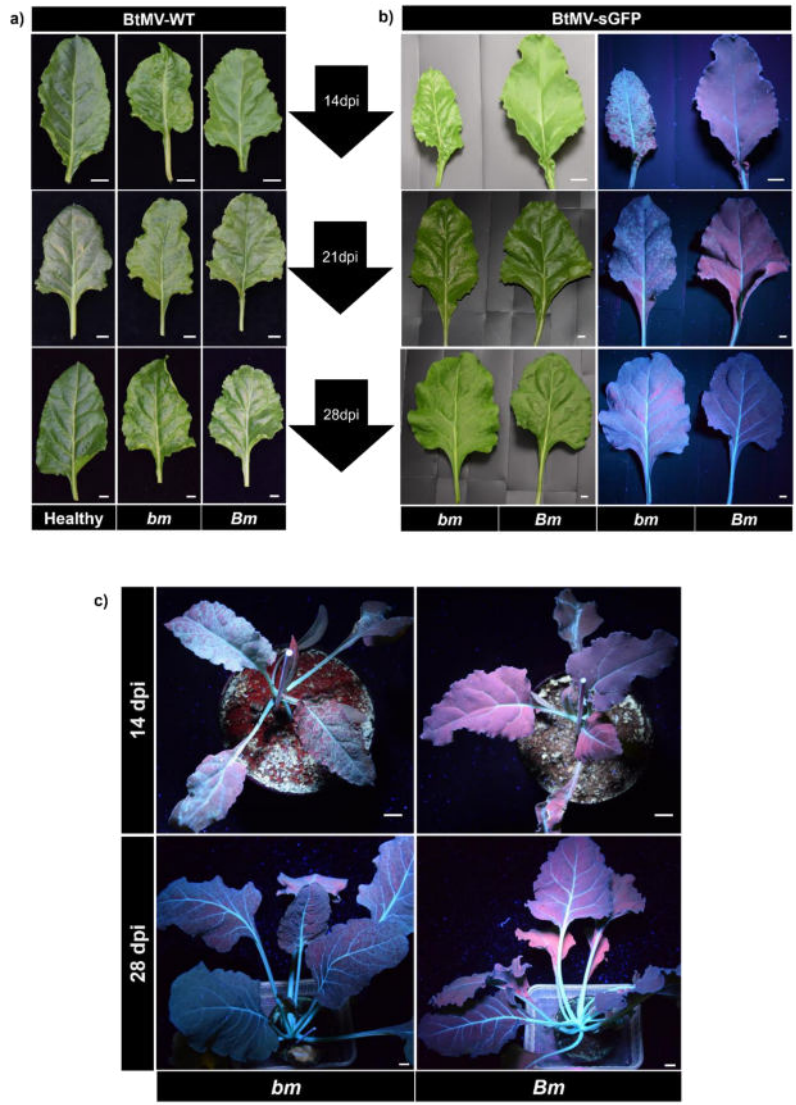

Figure 4: a) Symptom development caused by BtMV after mechanical inoculation in sugar beet plants carrying the *bm*- or *Bm*-allele. Shown are representative leaves displaying typical symptoms at 14-, 21- and 28-days postinoculation (dpi). **b)** Symptom development over time in plans of the same sugar beet lines inoculated with BtMV-sGFP under white/ UV-light. **c)** Symptom development and virus distribution of BtMV-sGFP infected plants carrying the *Bm/bm*-allele, respectively under UV-light displaying the green-GFP fluorescence. White bars= 1cm.

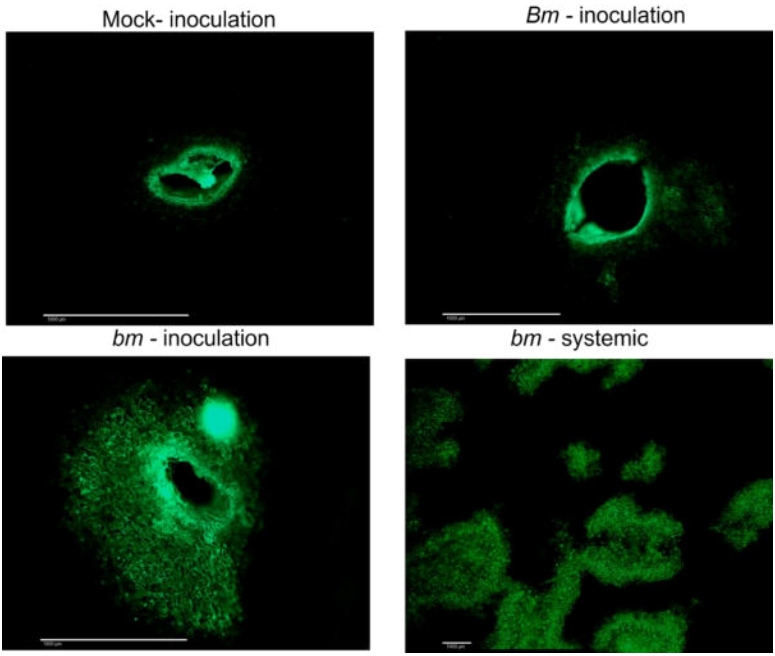

Figure 5: Epifluorescence pictures of *Bm/bm*-allele carrying sugar beet leaves trying to detect GFP fluorescence after prick inoculation without *Agrobacterium tumefaciens* (Mock) or with *A. tumefaciens* containing pDIVA-BtMV-sGFP. Shown are the damages and the virus spread of BtMV-sGFP starting from the infection foci (inoculation) or after systemic spread if applicable at 10 days postinoculation. White bars= 1000 µm.

The relative BtMV content increased significantly in *bm*-plants on a weekly basis except the period from 14 to 21 dpi. No amplification was detected in *Bm*-carrying plants at 7 dpi, in contrast to that, in *bm*-plants BtMV already accumulated systemically. With first symptom appearance in systemically infected leaves of *Bm*-plants at 14 dpi, BtMV was detected using RT-qPCR, still relative BtMV content was significantly lower compared to *bm*-plants. This pattern was repeatedly observed during the remaining two-week period with low and unchanged relative BtMV content in *Bm*-plants, significantly lower than in *bm*-plants. (Figure 6a).

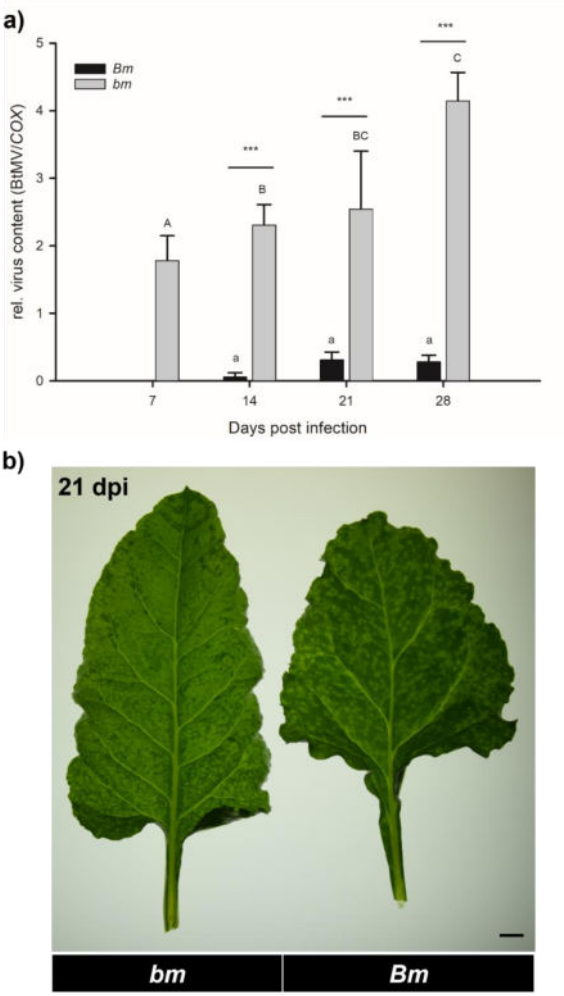

Figure 6: a) Relative BtMV content in comparison to the internal reference gene expression (COX) in systemically infected leaves of sugar beets carrying either the *bm*- or *Bm*- allele tested by RT-qPCR. Bars show means with standard deviation of relative BtMV content from five biological replicates. No amplification was observed in Bm- plants at 7 days postinoculation (dpi). Different upper/lower case letters indicate significantly different virus contents between timepoints for each virus identified by two-way ANOVA with a post-hoc Tukey test ($p < 0.05$; n = 5). Differences in virus accumulation between the two alleles for each timepoint are indicated with asterisks (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; n.s. = not significant; n=5). **b)** Typical symptoms of BtMV in dependency to the respective resistance allele at 21 dpi. Black bar= 1cm.

Discussion

Recessive resistance mediated by a loss of interaction between eIF and potyviral VPgs is one of the most often identified plant virus resistances and has been extensively characterized in the past. Still the underlying molecular mechanism mostly remain obscure (Diaz-Pendon *et al.* 2004; Mäkinen, 2020; Truniger and Aranda, 2009). Therefore, we wanted to investigate if a recessive resistance can be implemented against BtMV in *B. vulgaris*. Even though we were able to identify interactions between BtMV-VPg and Bv-eIF(iso)4E in BiFC and YTH, no influence on infectivity and symptom expression was observed in *Bv-eIF(iso)4E*^{KO}-plants or in plants of any of the other tested single knockout lines (Table 1). An interaction between BtMV-VPg and Bv-eIF4E-like was only found in YTH, while no resistance was obtained in plants carrying a single KO of *Bv-eIF4E-like* or a double KO of *Bv-eIF(iso)4E* + *Bv-eIF4E-like,* therefore we assume that the YTH results were false positive. As a result of this, we concluded that BtMV can colonize its host effectively independent of Bv-eIF(iso)4E despite a physical interaction with the BtMV-VPg. Reasons for this could be as manifold as the proposed molecular mechanisms behind an eIF-mediated recessive resistance (Truniger and Aranda, 2009). With the experiments performed in this study it remains unclear to what extend and for which exact functions Bv-eIF(iso)4E is involved in BtMV infection, still we suspect a redundant interaction with other Bv-eIFs nullifying the KO of *Bv-eIF(iso)4E*. An explanation for this could be, similar to our previous work on BMYV (Rollwage *et al.* 2024), a simultaneous interaction between potyviral VPg and Bv-eIF(iso)4E and Bv-eIF4E for translation initiation. However, in the case of BtMV-VPg we were unable to detect such an interaction, still it is conceivable that an interaction between BtMV-VPg and Bv-eIF4E is disguised by the autoactivation of the reporter genes in YTH. The involvement of a not yet identified/described protein is even more probable, as neither do the BiFC results

suggest an interaction between BtMV-VPg and Bv-eIF4E, although false-negative results are possible in this assay too. To obtain a new recessive resistance mechanism against BtMV in beets a broader approach to identify interaction partners should be used in the future screening whole genomic libraires, not using a biased candidate gene approach.

With the (re-)emergence of VY in European sugar beet growth areas, an approach with immediate impact for diseases management is urgently required. However, due to a relative moderate negative yield effect, single BtMV infections are currently not regarded as an economical threat itself. In sugar beet production the synergistic effects of BtMV during co-infection with other viruses is feared (Heathcote, 1973; Hossain *et al.* 2021; Stevens *et al.* 2005b; Wintermantel, 2005). By significantly reducing the amount of natural virus inoculum using the incomplete *Bm*-gene, BtMV in single and co-infection could be controlled to an economical acceptable level (Nutter, 1993).

To allow a better understanding of the resistance and the virus distribution we successfully labelled the BtMV cDNA full-length clone with sGFP, allowing to follow the virus infection at a single cell level using a fluorescence microscope or on a full plant level by UV-illumination. Even though the labelled clone is expected to replicate slower in the host plant and by that produces a lower viral load (Rollwage *et al.* 2023), the disease severity, virus spread and distribution on the discriminating plant material was easier to evaluate by naked eye using the fluorescence labelled clone. Intriguingly, *Bm*-plants only showed single spots on their leaves which are in common with the symptoms under white light, however as the plants grew older, no GFP signal was observed in *Bm*-plants indicating a recovery from the viral disease. Lewellen (1973) only described a recovering phenotype for the susceptible *bm*-plants, which was not found in our study as well as in Friesen *et al.* (2006). Recovery from viral diseases is often thought to be conferred by RNA interference (RNAi) within a host plant, resulting

in reduction of viral replication level in previously colonized tissue including recovery from symptoms. The lower the virus titre is within plants showing recovery, the faster the recovery itself is expected (Ghoshal and Sanfaçon, 2015). Due to the lower viral load of the BtMV-sGFP it can be suggested that only *Bm*-plants infected with the recombinant virus showed a recovery within four weeks which took longer in wildtype plants. Furthermore, we could show that BtMV is still able to spread from cell-to-cell and systemically infected *Bm*-plants, despite a delay of at least 10 days compared to *bm*-plants. Noticeable the delay was observed at the infection foci itself, indicating an involvement of *Bm* in cell-to-cell movement or replication efficiency and not the long-distance movement.

The observed symptom development is in common with the relative virus load determined by RT-qPCR, as *Bm*-plants without symptoms at 7 dpi proved to be not systemically infected. Over the course of four weeks the virus content remained low in *Bm*-plants, without increasing significantly, indicating an impairment of effective virus colonialization, while the BtMV content in *bm*-plants continuously increased. Potential reasons for this could be the already mentioned direct impairment of viral cell-to-cell movement or a lowered replication confining the infection (Ronde *et al.* 2014). The recovery process could then be mediated by the RNAi machinery (Ghoshal and Sanfaçon, 2015). A combination of the respective molecular mechanism could also be responsible for the significantly lower viral load and by that decreased symptom expression. With the data presented here, the *Bm*-resistance proved to be effective in controlling BtMV infections significantly, while the exact molecular mechanism and the impact on field performance for the *Bm*-gene remain unclear. However, to investigate the influence of the *Bm*-gene on yield stability in single or mixed infection, the gene should be fine mapped in the future to allow a fast and reliable integration of the gene into elite plant material followed by testing under field conditions (Collard *et al.* 2005).

For this a segregating population has to be screened in a fast and reliable manner, ideally distinguishing also intermediate phenotypes if applicable. Labelled cDNA clones like the here produced BtMV-sGFP or our previously published BtMV-MYB clone, which induces betalain production in leaves (Rollwage *et al.* 2023), provide an excellent opportunity for this task, without needing expensive and time-consuming laboratory work. Finally, these results led us to the conclusion that fine mapping *Bm*-resistance is worth trying, as the newly developed reverse genetic systems for BtMV provide a way to speed up the selection procedure without causing additional costs. If the fine mapping is successful, the field performance of *Bm*-carrying elite cultivars must be evaluated in the future. However, beside our previously proposed polerovirus-eIF resistance (Rollwage *et al.* 2024), it may provide an additional resistance mechanism to control VY in sugar beets.

Experimental procedures

Yeast two hybrid assay

A LexA based yeast two hybrid assay (YTH) (MoBiTec; Göttingen) was performed as described in Muellender *et al.* (2021) to detect potential protein-protein-interaction (PPI) between BtMV VPg and sugar beet eIFs. For sugar beet eIF CDS cloning, total RNA was extracted from sugar beets infected with BtMV (DSMZ PV-1228; GenBank accession no. MT815987); using the Nucleospin RNA plant mini kit (Macherey-Nagel; Düren). cDNA synthesis was performed with random hexamer primers, using RevertAid H Minus reverse transcriptase (Thermo Fisher Scientific; Waltham), according to the manufacturer's instructions. All cloning steps were performed by standard restriction enzyme cloning (Thermo Fisher Scientific; Waltham), resulting in in-frame fusions of activation/binding domain and protein of interest, respectively. Each obtained plasmid was confirmed by restriction enzyme digest and Sanger sequencing

of the complete insert. The coding sequence (CDS) of sugar beet eIF-genes *Bv-eIF4G1, Bv-eIF(iso)4G1, Bv-eIF(iso)4G2, Bv-eIF4E, Bv-eIF(iso)4E* and *Bv-eIF4E-like* were cloned into pEG202 to be translationally fused to CDS1 LexA DNA binding domain, while the BtMV VPg sequence (nucleotide 5731 to 6303 with a stop codon added) was cloned into pJG4-5 containing the C-terminal B42 transcription activator domain- hemagglutinin (HA-) epitope. Positive controls as well as the negative controls were supplied by MoBiTec. *S. cerevisae* (EGY48) cells were super transformed by lithium acetate transformation of the plasmids (Gietz and Schiestl, 2007). Transformants were resuspended in water and serial diluted (10^0 -1 x 10^{-4}). 5 µL of each dilution was spotted on petri dishes with culture media, incubated at 30 °C for 3 days (control medium) or 5 days (selection medium), respectively.

Bimolecular fluorescence complementation

BiFC experiment was performed according to Zilian and Maiss (2011) using the self-interaction of plum pox virus (PPV) coat protein as a positive control. The shortened PPV coat protein CP3, unable to self-interact, was used as negative control. Bv-eIF4E, Bv-eIF(iso)4E and Bv-eIF4E-like were translationally fused at their C-terminus to the N-terminal part of mRFP (mRFPN). The C-terminal part of mRFP (mRFPC) was fused to the N-terminus of the BtMV-VPg. Cloning was performed by Gibson isothermal assembly (Gibson *et al.* 2009). Each plasmid assembly was confirmed by restriction enzyme digest and Sanger sequencing of the insert. *Agrobacterium tumefaciens* C58C1 cells were transformed with the plasmid constructs using electroporation at 1440 V. *A. tumefaciens* C58C1 containing the different plasmids were grown overnight at 28°C. OD_{600} was adjusted to 0.5 in inoculation buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 µM acetosyringone). After three hours of incubation at room temperature, leaves of approximately four weeks old *Nicotiana*

benthamiana were co-infiltrated with the respective eIF-VPg combination and the tomato bushy stunt virus P19 silencing suppressor (Voinnet *et al.* 2003) into leaves. The infiltration patches were assessed for mRFP fluorescence signal 3 to 5 days postinoculation (dpi). The mRFP fluorescence was visualized with the TCS-SP5 confocal laser-scanning microscope (CLSM) (Leica Microsystems; Wetzlar). Excitation/emission wavelengths for mRFP were 584 nm/ 600 - 610 nm. All confocal images were processed with the LAS-AF software version 2.6.3.8173 (Leica Microsystems; Wetzlar).

Integration of reporter genes into pDIVA-BtMV1228

The infectious BtMV cDNA full-length clone pDIVA-BtMV1228 was PCR-amplified in two overlapping fragments using the primers PDIVA4 + M14 and M15 + PDIVA6 respectively (Supplementary table S1). With the primer M15 a duplication of the BtMV NIa/NIb-cleavage site (EVVEQ/G) was introduced 5' to the HC-Pro coding sequence (Rollwage *et al.* 2023). The synthetic green fluorescent protein S65T (sGFP) coding sequence was amplified from plasmid pNMD05535-sGFP (kindly provided by Stefano Torti; Nomad Bioscience GmBH; Halle), using primers BsGFP1 + BsGFP2 (Supplementary table S1). Both primer pairs added appropriate overlaps to the inserts, allowing an integration of the reporter between P1 and HC-Pro coding sequence in pDIVA-BtMV1228 using Gibson assembly.

BtMV infection assays

In-vitro grown sugar beet plantlets with specific single or multiple *eIF* knockouts (Rollwage *et al.* 2024) showing interaction with BtMV-VPg, were transplanted to soil, slowly adapted, and further cultivated under controlled greenhouse conditions

(24°C/14 h light, 18°C/10 h dark photoperiod). Leaves of infected and symptomatic BtMV plants were grinded 1:10 (w/v) in 10 mM phosphate buffer (pH 7.3) for mechanical inoculation. Infectious plant sap was inoculated together with Celite® 535 on 14 days old seedlings of beets. Plants were scored every two days and considered infected if they displayed mosaic symptoms within a four-week period after inoculation. As eIF-knockout plants did not result in a BtMV resistant phenotypes the dominant resistance *Bm*-allele was further characterized. For inoculation, cotyledons of 14 days old seedlings of sugar beet lines 'SV-IfZ100' (*Bm*) and 'SV-IfZ101' (*bm*), differing in the presence of the *Bm*-allele, were infiltrated with *Agrobacterium tumefaciens* C58C1 (agro-inoculation). For this, *A. tumefaciens* C58C1 cells transformed with the pDIVA-BtMV-sGFP plasmid were grown overnight at 28°C. OD_{600} and adjusted to 0.5 (*N. benthamiana*) or 1.5 (*B. vulgaris*) in inoculation buffer, respectively. Additionally, first true leaves *of N. benthamiana* were agro-inoculated 21 days after emergence (Laufer *et al.* 2018b). Virus symptom development was evaluated daily over a period of four weeks. To investigate virus distribution throughout whole plants, a hand-held long wave UV-lamp (Black Ray model B 100 AP; 100 W; UV Products) was used to visualize GFP-fluorescence and captured by digital camera (Canon; Tokyo).

Alternatively, for better evaluation of single infection foci, leaves were pricked with syringe needles covered with *A. tumefaciens* C58C1 containing pDIVA-BtMV-sGFP directly from the agar plate. The sGFP fluorescence was visualized with an Axio Scope.A1 epifluorescence microscope (Zeiss; Jena). Excitation/emission wavelengths for sGFP were 490 nm/ 500 - 520 nm. All epifluorescence images were processed with Motic Image plus 3.0 (Motic; Wetzlar).

BtMV quantification with real-time PCR (RT-qPCR)

Total RNA was extracted from virus infected sugar beets with or without *Bm*-allele on a weekly basis with GeneJET Plant RNA purification kit (Thermo Fisher Scientific; Waltham). All subsequent RT-qPCR reactions were carried out using the CFX96 Real time system (Bio-Rad). All used primers for gene expression analysis can be found in supplementary table S1. Viral load in BtMV infected plants was quantified by one step RT-qPCR using 100 ng of total plant RNA. For cDNA synthesis, Superscript™ IV reverse transcriptase (Thermo Fisher Scientific; Waltham) was used. In addition to that 2x Maxima Probe qPCR mix (Thermo Fisher Scientific; Waltham) with 400 nM primers BtMVqs1, BtMVqas1 and the Fam-BHQ1 labelled probe BtMVq-p1 were added to the reaction mix. *Beet cytochrome oxidase* (COX) was used as a housekeeping gene to confirm integrity of samples with 400 nM of primers COX-F, COX-R and the Fam-BHQ1 labelled probe COX-P-FAM as described (Mahillon *et al.* 2022). Cycler conditions were 3 minutes 95°C; 10 minutes 52°C; 10 minutes 95°C for cDNA synthesis, followed by 40 cycles 15 s 95°C; 60 s 60°C. Gene expression data was analysed using the delta Ct method (Schmittgen and Livak, 2008).

Statistical analysis

Relative BtMV content was calculated by delta Ct method with their log-transformed values being utilized for statistical analysis using SigmaPlot 14.5 (Systat Software). Mean values are displayed with standard deviation (SD). Mean values of expression data between different viruses and timepoints were tested for differences by a two-way ANOVA with a subsequent post-hoc Tukey test ($p \leq 0.05$).

Authors contribution

MV and LR contributed to the conception and design of the study. LR performed cloning, YTH, BiFC, the greenhouse bioassay and wrote the first draft of the manuscript. Both authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supporting Information

Supplementary Table S1: Primers used in this study for generation of pDIVA-BtMV-sGFP. To distinguish between vector/GFP and viral sequence, the vector/GFP sequences are italicized. Furthermore, RT-qPCR primers/probe are shown, which were used to quantify BtMV content.

Primer	Sequence 5' → 3'
M14	ACTACTGTAGTGCATGGTTCTACCAAC
M15	GAAGTAGTCGAGCAGGGAGAGGAAAGATTCTTTGCTGG
PDIVA4	*CGGATTGTCCCTATACGAATTCATTAGACAGCCGCTTAGC*
PDIVA6	*GCTAAGCGGCTGTCTAATGAATTCGTATAGGGACAATCCG*
BsGFP1	GAACCATGCACTACAGTAGT*ATGGTGAGCAAGGGCGAGG AG*
BsGFP2	TCTCCCTGCTCGACTACTTC*CTTGTACAGCTCGTCCATGC CG*
RT-qPCR Primer	
BtMVqs1	GATGCAGCAGAAGCGTATATTGA
BtMVqas1	TGTCTCTCAGATTTCTCTGAGC
BtMVq-p1	[FAM]CAACAGAGAAAGGCCATACATGCC[BHQ1]
COX-F	CGTCGCATTCCCGATTATCCA
COX-R	CAACTACAGAGATATAAGAGC
COX-P-FAM	[FAM]TGCTTACGCAGGATGGAATGCCCT[BHQ1]

6. General discussion

6.1 Bv-eIFs - new resistance genes to control virus yellows in sugar beet

Till 2030 a reduction of 50 % in used chemical plant protection is desired within the European Union (European Commission, 2022). Therefore, it is highly unlikely that a substitute pesticide for neonicotinoids will be available soon, if ever. For this reason, an inherited genetic resistance towards members of the VY appears to be the only possibility to protect sugar beet yields from losses caused by the viral disease. At the begin of this thesis no effective control for VY was known. Out of this reason this thesis aims to identify resistances towards members of the VY and provide tools to simplify the breeding procedure.

Nowadays (2023) first sugar beet varieties providing increased VY tolerance, suffering from less severe yield losses when infected, are registered and available to the market in Germany (Bundessortenamt, 2023), however, no resistance source that reduce or limit virus replication and/or movement has been described so far. Some of the most often used resistance genes in plant breeding are mutations of the host plants eIF, which confer a resistance to VPg carrying viruses mostly potyviruses via incompatibility (Truniger and Aranda, 2009). An eIF-based recessive resistance has previously not been found in *B. vulgaris*. However, my co-workers and I were the first to identify and describe an eIF-based recessive resistance against a member of VY disease in *B. vulgaris* by showing that Bv-eIF(iso)4E is a susceptibility factor during BChV infection. Furthermore, interaction assays indicate that BMYV directly interacts with both eIF isoforms Bv-eIF4E and Bv-eIF(iso)4E. Unfortunately, we were unable to knockout both eIFs simultaneously and could not prove that these are the sole interaction partners important for BMYV replication. For BChV on the other hand we could show that a *Bv-eIF(iso)4E* knockout results in a resistance with lowered viral titre

and decreased infection rate. Hence, I propose that the *Bv-eIF4E* isoforms are important candidate genes for VY resistance breeding. But there are questions, which remain obscure and should be investigated further before this resistance can be used commercially.

The current framework on genome edited plants in the European Union forbids their use in commercial plant production (European Commission, 2021), this makes genome editing currently unusable as a breeding tool. In addition to that, a knockout of single/multiple eIF genes in sugar beet is expected to be disadvantageous for the plant's fitness because of the central role of eIF-isoforms in the cellular mRNA translation. The eIF(iso)4E and eIF4E both preferably initiate translation of specific mRNA types (Browning and Bailey-Serres, 2015). Most often such preferences indicate an evolutionary advantage for the respective isoform and cannot be compensated without negative effects of any kind (Mayberry *et al.* 2009; Mayberry *et al.* 2011). Even though no obvious phenotype can be observed in most eIF knockout plants, similar to our own observation in eIF-knockout sugar beets, multifactorial traits such as yield have not been assessed. Yield penalties caused by eIF knockouts could be hidden negative effects and can only be assessed when such a mutation is integrated into elite cultivars. Considering that a null mutation would have no negative influence on plant fitness, mutations resulting in a recessive resistance e.g., by a preliminary stop codon, would occur more often within a plant population. Few examples like the *lsp1-1* and *lsp1-2* allele in *A. thaliana* are known to result in a preliminary stop codon of eIF(iso)4E conferring resistance towards TuMV (Lellis *et al.* 2002). However, there are no reports on how eIF-resistance alleles with a preliminary stop codon influence multifactorial traits before introduction into elite cultivars as they have not been assessed. Moreover, as shown by Zafirov *et al.* (2021), a knockout of one eIF isoform increases disease severity to viruses relying on the other unaffected

eIF-isoform. An increased susceptibility as a result of specific knockouts was not found for the beet infecting poleroviruses BMYV and BChV. Still, other viruses, which may rely on eIFs during their translation initiation because of a VPg or potential cap structure at their 5'end, have not been tested in a quantitative way such as BtMV or have not been tested at all such as BYV. Out of this reason the influence of knockouts on susceptibility towards other viruses could not be evaluated any further. As stated in manuscript I, the underlying molecular resistance mechanism of the eIF-mediated resistance against poleroviruses in *Beta vulgaris* remains unknown, as it is unclear if the BChV resistance functions as a full infection resistance or if BChV would be able to replicate within an isolated protoplast. Nonetheless, identification of a *Beta vulgaris* eIF variant conferring a loss of interaction to the VPg could result in resistance and is highly desirable. This is supported by the mutational analysis of the cap binding amino acids in manuscript I, which showed that when mutated, the interaction between eIF and VPg can be abolished. With a directed approach combining mutational analysis and cap complementation assays, it could be possible to identify amino acids involved in VPg binding, which are not involved in cellular mRNA cap binding. Here two possibilities exist, either screening systemically the Bv-eIFs stepwise from N- to C-terminus and subsequently screen germplasm for mutations at this specific site or alternatively perform a pre-screen of known/naturally available mutations within the respective Bv-eIFs and only test those for loss of interaction before selfing these plants to obtain homozygous material. The first approach would allow a more universal solution, while the second one would be a more cost-effective way of pre-screening the available variation of e.g., a breeder's germplasm. It has to be emphasized that such methods can only act as pre-screen tools. The loss of interaction and the mediated resistance must be validated in plants homozygous for the eIF-mutation. Furthermore, the exact VPg sequence of poleroviruses remains unknown as multiple

approaches e.g., using virus purification or expression of the human influenza hemagglutinin (HA)-tagged poleroviral P1 *in planta* did not result in sufficient yields or correct processing products for the desired BMYV- and BChV-VPg (data not shown). With the exact VPg sequence the validity of PPI studies would be improved and false-positive/ false-negative results avoided. However, with the data obtained here, it appears to be likely that at least the poleroviral members of the VY can be controlled in the future by identifying non interacting Bv-eIF(iso)4E variants for BChV and additional non interacting Bv-eIF4E variants for BMYV.

From the PPI studies it would have been expected that an additional BtMV resistance could be obtained by *Bv-eIF(iso)4E*^{KO} (+ *Bv-eIF4E-like*^{KO}), unfortunately this was not the case, still no quantitative assessment of BtMV was performed as only the presence of symptoms has been recorded, however as already observed with BChV, a quantitative resistance could be a possibility as well. Therefore, the experiments with knockout plants should be repeated with BtMV, trying to mimic the natural BtMV inoculation by performing aphid inoculation for infection and using RT-qPCR to determine potential quantitative effects. The BtMV-MYB cDNA clone described in manuscript II could also help to visualize potential differences in viral load with the naked eye. Other reasons for the missing resistance could be the simultaneous recruitment of Bv-eIF4E and Bv-eIF(iso)4E by BtMV, which is not supported by the PPI experiments, but could be masked by effects such as the YTH autoactivation of Bv-eIF4E. But also, one/multiple not yet identified interaction partners to the VPg or other viral proteins could play a role in the potyviral replication cycle. Therefore, an unspecific YTH screen using a *B. vulgaris* cDNA library could provide more clarification on potential hidden interaction partners without having a selection bias for the screen could be identified. A similar experiment would be desirable for BYV, while no work has been published on recessive resistance for Closteroviruses, it appears tempting

that all three VY and BtMV could be controlled by the same mutation(s) at the same time. Indications for potential recruitment of beet eIFs by BYV is the +ssRNA genome, which must be translated to function properly, but even more the speculated presence of a methylated nucleotide cap at the genomes 5'end (Karasev *et al.* 1989). It remains unclear if the BYV genome is truly capped and if so, how it is capped. For this cap snatching could be an explanation, which only has been reported for negative stranded RNA viruses and requires an endonuclease (Applied Plant Virology, 2020; Hull, 2014; Xu *et al.* 2022). No encoded endonuclease with this function is known for BVY (Agranovsky *et al.* 1994) but if it would be able to perform cap snatching, the capped genome would be indifferent to cellular mRNA, making its interaction with the eIF4Es indistinguishable. In this case only a null mutation or knockout of the respective gene would confer resistance towards BYV, as the cap binding amino acids could not be modified to only repress the interaction with the BYV genome while remaining its mRNA binding abilities. Therefore, the trade-off between losses caused by BYV and potential yield penalty caused by the specific null mutations needs careful evaluation. Moreover, it remains unclear whether BYV would be able to interchangeably use the different eIF isoforms making it uncontrollable by an eIF-mediated recessive resistance, which would shift the selection focus rather towards identification of a dominant resistance.

The durability of an eIF-mediated polerovirus resistance in sugar beets is difficult to assess, as it is impossible to predict the durability of a resistance gene in advance. However, recessive resistance is often thought to be more durable than dominant resistance. But work on resistance breaking isolates of e.g., cucumber vein yellowing virus (CVYV) showed that single mutations within a VPg allowed to overcome eIF-mediated resistances (Desbiez *et al.* 2022). It is unclear whether the original compatible reaction is restored in these events or if the VPg mutation allows the virus

to recruit the previously unused eIF isoform (Truniger and Aranda, 2009). The durability is highly influenced by the mutation rate of the respective viruses, which is expected to be increased for viruses than in higher organisms as the viral RdRp has an elevated error rate due to a lack of proof reading (Elena *et al.* 2008). Next to durability is the question on how broad an eIF-mediated resistance will function. With the here obtained data it is unknown if the other beet infecting poleroviruses BWYV and BLYV (Yoshida and Tamada, 2019) could be controlled by *Bv-eIF(iso)4E* (+ *Bv-eIF4E*) as no interaction data or bioassays have been performed yet. BWYV and BLYV have not been reported in Europe so far, however with the high degree of globalization and interstate connectivity, it is likely that these viruses will migrate towards Europe in the future as they did in the past (Bright, 1999). Therefore, proactive research is recommended investigating the control potential of the other poleroviruses by eIFs. For this, experiments performed within this thesis could be extended by integrating the other viruses VPg sequence in PPI and measure the influence of eIF knockouts on each virus's accumulation. The possibility to control BWYV and BLYV is likely due to the high resemblance of poleroviruses in their translation, however combining the findings of manuscript I and Reinbold *et al.* (2013) observations on BMYV and TuYV, it is obvious that even close related viruses could depend on different eIFs for their translation initiation and the true interaction partner must be identified experimentally and cannot be predicted. Next to already known beet infecting poleroviruses a potential threat could be the host spill over of other previously non-beet infecting virus species such as TuYV. First reports mention that TuYV isolates have been detected with low frequencies in *Beta vulgaris*. Intriguingly beets previously displayed a non-host resistance towards TuYV, which may was overcome trough mutations in the P3+P5 readthrough protein (Filardo *et al.* 2021; Puthanveed *et al.* 2023). In a European wide virus monitoring performed from 2017-2019, a single TuYV infected sugar beet plant

has been identified in a field in the Netherlands in collaboration between Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and IfZ. The TuYV sequence obtained from this plant, showed a high level of polymorphisms compared to other TuYV non-beet infecting isolates in the estimated VPg sequence (Hossain *et al.* 2022). Therefore, further investigation between beet- and non-beet infecting isolates of TuYV focusing on eIF-VPg interaction are required to get a better understanding of the often uncharacterized non-host resistance, as incompatibility between plant and virus has been proposed as one of the mechanisms behind non-host resistance (Baruah *et al.* 2020; Mysore and Ryu, 2004). These experiments would also allow to obtain extended knowledge on the function of VPgs in host specificity. If differences in the interaction pattern of the TuYV-VPgs could be found, hot spots responsible for the host change could then help to determine domains or amino acids responsible for resistance breaking in other polerovirus species allowing an estimation of resistance durability. These works will benefit from the availability of infectious cDNA clones such as the ones produced in manuscript II and III. These clones can be easily mutated to understand the influence of all respective mutations.

Overall, the *Bv-eIF(iso)4E* appears to be an effective candidate gene for resistance breeding against BChV infection in *B. vulgaris*, while natural mutations conferring resistance still need to be identified. But the work performed within the frame of this thesis is the first proof that an eIF-mediated recessive resistance can be implemented against members of the VY disease in sugar beet. In addition, it is the first time a recessive resistance against a polerovirus in a non-model organism was identified. Lastly, while no resistance towards BMYV and BtMV was observed, the results, especially for BMYV, indicate that by pyramiding non-VPg-interacting Bv-eIF(iso)4E and Bv-eIF4E variants a resistance might be implemented.

6.2 *BvMYB1* a new reporter gene to evaluate virus resistances in *B. vulgaris*

Plant virology vastly profited from the availability of infectious cDNA clones. With the use of these reverse genetic systems an in-depth view on multiple factors of the host-virus interaction can be obtained. Here, mutations within the viral genome and their influence on e.g., replication, host specificity, vector transmission or intra plant distribution can be assessed in a fast and reliable manner (Tilsner and Oparka, 2010). These respective mutations can range from single nucleotide polymorphisms to exchanges of complete open reading frames or even genome halves to mimic natural occurring intra/inter-species recombination. With an increasing research need on host-virus interaction these cDNA clones get more important due to their broad usability. One of the most often used techniques is the labelling of cDNA clones with reporter genes, like fluorescent proteins such as GFP or other reporter genes such as β-glucuronidase (GUS) (Baulcombe *et al.* 1995; Cruz *et al.* 1996; Dolja *et al.* 1992). However, these labelling techniques are either relying on specialized equipment for visualization or are invasive methods, making a live imaging impossible. Bedoya *et al.* (2012) were the first to visualize virus spread for the naked eye by using anthocyanin inducing transcription factors, which resulted in leaf pigmentation. This method has been adapted to manipulate carotenoid biosynthesis pathway as well (Majer *et al.* 2017). However, as *B. vulgaris* is a member of the order *Caryophyllales*, it exclusively produces the red pigment betalain instead of anthocyanin which is regulated completely different (Hatlestad *et al.* 2015; Stafford, 1994). In manuscript II we report for the first-time visualization of viral infection to the naked eye in plants of the order *Caryophyllales.* For this an infectious cDNA clone of BtMV was modified to heterologously express the *BvMYB1* transcription factor, which subsequently manipulated the betalain biosynthesis. With this manuscript me and my colleagues

provide the first reporter-gene system to visualize virus spread in beets without the need of any further technical equipment. Exemplified using BtMV, this tool can be used to either study host-virus interaction further or it can be directly used to improve resistance screenings. As shown in manuscript II the betalain biosynthesis and virus content highly correlated, hence if the visible red pigmentation increases the expected virus content within a leaf is higher. Traditionally, resistance breeding consists of an expert judgment assessing e.g., infection rates and disease severity, which have to be verified by molecular biological techniques. However, often viruses produce symptoms hard to distinguish, this could be improved by the red pigmentation. Also, the cDNA clone allows to visualize different speeds of systemic infection and unequal virus distribution in each plant. Subsequently, the breeding step of disease rating could be (partially) automated in the future. Using non-invasive image processing it appears likely that the pigmentated surface and its intensity correlate with the virus content. Hence, this method would not rely on expensive multispectral cameras and analysis making it more affordable for screens. One of the biggest advantages besides symptom scoring automatization is that the indirect indication of infection with hints on the virus load vastly decreases the amount of molecular biological laboratory work. If an algorithm can be developed to estimate viral loads, methods such as ELISA or RT-qPCR may will be obsolete in the future. This step often limits the throughput of plants to be screened due to the need of highly skilled workers and the high costs per single sample. However, the *BvMYB1* reporter has also its limitations which might limit it´s practical application. Through the integration of the transcription factor the virus content significantly decreased reducing the comparability towards a natural infection. Additionally, the plants to be tested should be able to produce betalains in general but not constitutively in all tissues. Therefore, plants should carry the *R*-allele responsible for the red colour of the hypocotyl encoding a functional *BvCYP76AD1* and at the same

time not produce *BvMYB1* from the *Y*-locus (Hatlestad *et al.* 2012; Hatlestad *et al.* 2015). If the plants do not have a *R* and *y*-genotype, the lack of red pigments is no indicator for a resistance, as further mutations influencing the betalain biosynthesis pathway can influence the pigmentation, thus a potential resistance has always to be confirmed by a more refined molecular technique. Additionally, the recombinant virus falls under the law for genetically modified organisms in Germany, hence it cannot be used in field trails and only in specialized labs and greenhouses, which requires a high initial investment and maintenance cost limiting the amount of plants to be screened. For this reason, the inoculation method itself must be improved in the future to allow an infection e.g., by spray inoculation instead of using needleless syringes (Hahn *et al.* 2015).

BtMV is often regarded as neglectable due to comparable low yield losses of ~ 10 % instead of the 20 – 40 % caused by the VY members (Hossain *et al.* 2021; Shepard *et al.* 1964; Stevens *et al.* 2005b; Wintermantel, 2005) and breeding for resistance is not regarded as first priority. Hence, labelling other beet infecting viruses of higher economic importance would be desirable. However, because of the poleroviruses and closteroviruses genome organisations the integration of the *BvMYB1* transcription factor will be more difficult. The expression strategy of the potyvirus BtMV is the production of one single polyprotein from a single ORF, which is then subsequently cleaved and processed into the final proteins. Using the natural P1-HC-pro cleavage site and an additional NIa/NIb cleavage site in the BtMV full-length clone presented in manuscript II, the proper release of the BvMYB1 transcription factor from the polyprotein was ensured without any additional fusion proteins. Thus, the BvMYB1 was able to localize to the nucleus promoting betalain biosynthesis. On the other hand, labelling approaches for the polerovirus TuYV with GFP, suggest to require a partial deletion instead of fusing a fluorescence protein to the P3 + P5 read through protein

(Boissinot *et al.* 2017). With this, the virion itself is decorated with the fluorescence protein as the read through protein is a minor capsid protein exposing the GFP at the virions outer surface, a similar approach was used for BNYVV (Laufer *et al.* 2018a). Hence, it appears unlikely that such a fusion-protein is still able to localize to the nucleus and additionally induces transcription of betalain biosynthesis genes. For the closterovirus BYV GFP-labelled cDNA clones, which are infectious in *B. vulgaris,* have not been reported. However, the expression strategy of Peremyslov *et al.* (1999) inducing an additional GFP-ORF in the BYV genome between major and minor coat protein, driven by the subgenomic BYV-CP promoter, while the BYV-CP itself is driven by a promoter derived from beet yellow stunt closterovirus, would ensure proper expression of the BvMYB1 factor without fusion to other proteins. If this strategy could be transferred to poleroviruses a labelling of the respective virus could be possible, but it always remains to be seen what influence on infectivity the integration of additional ~750 bp have on the virus replication and distribution. Also, the presence of the virus expressed *BvMYB1* RNA sequence could promote the RNAi related anti-viral response within the host plant. Unfortunately, the currently available cDNA full-length clones of poleroviral VY members have a low infection rate of ~ 30 % in *B. vulgaris* (Hasan, 2004; Peremyslov *et al.* 1998; Stephan and Maiss, 2006; Wetzel *et al.* 2018), except for the BMYV cDNA clone published by Klein *et al.* (2014). However, to monitor resistances an infection rate of 100 % is needed, to rule out any miss judgement of potential resistance due to a missing initial infection. Out of this reason, the use of *BvMYB1* labelled cDNA clones for resistance screens is limited as long as the infection rates cannot be increased to levels of the BtMV cDNA clone. For research purpose and exemplary visualization, *BvMYB1*-labelling of cDNA clones with low infection rates still can be beneficial. Labelled clones, either with fluorescence proteins or *BvMYB1*, have their advantages and disadvantages respectively. Nonetheless, they could help

to further characterize the eIF-mediated resistance identified within manuscript I to see whether the resistance impairs virus transport or already acting on a single cell level limiting viral genome translation directly.

6.3 *Bm*-resistance to control BtMV

Intriguingly, the eIF-based plant virus resistance was identified in multiple hosts and virus species most often against potyviruses. Therefore, when performing the experiments for manuscript I, we hypothesized that *B. vulgaris* eIFs and the potyviral BtMV-VPg interact with each other, and eIF-knockouts allow to implement a recessive resistance. This was expected to be a proof of concept for the first recessive resistance in sugar beet. While we could find an interaction between the BtMV-VPg and Bv-eIF(iso)4E in PPI-assays, we were unable to show by symptom evaluation that a (qualitative) BtMV complete resistance to infection was obtained. As already mentioned above, no quantitative assessment was performed. Hence, a quantitative resistance could be generated and hidden and should be re-assessed by RT-qPCR in future experiments. Finally, it was concluded that a recessive resistance against BtMV was unobtainable by the available eIF-knockout plants in manuscript I. Arguably a domain mapping and combination of VPg non-interacting eIFs within one plant could be performed in analogy to a putative polerovirus resistance strategy. However, this thesis aims to identify further solutions against VY and the associated BtMV. Therefore, the poorly described *Bm*-resistance gene was further investigated in manuscript III. *Bm*-carrying plants showed significant slower systemic virus accumulation with lower accumulation compared to *bm*-carrying plants, indicating that the *Bm*-gene still could represent a potential resistance gene towards BtMV in sugar beets 50 years after its first description by Lewellen (1973). Unfortunately, the differences in virus accumulation could not be visualized by the BtMV-MYB cDNA clone from manuscript

II, as the available *Bm*-plants were carrying the *r*-gene making the plants unable to produce BvCYP76AD1. To fine map the loci of the *Bm*-gene, a segregating progeny population must be obtained and geno-/pheno-typed to narrow it down. For the phenotyping procedure the BtMV-MYB cDNA clone would come in handy, as a fast and reliable way for visualization, especially if quantitative effects occur, which could not be found in manuscript III, as only one homogenous line was investigated. Neither was a quantitative effect assessed in the progenies used by Lewellen (1973) or Friesen *et al.* (2006) as only symptom development was the recorded trait to distinguish resistant and susceptible plants. To solve the limitation of the *r*-gene presence, an *R*-allele carrying pollen donor should be used during the necessary crosses to obtain a segregating population (Hatlestad *et al.* 2012). The progeny then can be screened by the BtMV-MYB cDNA clone, and as a side effect, true progeny of the testcross can be identified by the presence of a red hypocotyl. Also, from a virologist's point of view it appears tempting to further investigate the true nature of the *Bm*-resistance and how the viral infection is perturbed. For this, the BtMV-sGFP cDNA clone from manuscript III, allowed first observations on a single cell level. The BtMV-sGFP infection should be repeated and investigated in a daily routine with e.g., CLSM to further distinguish the impairment of cell-to-cell or long-distance movement. Furthermore, single cell accumulation should be monitored by comparing the fluorescence intensity of BtMV-sGFP in a comparative infection assay with isolated protoplasts from susceptible and resistant plants respectively. If a protoplast assay is too artificial, the cDNA clone of BtMV-sGFP could be modified to lack its CP. Dai *et al.* (2020) demonstrated that TuMV is able to replicate in single cells but not spread from cell to cell when lacking its CP similar mutations in a labelled cDNA clone of BtMV would allow a comparative assay without the need of protoplast isolation. As no indications for a HR was found in the leaves, neither a full resistance, another mechanism limiting the virus accumulation

must be active in *Bm*-plants. Here, multiple explanations are thinkable, either a limitation of virus replication, which could indirectly influence the observed slower cell-to-cell and long-distance movement (Ronde *et al.* 2014). Famous examples for dominant resistance genes in *A. thaliana,* which influence the long-distance transport of TEV, are the three restricted TEV movement (*RTM*) genes *RTM1*, *RTM2*, *RTM3* (Chisholm *et al.* 2001). Subsequently, the sugar beet genome could be screened *in silico* for homologues to *RTM1-3* with main focus on chromosome I, where the *Bm*-gene is localised (Friesen *et al.* 2006). However, each of the movement limitations could explain the observed phenotype on their own neither can a (partial) influence of RNAi be excluded. If the *Bm*-loci could be narrowed down, this would allow to further characterize the nature of the resistance gene. Finally, if the *Bm*-gene would be unravelled, it could be integrated into elite cultivars to investigate its influence on multifactorial traits such as yield and ideally combined with non-VPg-interacting eIFs to prevent poty-and polerovirus infection in sugar beets. However, it must be mentioned that the yield penalty caused by introgression of the *Bm*-gene has to be below the losses caused by a BtMV infection itself.

6.4 Closing remarks

Summarizing the main findings of this thesis, my colleagues and I were able to identify and describe resistance mechanisms against 3 out of 4 viruses belonging to the VY disease or being associated with it. However, for practical use, the performance of the potential resistances must be investigated in elite cultivars to exclude yield penalties. Furthermore, with the newly developed BtMV-MYB cDNA clone a useful tool for breeding as well as research is provided without relying on expensive technical equipment. However, with the here presented data more questions arise as these results can only be defined as the starting point for further research. Many open

questions remain, as no natural eIF-variant that confers polerovirus resistance in sugar beet has been identified yet. Still, by proving the suitability of a recessive resistance in this pathogen-host system eIFs will be candidate genes for breeding progress to control the VY disease without the need for chemical plant protection. While the *Bm*-resistance is already known, it must be further fine mapped to be of practical use in the future. Additionally, no resistance mechanism against the prevalent BVY has been identified in this thesis neither have other beet infecting poleroviruses been investigated as they have not been reported yet in Europe. Lastly it must be concluded that the VY disease in sugar beet cultivation will be an issue for years to come even with the research progress presented in this thesis. However, this work is an excellent starting point for future research and contributes to solve one of the biggest challenges in current sugar beet production.

7. References

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Publications

Rollwage, Lukas; Maiss, Edgar; Menzel, Wulf; Hossain, Roxana; Varrelmann, Mark (2023): Beet mosaic virus expression of a betalain transcription factor allows visual virus tracking in Beta vulgaris. In: *Molecular plant pathology*. DOI: 10.1111/mpp.13372.

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10. Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.
Weiter erkläre ich, dass ich mich an keiner anderen Hochschule um einen Doktorgrad beworben habe.

Göttingen, den

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2. Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, den

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