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The sugar beet multi-talented resistance protein Rz2

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II. List of Abbreviations

$(A)_n$	polyadenylation
+ssRNA	Plus-orientated single-stranded RNA
A. thaliana	*Arabidopsis thaliana*
A.tumefaciens	*Agrobacterium tumefaciens*
A_{405}	absorption at 405 nm
ADP	adenosine diphosphate
ADR	ACTIVATED DISEASE RESISTANCE
AGO	ARGONAUT protein
agroinfiltration	*Agrobacterium tumefaciens* mediated infiltration
agroinoculation	*Agrobacterium tumefaciens* mediated inoculation
ATP	adenosine triphosphate
avr	avirulence determinant
B. macrocarpa	*Beta vulgaris* subsp. *macrocarpa*
B. maritima	*Beta vulgaris* subsp*. maritima*
B. vulgaris	*Beta vulgaris* ssp. *vulgaris*
BAK1	BRI1 ASSOCIATED RECEPTOR KINASE1
BiFC	Bimolecular fluorescence complementation assay
BNYVV	*Beet necrotic yellow vein virus*
BRI1	BRASSINOSTEROID INSENSITIVE1
BSBMV	*Beet soil-borne mosaic virus*
BSBV	*Beet soil-borne virus*
BSMV	*Barley stripe mosaic virus*
Bsr-1	*BARLEY STRIPE RESISTANCE GENE 1*
BVQ	*Beet virus Q*
C. quinoa	*Chenopodium quinoa*
CaMV	*Cauliflower mosaic virus*
Cas9	CRISPR-associated endonucleases 9
CC	coiled-coil domain
cDNA	complementary DNA
cM	centimorgan
CMV	*Cucumber mosaic virus*
CNL	CC-NLR
CP	coat protein
CPKs	calcium-dependent protein kinases
CP-RT	coat protein read through
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats

DAB	3,3´-diaminobenzidine
DAS-ELISA	Double-antibody sandwich ELISA
DBS	double strand break
DNA	deoxyribonucleic acid
DOB	dropout base medium
dpi	days post infection
DsRed	*Discosoma* coral RFP
dsRNA	double stranded RNA
E	glutamic acid
E. coli	*Escherichia coli*
EDS1 / 5	ENHANCED DISEASE SUSECPTIBILTY 1 / 5
ELISA	enzyme-linked immunosorbent assay
ER	extreme resistance
ETI	effector triggered immunity
Fib2	FIBRILLARIN 2
GFP	GREEN FLUORESCENT PROTEIN
GHKL ATPase	Gyrase, Hsp90, Histidine kinase, MutL
Glk1	Golden2-like transcription factor
H	histidine
HA tag	human influenza hemagglutinin tag
HDR	homology-directed repair
HR	hypersensitive response
hrs	hours
HSP90	HEAT SHOCK PROTEIN 90
I	isoleucine
ID	integrated domain
JAX1	JACALIN-TYPE LECTIN REQUIRED FOR POTEXVIRUS RESISTANCE1
K	lysine
kb	kilobase
kbp	kilobase pair
kDA	kilodalton
L	leucine
LRR domain	leucine-rich repeat domain
MAMPs	microbial-associated molecular patterns
MAPK	mitogen-activated protein kinase
MLA	*Mildew locus A*
MP	movement protein

mRFP	monomeric RFP
N	asparagine
N. benthamiana	*Nicotiana benthamiana*
NB domain	nucleotide-binding domain
NB-ARC	nucleotide-binding domain shared with APAF-1, various R-proteins and CED-4
NGS	Next Generation Sequencing
NHEJ	non-homologous end joining
NIK1	NSP INTERACTING KINASE
NLR	nucleotide-binding site and a leucine-rich-repeat domain protein
NLR-ID	NLR with in integrated domain
nm	nanometer
NPTII	neomycin phosphotransferase II gene
NRC	NLR-REQUIRED FOR CELL DEATH
NRG	N REQUIREMENT GENE
NRIP1	N receptor interaction protein 1
NSP	NUCELAR SHUTTLE PROTEIN
nt	nucleotide
OD_{600}	optical density at 600 nm
ORF	open reading frame
P	proline
P. betae	*Polymyxa betae*
P. syringae	*Pseudomonas syringae*
PAD4	PHYTOALEXIN DEFICIENT 4
PAMPs	pathogen-associated molecular patterns
PBL2	AvrPPHB SUSCEPTIBLE1-LIKE2
PCR	polymerase chain reaction
Pd	plasmodesmata
PDS4	PHYTOALEXIN DEFICIENT 4
PMTV	*Potato mop-top virus*
POI	protein of interest
PPR	pattern recognition receptors
PPV	*Plum pox virus*
PR genes	pathogen-related genes
PTI	pathogen-triggered immunity
PVX	*Potato virus X*
PVY	*Potato virus Y*
qRT-PCR	quantitative reverse-transcription-PCR

R	arginine
R gene	resistance gene
R proteins	resistance proteins
RanGap2	RanGTPase -activation protein 2
RAR1	REQUIERED FOR MLA12 RESISTANCE1
RdRp	RNA-dependent RNA-polymerase
RFP	RED FLUORESCENT PROTEIN
RISC	RNA-inducing silencing complexes
RITS	RNA induced Transcriptional Silencing complex
RKS1	RESISTANCE RELATED KINASE 1
RNA	ribonucleic acid
RNAi	RNA silencing
RNP	ribonucleinprotein
ROS	reactive-oxygen species
RPW8	RESISTANCE TO POWDERY MILDEW 8
RT-PCR	reverse-transcription PCR
S	serine
SA	salicylic acid
SAG101	SENESCENCE-ASSOCIATED GENE 101
SBP	SQUAMOSA PROMOTER BINDING PROTEIN
SD	standard deviation
sec	seconds
SERK3	SOMATIC EMBRYOGENESIS RECEPTORLIKE KINASE 3
SGT1	SUPPRESOR OF THE G2 ALLELE OF SKP1
siRNA	short-interfering RNA
smRSGFP	short-lived, red shifted GFP
SNP	single nucleotide polymorphism
SPL6	SQUAMOSA PROMOTOR BINDING PROTEIN LIKE 6
STAND	signal-transducing ATPase with numerous domains
T	threonine
T. *expansa*	*Tetragonia expansa*
TCV	*Turnip crinkle virus*
TGB	triple gene block
TGB1	triple gene block protein 1
TGB2	triple gene block protein 2
TGB3	triple gene block protein 2
TIR	Toll/interleuikin-1 receptor

TMV	*Tobacco mosaic virus*
TNL	TIR-NLR
TSWV	*Tomato spotted wilt virus*
U	uracil
V	valine
VPE	vacuolar proteases
vRNA	viral RNA
VSR	viral suppressors of RNA silencing
W	tryptophan
Y	tyrosine
YTH	Yeast two-hybrid system
ZAR1	HOPZ-ACTIVATED RESISTANCE1

CHAPTER 1: General Introduction

In times of a global pandemic caused by the SARS-CoV-2 virus, there is increasing awareness that viral agents can cause serious diseases. But not only humans are facing a high number of viral diseases, plants are also affected. In agriculture an estimated crop loss of 20-30% occurs every year due to pests and pathogen attack, with a share of 3% for viral diseases (Oerke and Dehne, 2004; Savary *et al.* 2019). However, it is assumed that crop losses caused by viruses are much more severe than reported and Loebenstein (2008) estimates the reduction of crop yield due to viral infection up to 10-15% (Hull, 2014). According to Hull (2014), viruses are defined as followed:

> "A virus is a set of one or more nucleic acid template molecules, either RNA or DNA, normally encased in a protective coat or coats of protein or lipoprotein, that is able to organize its own replication only within suitable host cells. It can usually be horizontally transmitted between hosts. Within such cells, virus replication is (i) dependent on the host's protein synthesizing machinery, (ii) organized from pools of the required materials rather than by binary fission, (iii) located at sites that are not separated from the host cell contents by a continuous lipoprotein bilayer membrane, and (iv) continually giving rise to variants through various kinds of change in the viral nucleic acid".

Viral symptoms in case of plants range from leaf symptoms such as mosaic patterns, chlorotic and necrotic lesions, yellowing, vein clearing, leaf rolling or curling, to systemic symptoms with a reduction in plant growth and deformation, and can result in a decreased yield or crop quality. In contrast to infection by fungi, bacteria, or nematodes, there are no direct control mechanisms for virus invasion into the host plant. Even though measurements which reduce the source of infection by controlling the vector or phytosanitary actions exist, the only effective method to control viral infections are resistance genes, preventing virus replication, genome expression, movement inside the host and transmission of the virus.

1.1 Plant Immunity

Compared to humans or animals, where the immune system can adapt to the pathogen, plants are relying on an innate immunity based on systemic signaling and specialized proteins which sense evading pathogens and therefore restrict colonization of the host plant and protecting themselves from damage (Dodds and Rathjen, 2010). Beginning with Flor´s gene-for gene hypothesis, which postulated that a single dominant plant gene recognizes a single pathogen-derived gene (Flor, 1971), enormous knowledge has been gained in the field of plant resistance in the last 50 years.

Proteins leading to a restriction of the pathogen are called resistance proteins (R proteins) and these proteins can sense all types of pathogens (Monteiro and Nishimura, 2018). The plant immune system can be divided into extracellular and intracellular immunity, depending on the site where the pathogen-derived molecule is detected. The first layer of plant immunity, the

pathogen-triggered immunity (PTI), consists of pattern recognition receptors (PPR) located on the outer cell membrane, which can sense pathogen-associated (also named microbial-associated) molecular patterns (PAMPs or MAMPs) (Figure 1). PAMPs are defined as evolutionary conserved structures that are located on the surface of microbes and are not present in plants. The most important PAMPs known are flagellin and lipopolysaccharides from bacteria, glucans from fungi and oomycetes, and ascarosides from nematodes (Manosalva *et al.* 2015). Also wounding of the plant cell by feeding insects are recognized by PPRs. Reviewed in Zipfel (2008), PPRs can be classified in transmembrane receptor kinases or transmembrane receptor-like proteins. After recognition, intracellular responses like ion fluxes across the membrane, mitogen-activated protein kinase (MAPK) or calcium-dependent protein kinases (CPKs) activation, production of reactive-oxygen species (ROS), gene expression changes, defense hormone accumulation and signaling as well as cell wall reinforcement are enabled (Zipfel, 2008). In PTI, a common mechanism comprised of a core receptor and an associated kinase forming the primary receptor and by interactions with additional regulators, the receptor enables recognition and downstream signaling (Li *et al.* 2020).

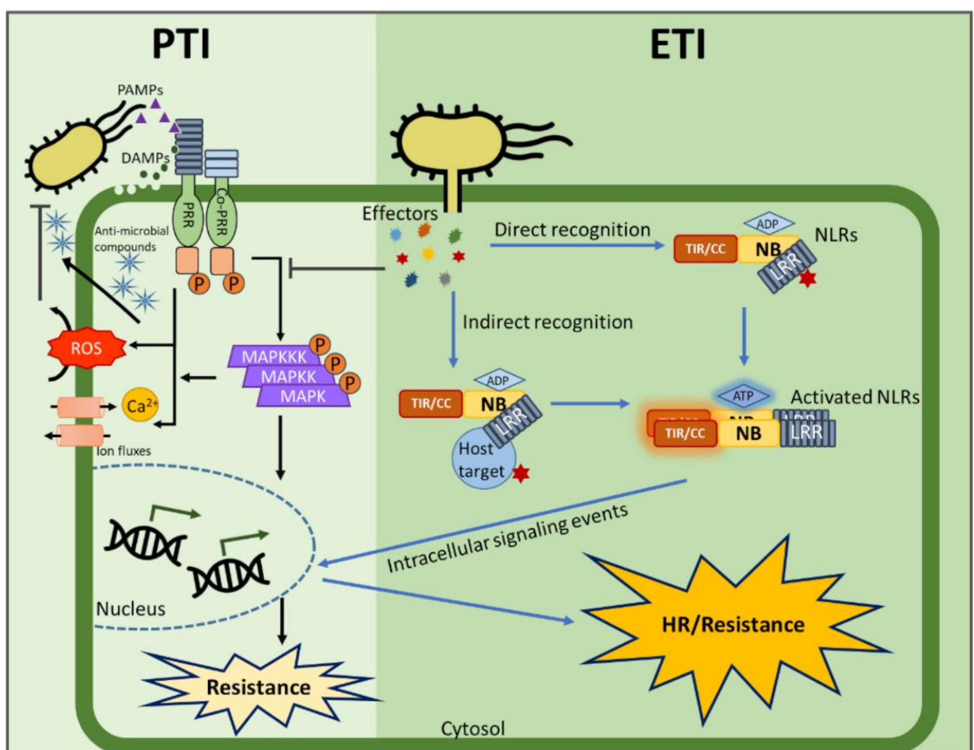

FIGURE 1 Overview of plant immunity. The first layer of resistance, the pattern-triggered immunity (PTI, indicated by black arrows), is activated by pattern recognition receptors (PRRs) perceive pathogen-associated molecular patterns, followed by resistance responses, including production of reactive oxygen species (ROS), calcium influx (Ca2+), activation of mitogen-activated kinases (MAPK). Once pathogens release effectors in the cytosol, the second layer of plant immunity, the effector-triggered immunity (ETI), is

activated (indicated by blue arrows). Specialized proteins, encoding nucleotide-binding (NB) and leucine-rich-repeat (LRR) with varying N-terminal domains (NLRs), sense effectors by direct or indirect binding. Upon binding, an ADP to ATP exchange supports a conformation change in the NLR and downstream signaling events occur, usually leading to a hypersensitive response (HR) or preventing host colonization in alternative ways (modified after Nguyen *et al.* (2021)).

Pathogens are able to evade PTI and activate the second layer of immunity, the effector-triggered immunity (ETI) by secreting effector proteins (Figure 1) (Jones and Dangl, 2006). Effector proteins are able to interfere with PTI by mimicking or inhibiting host cellular functions, reviewed in Dodds and Rathjen (2010). In ETI, specialized resistance proteins (R proteins) can sense these effectors and a resistance response follows. In most cases, resistance genes (*R* genes) encode proteins possessing a nucleotide-binding site (NB site) and a leucine-rich-repeat domain (LRR domain) (NLR). According to their N-terminal part, they can be subdivided in proteins encoding a coiled-coil domain (CC) or a Toll/interleukin-1 receptor (TIR). Recognition of the effector or avirulence determinant (*avr*) in the intracellular space by NLRs can be directly or indirectly, followed by downstream signaling activation. Comparable to PTI, upon activation of R proteins, several downstream responses, like activation of MAPK, production of ROS, calcium ion (Ca^{2+}) influx, accumulation of phytohormones like salicylic acid (SA) as well as reprogramming of protein transcription and expression of pathogen-related (*PR*) genes are activated. But in contrast to PTI, *R* gene-mediated resistance is most often associated with the development of a hypersensitive response (HR), a form of programmed cell death leading to the restriction of pathogen movement and multiplication. Moreover, by the accumulation of SA, defense signaling is activated in distal tissue of infection resulting in systemic acquired resistance. One special form of programmed cell death is the development of an extreme resistance (ER) as it was observed in case of *Rx1*-mediated resistance against *Potato virus* X (PVX) (Bendahmane *et al.* 1999; Kohm *et al.* 1993). While during HR a local cell death at the site of infection occurs, ER restricts viral invasion without exhibiting visible symptoms (Bendahmane *et al.* 1999).
According to the zigzag model stated by Jones and Dangl (2006) pathogens are able to evade ETI by alternating the *avr* to circumvent recognition. Followed by *R* gene selection which can sense the alternative effector and resistance is activated again. NLRs can sense effectors in direct interaction, reported for CC-NLR (CNL) Pi-ta which recognized the fungal effector AvrPita from *Magnaporthe grisea* (Jia *et al.* 2000) and the *Sw-5b* gene against *Tomato spotted wilt virus* (TSWV) (described in more detail in section 1.1.3) (Brommonschenkel *et al.* 2000; Li *et al.* 2019; Zhu *et al.* 2017). But most of the times, recognition by NLRs is achieved by indirect recognition. Hereby, several different models for indirect recognition by NLRs exist, for example the guard and the decoy model (Figure 2)

(Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). In both models, NLRs interact with a host protein which is modified by the pathogen effector through degradation, phosphorylation or transcriptional changes (Bentham *et al.* 2020). The difference between the two models is that guard proteins are important for additional host cell function, whereas decoys only mimic effector targets without additional function for cell survival. A main advantage of the indirect recognition is that a single R protein can sense multiple effectors if those target and modify the same host protein (Dangl and Jones, 2001). In addition to direct and indirect recognition, several NLRs work in pairs, for example for RRS1/RPS4 from *Arabidopsis thaliana* (*A. thaliana*) or RGA4/RGA5 derived from rice (Cesari *et al.* 2013; Sarris *et al.* 2015). In both examples, one of the NLRs possesses an integrated domain (ID) for effector binding that activates the second NLR after pathogen recognition to trigger an immune response (Figure 2). This model can also be referred as helper and sensor model (Baggs *et al.* 2017). NLR pairs often work in negative regulation and the helper NLR is released by recognition of the pathogen by the sensing NLR (Wu *et al.* 2018). While sensor NLRs are specific for the pathogen to be recognized, there are several reports that helper NLRs are conserved within most plants (Cappello *et al.* 2016). Two well studied helper NLRs are the subfamilies ACTIVATED DISEASE RESISTANCE (ADR) and N REQUIREMENT GENE (NRG) and their homologs, respectively (Bonardi *et al.* 2011; Collier *et al.* 2011; Peart *et al.* 2005). Both protein families are thought to play an important role in NLR function, while ADRs are specifically involved in SA accumulation and NRGs are involved in induction of cell death (in particular for TIR-NLR (TNL) triggered resistance) (Castel *et al.* 2019; Qi *et al.* 2018). Interestingly, all known helper NLRs represent CNLs that most often lack an interaction site in the NB region (Cesari *et al.* 2014; Cui *et al.* 2015). More details on signaling are considered in section 1.1.3.

Although it has been assumed for a long time that PTI and ETI are uncoupled resistance pathways, recent studies suggested that ETI is dependent on the PTI machinery (Ngou *et al.* 2021; Yuan *et al.* 2021). Both studies investigated the role of PTI during ETI and showed that ETI alone is not sufficient to restrict pathogen invasion. Additionally, it was demonstrated that intracellular immunity is enhanced by cell-surface receptors.

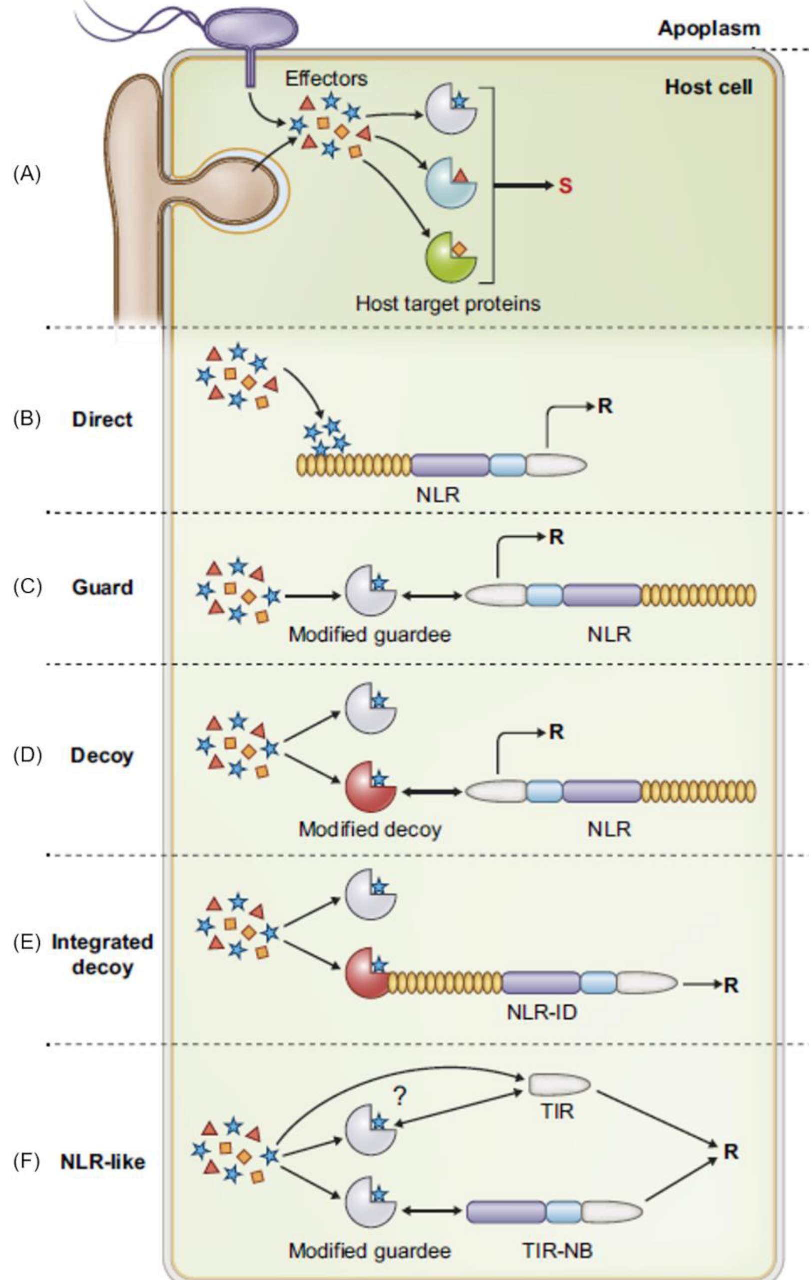

FIGURE 2 Different pathways of pathogen effector recognition by R proteins. After release of the effectors, they can interact with different host plant proteins and support susceptibility (A). In direct interaction (B), the effector is bound by the R protein at its interaction domain and triggers a resistance response. During the Guard (C) and the Decoy (D) pathway, the effector binds to a host protein, either a guardee or decoy protein, which also can be integrated in the R protein (E) itself. Binding by the effector leads to a modification of the guardee/decoy which is sensed by the R protein. In the NLR-like pathway (F) resistance is activated by truncated NLR proteins acting as regulator of resistance either by direct binding, interaction with guradees or decoys, or unknown pathways (modified after Cesari (2018).

Besides the active plant resistance mediated by *R* genes, a second resistance is known based on recessive resistance. As plant viruses are dependent on the cellular machinery of the host, they require host proteins to replicate their genome, move from cell to cell to colonize the host plant, and to interact with their vectors to be transmitted (Truniger and Aranda, 2009). Recessive resistance relies on these factors as absence or mutated host proteins lead to loss-of-susceptibility (Truniger and Aranda, 2009). Although recessive resistance was also reported for other plant pathogens as viruses, half of the known plant virus resistance genes are based on recessive resistance (Kang *et al.* 2005). The best studied mechanism is the interaction between several potyviruses and the eukaryotic translation initiation factor *eIF4E* and their isoforms (Truniger and Aranda, 2009). Reviewed in Hashimoto *et al.* (2016), resistance mediated by *eIF4E* can be found in several crop cultivars including pepper, lettuce, and tomato, and is active not only against members of the genus potyviruses. Interaction between plant virus cap structures and *eIF4E* indicate that some plant viruses use these translation system and missing interaction leads to resistance.

1.1.1 Structure and signaling mediated by nucleotide-binding leucin-rich repeat proteins

NLRs are multidomain proteins which share multiple conserved structures. Only a few NLRs are reported with divergent N-terminal parts, for example RESISTANCE TO POWDERY MILDEW 8 (RPW8) possessing an atypical CC domain (Collier *et al.* 2011). Recent studies indicated raising evidence that NLRs possessing an integrated domain (NLR-ID) are more prevalent than expected and thought to be involved in sensing pathogens (reviewed in Araújo *et al.* (2019). Both, TIR and CC domains are considered to be involved in protein-protein interaction and therefore important for signaling. Studies showed that the CC domain of several R proteins are involved in intermolecular interactions and important for activation or signal transduction leading to cell death, as summarized by Kapos *et al.* (2019). Furthermore, the CC domain of the Sw-5b R protein from tomato against TSWV virus was reported to be involved into nuclear translocation (Oliveira *et al.* 2016). Additionally, both TIR and CC domains are self-associating and can lead to effector-independent cell death when isolated expressed, as it was reported for barley MILDEW LOCUS A (MLA) protein (Maekawa *et al.* 2011) and the TIR domain of RPP1 from *A. thaliana* (Schreiber *et al.* 2016). The central domain, consisting of a nucleotide-binding (NB) adaptor, is the most conserved part of NLRs. The NB domain serves as an ADP-ATP switch that regulates the activation of NLRs (ADP-binding “off” state and ATP-binding “on” state) and belongs to the class of signal-transducing ATPase with numerous domains (STAND) protein family. Similar proteins involved in immunity are also found in humans (apoptotic protease-activating factor 1) and nematodes (cell-death protein 4), leading to the name NB-ARC domain (Leipe *et al.* 2004). The functional P-loop in the NB domain was further reported to play a central role in proper regulation of R proteins as

mutations in this region resulted in a constitutive gain-of function in absence of the *avr* (Bendahmane *et al.* 2002). Of importance here is that NLR working in pairs, one of the NLRs can lack functional motifs in the NB region without influencing the function of the complex (Cesari *et al.* 2014). The C-terminal part of R proteins consists of a leucine-rich-repeat domain (LRR). It was reported that the LRR domain maintained the auto-inhibitor state of NLRs and deletion of the LRR domain resulted in auto-activity (Bendahmane *et al.* 2002). In contrast to *Sw-5b*, where deletion of the LRR domain did not lead to HR, but deletion of the N-terminal did. (Oliveira *et al.* 2016). Several studies reported that the LRR domain interacted with the effector, and upon effector binding, a conformational change enabled ADP to ATP exchange (Deslandes *et al.* 2003; Jia *et al.* 2000).

Although there are increasing studies on signaling and function of NLRs, and their role in immunity is not always clear, one common meaning is prevalent. For proper function of NLRs, a well-balanced interaction between the functional domains is important for sensitive signaling and rapid resistance response while in absence of the pathogen no undesired activation should occur (Zhang *et al.* 2017). But in general, the LRR domain is responsible for specific recognition of the effector, the NB domain for conformation changes and the N-terminal part for signaling and activation of downstream processes (Gantner *et al.* 2019).

1.1.2 Antiviral immunity

Viruses are intracellular obligate pathogens that are dependent on the host cellular machinery to replicate their genome and to be able to move not only in the host but also to become transmitted. Their genome consists either of DNA or RNA and encodes a minimal number of proteins, which often possess multifunctional properties. For example, the HCPro protein derived from potyviruses is responsible for aphid transmission, suppressor of RNA silencing and possess protease activity (Valli *et al.* 2018). Although it is assumed that PTI is not common in antiviral defense as they are more likely in the intracellular space, several studies indicated an outer cellular viral defense machinery (Macho and Lozano-Duran, 2019; Nicaise and Candresse, 2017; Niehl *et al.* 2016; Teixeira *et al.* 2019). The most promising antiviral PTI displays RNA silencing (RNAi). The elicitor of RNA silencing is double-stranded RNA (dsRNA) formed during replication of RNA and DNA genomes. Dicer-like proteins cleave these dsRNA in short-interfering RNAs (siRNA) with a length of 21 nucleotides (nt). siRNAs are loaded into ARGONAUT (AGO), a component of the RNA-inducing silencing complexes (RISC) or RNA induced Transcriptional Silencing complex (RITS). Subsequently, degradation of the same or similar dsRNAs or post-transcriptional repression (mediated by RISC) in a sequence-specific manner is achieved (reviewed in Csorba *et al.* (2015)). To counteract against RNA silencing, viruses evolved proteins that act against RNA silencing (viral suppressors of RNA silencing, VSR). The first identified VSR is the 2b from *Cucumber mosaic virus* (CMV) (Ding *et al.* 1996). Meanwhile, it is known that viruses encode at least one VSR and suppression is obtained at

different stages of RNA silencing, for example blocking initiation of VSR, interaction with AGO or other important proteins in RNA silencing, binding of siRNA or blocking RISC, reviewed in Csorba *et al.* (2015)

In addition to act as elicitor of RNA silencing, dsRNA has been demonstrated to activate downstream signaling comparable to PTI and independent on RNA silencing (Niehl *et al.* 2016). Important for viral PTI are co-receptors like BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE1 (BAK1) (also named SOMATIC EMBRYOGENESIS RECEPTORLIKE KINASE 3, SERK3), as silencing of the receptor resulted in an increased virus susceptibility in *A. thaliana* (Kørner *et al.* 2013). Following recognition, comparable mechanisms known from PTI against other pathogens, like hormone signaling, activation of MAP kinases, production of ROS and induction of defense gene expression are activated (Kørner *et al.* 2013; Nicaise and Candresse, 2017; Niehl *et al.* 2016). Furthermore, another antiviral defense mechanism works on suppression of the cellular protein synthesis, which was first found in viral defense against *Begomoviruses* (Mariano *et al.* 2004). The NUCELAR SHUTTLE PROTEIN (NSP) INTERACTING KINASE (NIK1) is the key component and a plasma membrane bound LRR serin/threonine kinase (Mariano *et al.* 2004). Upon viral recognition, NIK1 activated a signaling cascade by phosphorylation which resulted in repression of ribosomal proteins and thus cellular and viral protein synthesis was inhibited (reviewed in Wu *et al.* (2019).

1.1.3 Recognition of viral effectors and activation of downstream signaling

Despite RNA silencing and PTI mediated by dsRNA, control of virus infection is mainly mediated by expression of *R* genes. Following recognition of the *avr* determinant elicited by the virus, downstream signaling pathways are activated, resulting in a HR. Although downstream signaling also occurs after recognition of other biotic stress factors, in the following the focus will be placed on viral detection.

For viral effector recognition, several studies investigated viral effectors signaling either by direct or indirect interaction. One example is the recognition of the cell-to-cell movement protein NSm of TSWV by the corresponding resistance gene *Sw-5b* from tomato (Figure 3) (Brommonschenkel *et al.* 2000; Hallwass *et al.* 2014; Peiró *et al.* 2014). Sw-5b interacts directly with NSm by binding a 21 amino acid sequence through its NB-ARC-LRR domain and additionally an N-terminal domain at the CC-part (called SD domain) indicating a multilayered recognition mechanism (Li *et al.* 2019; Zhu *et al.* 2017).

Recognition of *Tobacco mosaic virus* (TMV) by the TNL N happens by indirect recognition of the helicase domain of the virus (p50) through the N-terminal TIR domain (Burch-Smith *et al.* 2007). p50 interacts with the N RECEPTOR INTERACTION PROTEIN 1 (NRIP1), a chloroplast-localized sulfurtransferase (Caplan *et al.* 2008). Interaction with p50 leads to

relocalization of the complex to the cytoplasm and nucleus, where N recognizes the p50-NRIP1 complex (Caplan *et al.* 2008).

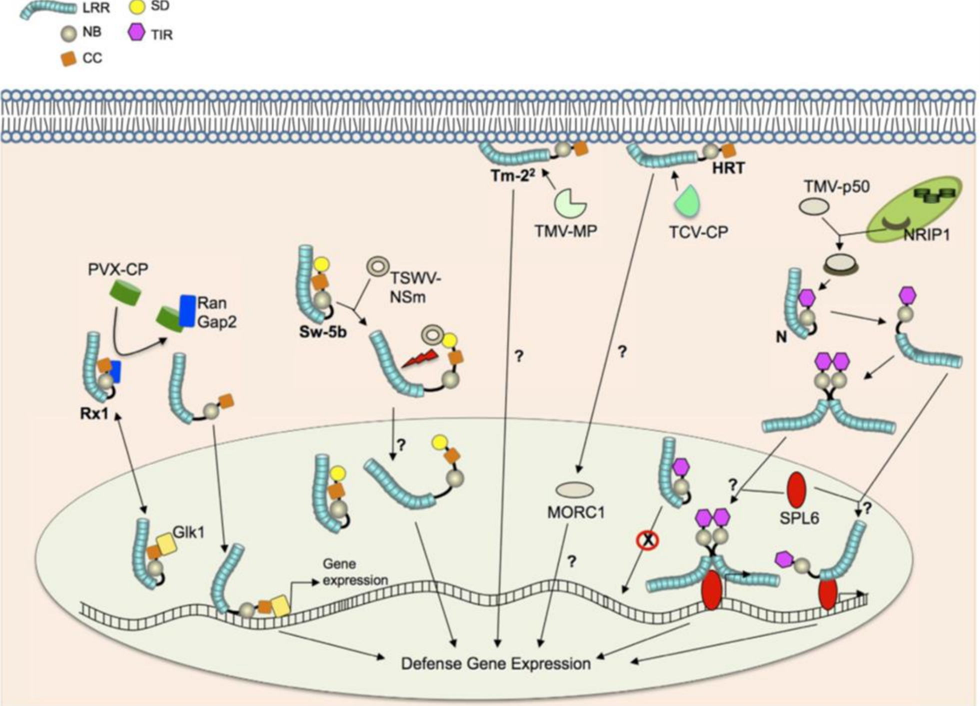

FIGURE 3 Recognition of viral effectors by NLRs and different cofactors leading to defense gene expression. RanGap2 interacts with PVX-CP and upon recognition of the complex by Rx1 leads to a conformation change in the R protein. Activated Rx1 can now interact with Glk1 and enables defense gene expression. Binding of the *avr* determinant TSWV-NSm through the SD and the LRR domain leads to activation of expression. Both plasma-membrane bound R proteins, Tm-2^2 and HRT interact with their corresponding *avr* determinants, TMV movement protein (MP) and TCV coat-protein (CP) resulting in gene expression activation with cooperation with unknown factors in case of Tm2^2 and MORC1 in case of HRT, respectively. The *avr* determinant released by TMV, p50, is recognized by NRIP1 and enables recognition by the R protein N. Consequently, conformational alternations of N and interaction with the transcription actor SPL6 result in transcription activation of defense genes (Figure from Meier *et al.* (2019)).

After recognition of the pathogen and activation of the NLR, downstream signaling are initiated that led to a defense mechanism. Different components in the signaling are used by several NLRs, like the ENHANCED DISEASE SUSECPTIBILTY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PDS4), SENESCENCE-ASSOCIATED GENE 101 (SAG101), HEAT SHOCK PROTEIN 90 (HSP90); SUPPRESOR OF THE G2 ALLELE OF SKP1 (SGT1), REQUIERED FOR MLA12 RESISTANCE1 (RAR1), and the helper NLRs, ADR1 and NRG1 (Aarts *et al.* 1998; Feys and Parker, 2000; Liu *et al.* 2002; Liu *et al.* 2004).

The *N* gene requires the complex of HSP92, SGT1, and RAR1 for proper function (Liu *et al.* 2004). Binding to the complex allows proper folding, accumulation, and regulation of the NLR. Upon binding the effector p50, SGT1 guides the complex to the nucleus (Burch-Smith *et al.* 2007), where the NB domain of N interacts with transcription factors to activate defense responses (Figure 3) (Padmanabhan *et al.* 2013). One of these transcription factors is SQUAMOSA PROMOTOR BINDING PROTEIN LIKE 6 (SPL6), essential for defense reaction against TMV (Padmanabhan *et al.* 2013). The potato derived resistance protein *Rx1* mediates resistance towards PVX by interaction with the viral coat protein (CP) resulting in extreme resistance (Bendahmane *et al.* 1995; Bendahmane *et al.* 1999). For elicitation of resistance, *Rx1* interacts with SGT1, HSP90, RAR1 and RanGTPase -activation protein 2 (RanGAP2) (Botër *et al.* 2007; Slootweg *et al.* 2010). RanGAP2 and SGT1 are responsible for nucleocytoplasmic distribution and at least RanGAP2 is involved in folding of *Rx1* to allow pathogen recognition (Tameling *et al.* 2010). For proper function of *Rx1*, the protein must be present both in the nucleus and in the cytosol, where recognition of the PVX CP takes place (Slootweg *et al.* 2010; Tameling *et al.* 2010). Upon interaction, the activated *Rx1* is able to interact with a transcription factor, Glk1 (Golden2-like transcription factor) and dense gene expression is started (Townsend *et al.* 2018). However, a complex formed of EDS1, PAD4, and SAG101 is involved in resistance against *Turnip crinkle virus* (TCV) in *A. thaliana* mediated by *HRT* (Zhu *et al.* 2011). The same study postulated that the complex is responsible for localization and therefore activation of signaling defense genes in the nucleus. Studies by Kang *et al.* (2008; 2012) reported that besides the complex of EDS1, PAD4, and SAG101, a protein belonging to the MORC proteins, CRT1, is necessary for *HRT*-mediated resistance. CRT1 possess a GHKL ATPase motif (Gyrase, Hsp90, Histidine kinase, MutL) and it thought to be important for several functions within plant immunity (Kang *et al.* 2012).

Signaling and downstream processes are highly diverse and further research in plant immunity is ongoing. It seems that there is no common rule for pathogen detection and signaling, and every plant-pathogen interaction has its uniqueness. The mentioned examples indicate that *R* gene-mediated resistance is dependent on diverse additional factors and there needs to be a balanced orchestrating of the single proteins involved in resistance. In this context, it is interesting to note that several proteins downstream of recognition are conserved in plant species and act also against different types of pathogen invasion as these are also important for signaling against fungi, bacteria, and oomycetes (Gantner *et al.* 2019; Gouveia *et al.* 2016; Lapin *et al.* 2019; Liebrand *et al.* 2014)

1.2 Sugar beet and Rhizomania

Yearly, almost 180.000 tons of white sugar are produced worldwide[1], 22% thereof from sugar beet. The main sugar beet growing areas are Europe, North America, and Central Asia (temperate zone), whereas sugarcane is grown in central and south America, Africa, Southeast Asia, and Australia (tropic zone) (Miedaner, 2018).

Beta vulgaris ssp. *vulgaris* (*B. vulgaris*) plants are biennial plants storing up to 20% sucrose in the roots under favorable conditions. Besides abiotic factors influencing beet quality, like water, nutrients, and agronomic practices, many biotic factors can reduce the sugar yield. *B. vulgaris* is a host for a wide range of fungal pathogens, like *Cercospora beticola*, *Rhizoctonia solani* or *Ramularia beticola*, nematodes, *Heterodera schachtii*, proteobacteria, like *Candidatus* Arsenophonus phytopathogenicus, insects or viruses, like *Beet necrotic yellow vein virus* (BNYVV), *Beet yellows virus* or *Beet curly top virus*.

According to Scholten and Lange (2000), the disease rhizomania, caused by the *Beet necrotic yellow vein virus* (BNYVV), is the most devastating disease in sugar beet cultivation if not controlled. Infection with BNYVV, transmitted by the soilborne protist *Polymyxa betae* (*P. betae*), can result in a reduction of the dry root weight by up to 57% (Rezaei *et al.* 2014) and up to 80% reduction in sugar yield (Peltier *et al.* 2008). The high damage potential of BNYVV infection can be explained by the worldwide distribution of BNYVV, the long persistence of the vector in the soil, the high yield losses, and missing control strategies despite resistant varieties (Biancardi and Tamada, 2016; McGrann *et al.* 2009; Peltier *et al.* 2008).

Infection with BNYVV in *B. vulgaris* plants results in excessive lateral root proliferation (Figure **4**) leading to a bearded tap root when plants are infected at an early growth stage and severe levels of the soilborne vector are present. In addition, plants infected with BNYVV have a reduced sugar content and processing quality, because of higher concentrations of sodium and reducing sugars (Biancardi and Tamada, 2016). Leaf symptoms in form of yellowing and necrotic veins can occur at the end of the growing period but are rather seldom and only occur when an early and severe infection has occurred (Biancardi and Tamada, 2016; Lewellen *et al.* 2003). When a BNYVV infection occurs later in the season or under low virus pressure, no symptoms are visible either on leaves or roots. Infested soil, irrigation water, and even wind blowing are reasonable ways to spread virus-transmitting *P. betae*, as well as farming machinery and infested seed materials like seed onions. Besides *B. vulgaris*, BNYVV infection is limited to plants of the family *Amaranthaceae*, including fodder beet and spinach (Heidel *et al.* 1997). Even though natural infection is dependent on the spread of *P. betae*, BNYVV can infect several host plants by mechanically inoculation. Species like *Beta macrocarpa* and

[1]https://www.zuckerverbaende.de/zuckermarkt/zahlen-und-fakten/weltzuckermarkt/erzeugung-verbrauch.html, 16.06.2021

Beta vulgaris subsp. *maritima* (*B. maritima*), *Chenopodium quinoa* (*C. quinoa), Tetragonia expansa* (*T. expansa*) and even *Nicotiana benthamiana* (*N. benthamiana*) develop local lesions and some plants species become systemically infected (reviewed in Biancardi and Tamada, 2016).

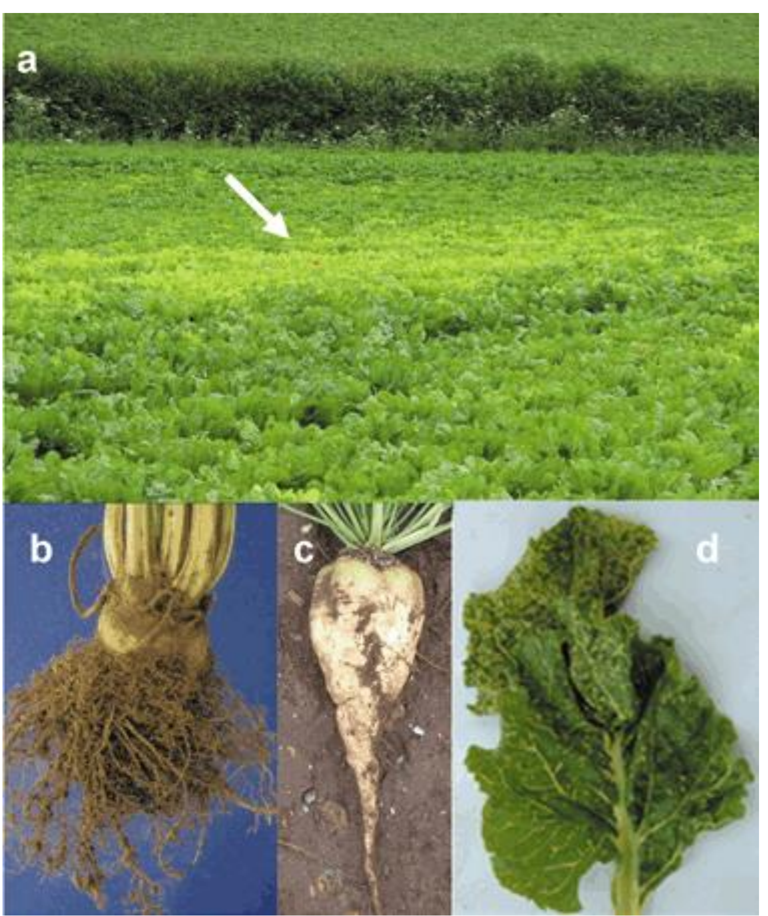

FIGURE 4 Symptom development of naturally infected *B. vulgaris* plants in field (a) and on single plant level. Lateral root proliferation can be observed on sugar beet root tissue (b) in comparison to a healthy plant (c). Rarely, leaf vein yellowing can also be observed in systemically infected host plants (d) (modified after McGrann *et al.* (2009)).

1.2.1 The *Beet necrotic yellow vein virus*

The *Beet necrotic yellow vein virus* belongs to the family of *Benyviridae* and consists of four to five, plus orientated single-stranded RNA components (+ssRNA) with a 5´cap and polyadenylation at the 3´end (Figure 5) (Peltier *et al.* 2008). Virus particles form a rigid rod-shaped structure and have a size between 80 nm and 390 nm according to the sequence length (Richards and Tamada, 1992; Tamada *et al.* 1989).

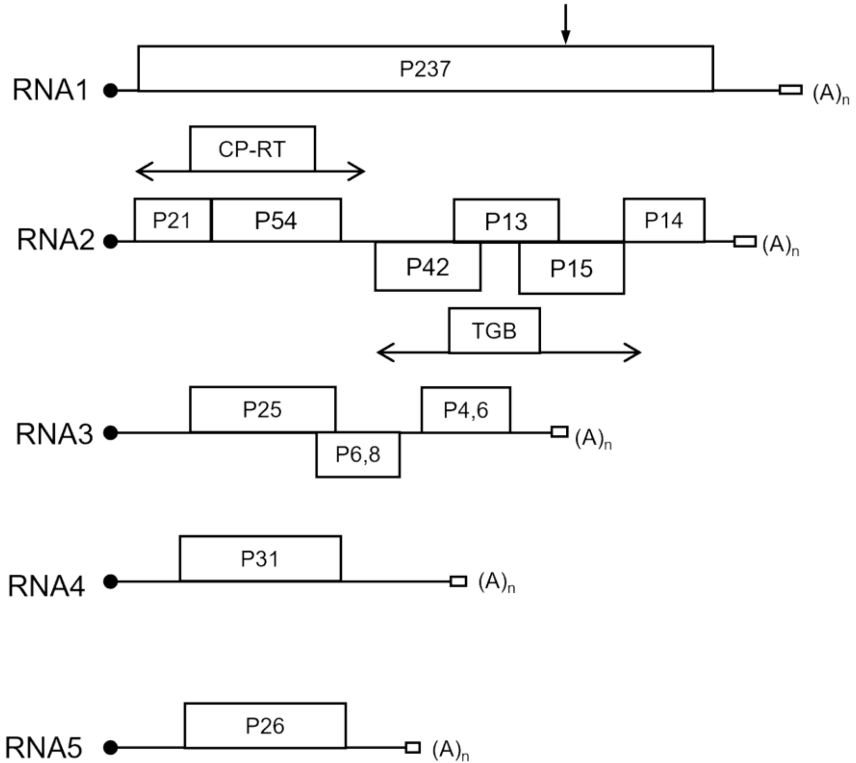

FIGURE 5 Genome organization of *Beet necrotic yellow vein virus*, consisting of four or five RNAs. 5´cap structures (black dots) and polyadenylation ($(A)_n$) at the 3´end are indicated. Open reading frames are shown by boxes with resulting protein size in kDa. The arrow indicates autocatalytically cleavage site for RNA1 encoded protein P237 resulting in two proteins with a size of 150 kDa and 66 kDa, respectively. (CP-RT = coat protein read through, TGB = triple gene block) (modified after Lee *et al.* 2001, Peltier *et al.* 2008 and Gilmer *et al.* 2017).

BNYVV RNA1 and RNA2 encode for house-keeping proteins. RNA1, 6746 nt in length, contains one open reading frame (ORF) encoding one polypeptide with a size of 237 kDa which is autocatalytically processed into a 150 kDa and 66 kDa protein (Figure 5) (Bouzoubaa *et al.* 1987; Hehn *et al.* 1997; Richards and Tamada, 1992). The 150 kDa protein contains a methyltransferase, a helicase, and a papain-like protein domain (Bouzoubaa *et al.* 1987). The RNA-dependent RNA polymerase (RdRp) is encoded on the 66 kDa region (Hehn *et al.* 1997). RNA2 of BNYVV is 4612 nt long and encodes a total of six ORFs involved in encapsidation, cell-to-cell and long-distance movement, vector transmission, and suppression of RNA silencing (Bouzoubaa *et al.* 1986, Peltier *et al.* 2008). The first ORF encodes the main CP with a size of 21 kDa. An amber stop codon following the first ORF allows the translation of a CP-readthrough protein (CP-RT, P54), playing a minor part during encapsidation (Haeberlé *et al.* 1994). The resulting P54 is also involved in vector transmission by its KTER motif (Tamada *et al.* 1996b; Tamada and Kusume, 1991). The following three overlapping ORFs encode the

triple gene block (TGB), important for cell-to-cell movement and are expressed from subgenomic RNAs (Bouzoubaa *et al.* 1986; Gilmer *et al.* 1992). The TGB consists of a 42 kDa (TGB1), a 13 kDa (TGB2), and a 15 kDa (TGB3) protein and similar proteins can be found in a variety of plant virus genus like *Potexviruses, Hordeiviruses, Pomoviruses,* and *Pecluviruses* (Verchot-Lubicz *et al.* 2010). Protein-protein interactions are required for the proper function of the TGB proteins. The last ORF on RNA2 is also translated from subgenomic RNA and encodes the P14, the viral silencing suppressor of RNA (VSR) and regulator of RNA2 accumulation (Dunoyer *et al.* 2002; Gilmer *et al.* 1992; Hehn *et al.* 1995). On RNA3 an ORF of about 1775 nt in length encodes the P25 which is mainly responsible for induction of rhizomania symptoms in sugar beet and long-distance movement in *Beta vulgaris* subsp. *macrocarpa* (*B. macrocarpa*) and *B. maritima* (Lauber *et al.* 1998; Peltier *et al.* 2012; Tamada *et al.* 1989). Furthermore, P25 is assigned as the *avr* determinant towards the monogenetic resistance gene *Rz1*, as a variable tetrad in P25 at amino acid position 67-70 is related to resistance-breaking abilities (Bornemann *et al.* 2015; Koenig *et al.* 2009; Liebe *et al.* 2019; Schirmer *et al.* 2005). Only recently, a study by Liebe *et al.* (2019) even demonstrated *in vitro* that a P25 variant with a deletion of the amino acid at position 179 is able to overcome *Rz1* resistance. Two additional proteins are encoded on RNA3, the P4.6 and N (Bouzoubaa *et al.* 1985; Jupin *et al.* 1992). Whereas the function of P4.6 is unknown, the N protein is involved in symptom expression on *T. expansa* leaf tissue (Jupin *et al.* 1992). RNA4 contains one ORF encoding the P31 which is required for vector transmission by *P. betae* (Bouzoubaa *et al.* 1985; Dunoyer *et al.* 2002, Rahim *et al.* 2007, Chiba *et al.* 2013). Furthermore, P31 was associated with silencing activity in roots of sugar beet plants and symptom expression in *N. benthamiana* (Rahim *et al.* 2007). Some BNYVV strains also contain a fifth RNA which harbors a single ORF. This ORF encodes a 26 kDa protein which shows size variation between single virus strains (Koenig *et al.* 1997b; Miyanishi *et al.* 1999). P26 is involved in severe symptom expression in sugar beet roots and leaf symptoms in *C. quinoa* (Link *et al.* 2005; Tamada *et al.* 1996a). Like P25, P26 is a nucleocytoplasmic shuttle protein (Link *et al.* 2005).

1.2.2 *Polymyxa betae* Keskin associated soil-borne viruses infecting sugar beet

The obligate biotroph plasmodiophorid, belonging to the order of protist, *Polymyxa betae* Keskin is the only known transmission vector of BNYVV and the virus can be present in all stages of the protist life cycle (Keskin, 1964). Infection of sugar beet plants occurs at the root level by zoospores entering the hair-roots or epidermal cells (Keskin, 1964). The virus is released and starts to replicate after being delivered into the plant cell by the vector (Biancardi and Tamada, 2016). *P. betae* takes up the virus from *in vivo* infected plant cells but the mechanism remains unknown (Biancardi and Tamada, 2016).

BNYVV is known to be the main causative agent for rhizomania, but besides BNYVV, a second *Benyvirus*, the *Beet soil-borne mosaic virus* (BSBMV), is also transmitted by *P. betae* and infects sugar beet plants. Unlike BNYVV, which occurs worldwide, BSBMV has only been detected in the US. BNYVV and BSBMV have a similar genome structure and share 23% to 92% homology on amino acid level (Lee *et al.* 2001). Infection with BSBMV results in mosaic pattern of the leaf tissue and mottling, whereas no root symptoms occur. Mixed infections of BNYVV and BSBMV were detected in the field but the study by Laufer *et al.* (2018) indicated that BSBMV and BNYVV remained spatially separate in the host plant (Heidel *et al.* 1997; Wisler *et al.* 2003; Workneh *et al.* 2003).
In addition to BNYVV and BSBMV, *P. betae* also transmits two viruses from the genus *Pomovirus*, *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ). Both viruses are present in mixed infection with BNYVV and at least BSBV is distributed in sugar beet fields throughout the world (Mahillon *et al.* 2021). Members of the genus *Pomovirus* have a tripartite RNA genome of about 6 kbp, 3-3.5 kbp and 2-2.5 kbp in size and are organized in rod-shaped segments (Barbarossa *et al.* 1992). RNA1 encodes two readthrough proteins involved in replication (Barbarossa *et al.* 1992). The CP and its CP-RT are encoded on RNA2, whereas the overlapping ORFs on RNA3 encode the TGB proteins (TGB1, TGB2 2 and TGB3) responsible for cell-to-cell movement (Koenig *et al.* 1996; Koenig *et al.* 1997a). Although BSBV most often occurs in mixed infection with BNYVV, the impact of BSBV infection alone was studied only a few times (McGrann *et al.* 2009). These studies reported about leaf and root symptoms after artificial infection, but the assumption that BSBV is a mild pathogen needs to be further addressed (Kaufmann *et al.* 1993; Mahillon *et al.* 2021; McGrann *et al.* 2009). The second virus belonging to the genus *Pomovirus*, BVQ, was considered to be a BSBV strain due to a similar host range, transmission and genome organization but was finally assigned as an independent virus species (Koenig *et al.* 1998). BVQ was detected in Europe and Iran and is suspected to be less distributed than BSBV (Farzadfar *et al.* 2005; Meunier *et al.* 2003). Distinction between the different sugar beet infecting soil-borne viruses can be obtained by using a Multiplex-PCR, developed by Meunier *et al.* (2003).

1.2.3 Viral strains and viral spread

Molecular analyses indicated four distinct BNYVV stains, A-, B-, J- and P-type. The most prevalent difference is the absence and presence of RNA5. While BNYVV A- and B-types only have four components, the J- and P-type contain an additional fifth RNA. According to Schirmer *et al.* (2005), A- and B-type strains can be differentiated by molecular divergence in the CP, P25 and P31 gene sequences. While the A-type is distributed worldwide, the B-type is limited to areas in Central and Northern Europe (Koenig and Lennefors, 2000). The P-type was firstly detected in an area around Pithiviers, France, and molecular analyses suggested a closer relation towards the A-type (Koenig *et al.* 1997b). The J-type, named after the occurrence in

Japan, is characterized as an individual strain due to its higher distinction towards the remaining strains. Besides Japan, BNYVV J-type is mainly present in China. The J-type is assumed to have evolved by reassortment or recombination because the CP from B-type are mixed with A-type proteins (Li *et al.* 2008; Miyanishi *et al.* 1999). In addition, Simon-Loriere and Holmes (2013) assumed that RNA5 has developed by gene duplication and that P25 and P26 share the same ancestor protein.

As the highest variability of BNYVV genomes occurs in Asia, it is suggested that the origin of BNYVV was in Asia and BNYVV spread from Asia to the world (Chiba *et al.* 2011). The same study also suggested, that BNYVV was already present before sugar beet cultivation at beginning of the 20th century was intensified. BNYVV was firstly detected in Europe in the 1950s in North Italy and the causative agent has been identified by Tamada and Baba in 1973. Soon after the first occurrence, BNYVV distributed in Europe within nearly 20 years. The first occurrence of rhizomania in Japan was reported in 1965 and in China 1978, whereas in the USA the first occurrence was reported 1981 (reviewed in Biancardi and Tamada, 2016). One reason for the rapid spread is the fact that low BNYVV levels were of small interest as they had no economic impact (Biancardi and Tamada, 2016) as well as the fact that soils collected in sugar factories during processing are reapplied to sugar beet fields. Additionally, short rotation for sugar beet cultivation, upcoming irrigation, or rainy seasons, and also climate change and with this changing soil temperatures favored BNYVV distribution (reviewed in Biancardi and Tamada (2016)). When the virus accumulation increased and infected fields were detected, the awareness raised, and action was needed.

1.2.4 BNYVV resistance breeding

Since rhizomania is a viral disease transmitted by a soil-borne protist, it is extremely difficult to control. Some agronomic measurements are helping to reduce the infection by *P. betae,* like ensuring good soil structures and drainage to avoid wet conditions, sowing at low temperatures to avoid early infections, elongating sugar beet rotation, avoid soil movement or pH values under 7.0 (Biancardi and Tamada, 2016). Moreover, soil fumigations were used to reduce *P. betae* content in the soil but due to environmental, practical, and economic issues, using chemicals were not appropriate.

With the first occurrence and the rapid spread of rhizomania disease in almost all sugar beet cultivation areas, the need for sufficient control strategies increased. The first rhizomania resistance gene was “Alba type”, coming from genotypes resistant towards Cercospora leaf spot (Biancardi *et al.* 2002). These varieties showed moderate effect on BNYVV infection and consequently were used for commercial breeding programs producing varieties with reduced BNYVV content when infected (Biancardi *et al.* 2002). The first monogenic resistant trait was “Holly” coming from the US-American Holly Sugar Company and the resistance source was named *Rz1* (Lewellen *et al.* 1987). Plants harboring *Rz1* allow virus replication without visible

viral symptoms (Scholten and Lange, 2000). Investigation of the expression level after BNYVV infection indicated reprogramming of genes involved in pathogenesis, such as oxidative enzymes and phytohormones (Larson *et al.* 2008) but the resistance mechanism behind *Rz1* is still unknown.

Further investigation identified other resistance genes in germplasm derived from *B. maritima* (L.) accessions WB42 and WB43, assigned as *Rz2* and *Rz3* (Lewellen *et al.* 1987; Whitney, 1989). Plants harboring the *Rz2* resistance gene even showed higher resistance towards BNYVV compared to *Rz1* carrying plants (Scholten *et al.* 1999). Both resistance genes are located on chromosome 3 with a distance of 25-30 cM. A third resistance gene, *Rz3*, is assumed to be located in proximity to *Rz2*, roughly 5 cM and studies indicated, that *Rz2* and *Rz3* can be drawn back to the same gene, symbolizing allelic series (Grimmer *et al.* 2008). The same is suggested for the later identified resistance genes *Rz4* and *Rz5*, localized in proximity to *Rz1* (Grimmer *et al.* 2007). In the year 2002, the first cultivars with *Rz1* and *Rz2* resistance were introduced in the USA and France and are currently used for sugar beet cultivation worldwide.

With the introduction of *Rz1* in commercial varieties as only source to control rhizomania, the emergence of resistant-breaking BNYVV strains was probable. The first strains overcoming *Rz1* resistance have been detected in France and the USA (Liu and Lewellen, 2007; Liu, H.-Y. *et al.* 2005). In France, the P-type strain of BNYVV, containing RNA5, has been associated with resistance breaking, whereas in the USA mutated A-type BNYVV strains have been reported. Lots of investigation in the upcoming years identified a variable tetrad in the P25 encoded on RNA3 to be responsible for resistance breaking (Acosta-Leal *et al.* 2008; Acosta-Leal *et al.* 2010; Bornemann *et al.* 2015; Chiba *et al.* 2011; Koenig *et al.* 2009; Pferdmenges *et al.* 2009). Variation in the amino acid positions 67-70 with 21 different combinations were found in field surveys in the Pithiviers area (Galein *et al.* 2018) confirming the P25 as avirulence gene towards *Rz1*. Moreover, sequence variability independent on amino acid sequence 67-70 were suspected to be involved in resistance breaking as only recently reported by Liebe *et al.* (2019) using a reverse genetic system to investigate BNYVV genome variability.

The fact that BNYVV P-type is able to circumvent *Rz1* resistance was first described 1997 by Tamada *et al*. Studies suggest that the presence of P26 is responsible for overcoming *Rz1* resistance rather than the amino acid tetrad in P25 (Chiba *et al.* 2011). The P26 encoded by RNA5 shares around 22% sequence homology to P25 and might explain the ability to evade resistance. For *Rz2* until now no resistance-breaking strains were identified although Galein *et al.* (2018) reported about elevated BNYVV virus content in plant tissue obtained from plants with *Rz1*/*Rz2* resistance level, this remains to be further investigated.

1.2.5 The resistance protein Rz2

Since the discovery of *B. maritima* (L.) accessions WB42 as resistance source, the resistance gene *Rz2* was used for breeding programs worldwide and used to cultivate sugar beets in region with BNYVV infestation. In 2017 the genetic background was identified as a classical *R* gene encoding an CC-NB-ARC-LRR protein by Capistrano-Gossmann *et al.* (2017). Using a mapping-by-sequencing approach of nearly 200 accessions of wild beets (crop wild relative population), it was possible to locate the gene. Moreover, differences in analyzed susceptible accessions, either by containing a premature stop-codon or a transposon insertion, resulting in susceptibility and confirmed *Rz2* as resistance gene (Figure 6) (Capistrano-Gossmann *et al.* 2017).

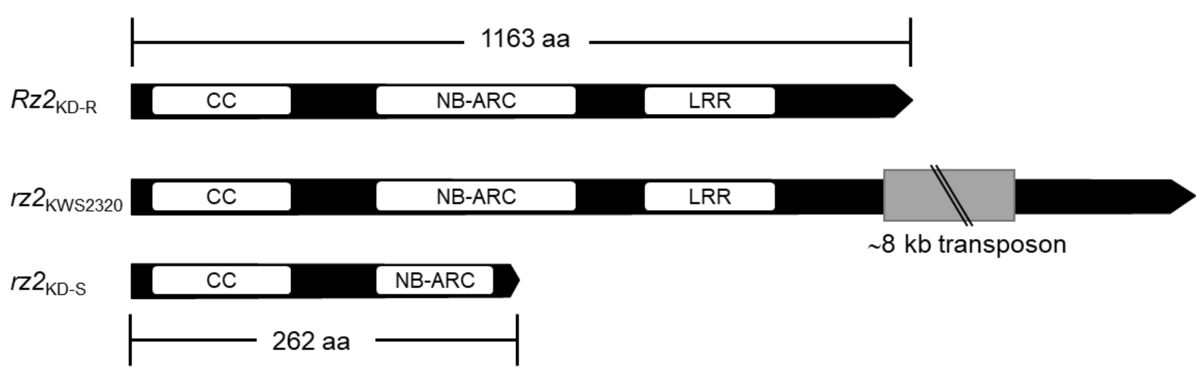

FIGURE 6 Schematic structure of the *Rz2* gene in different genotypes; *Rz2*$_{KD-R}$: resistant plants derived from Kalundborg; *rz2*$_{KWS2320}$ gene structure identified in susceptible sugar beet genotype KW$_{S2320}$ possessing a transposon insertion (grey box) and rz2$_{KD-S}$ found in susceptible plants derived from Kalundborg with premature stop codon. White boxes indicate NLR specific motifs in the gene (modified after Capistrano-Gossmann *et al.* (2017)).

CHAPTER 2: Research objectives

Sugar beet cultivation is highly dependent on effective control of BNYVV infection. Nowadays, the two monogenic dominant resistance genes *Rz1* and *Rz2* are used in commercial cultivars to allow growing sugar beet in areas with BNYVV infestation. In a previous study, the genetic background of *Rz2* was identified, whereas it remains unknown for *Rz1*. To improve BNYVV resistance, the molecular characteristics of the resistance gene *Rz2* are investigated.

First, a resistance test with homozygous breeding lines should confirm that *Rz2* represents an individual resistance gene. Subsequently, the expression level of *Rz2* should be investigated to determine whether the expression of *Rz2* is pathogen-induced or constitutive and whether tissue-specific expression occurs. Since no information is available about the ability of *Rz2* to also confer resistance against BSBMV, this should be investigated by mechanical inoculation of previously generated cDNA clones of BNYVV and BSBMV.

One major objective of the study is to identify the viral *avr* determinant recognized by *Rz2*. Therefore, individual BNYVV ORFs and the *Rz2* gene should be isolated and cloned into a binary plant expression vector under a constitutive promotor to ensure high protein expression. Subsequently, *Agrobacterium*-mediated transient coexpression experiments in *N. benthamiana* leaf tissue will be conducted to identify the *avr* determinant by monitoring resistance responses. Furthermore, the ability of *Rz2* to confer resistance against other soil-borne viruses transmitted by *P. betae,* like BSBMV and BSBV, shall be investigated.

A second aim of the thesis is to generate transgenic *N. benthamiana* plants as a model system. These plants will help to provide more detailed information on the mode of resistance mediated by *Rz2*, to obtain possible conclusions about the natural host-pathogen interaction between BNYVV and *B. vulgaris*. Plants will be generated using a leaf-disk transformation protocol and selected. Therefore, different infection assays, including mechanical inoculation and *Agrobacterium*-mediated transient infiltration, will be used to identify transgenic *N. benthamiana* plants resistance towards BNYVV infection.

Additionally, interaction studies should be performed for a deeper understanding of the interaction between *Rz2* and the *avr* gene. In this regard, two *in planta* systems and one *in vivo* assay using yeast cells will be applied to investigate protein-protein interaction. Finally, the ability of BNYVV to evade *Rz2* resistance will be investigated by searching for resistance-breaking variants of the virus using different sequencing approaches. Subsequently, the amino acid alternations identified in the *avr* determinant are integrated into the plant expression vector and tested for their ability to elicit a resistance response.

CHAPTER 3: Manuscript I

The *Beta vulgaris* derived resistance gene *Rz2* confers a broad-spectrum resistance against soil-borne sugar beet infecting viruses from different families by recognizing the triple gene block protein 1

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Keywords: *Avr* determinant, *Beet necrotic yellow vein virus*, hypersensitive response, *R* gene, TGB1

Author contributions:

VW carried out all experiments described in the manuscript, analyzed, and visualized the data and composed the first draft. Results reported in Fig. 1B were performed by MSc. student Jessica Lang under supervision of SL and VW.

Abstract

Sugar beet cultivation is dependent on an effective control of the *Beet necrotic yellow vein virus* (BNYVV, family *Benyviridae*) causing tremendous economic losses in sugar production. As the virus is transmitted by a soil-borne protist, the use of resistant cultivars is currently the only way to control the disease. The *Rz2* gene belongs to a family of proteins conferring resistance towards diverse pathogens in plants. These proteins contain coiled-coil and leucine-rich repeat domains (Capistrano-Gossmann et al., 2017). After artificial inoculation of homozygous *Rz2* resistant sugar beet lines, BNYVV and *Beet soil-borne mosaic virus* (BSBMV, family *Benyviridae*), were not detected. Analyzing the expression of *Rz2* in naturally infected plants indicated a specific and constitutive expression in root system. In a transient assay, co-expression of *Rz2* and the individual BNYVV encoded proteins revealed that only the combination of *Rz2* and triple gene block protein 1 (TGB1) resulted in an hypersensitive reaction (HR) like response. Furthermore, HR was also triggered by the TGB1 homologues from BSBMV as well as from the more distantly related virus, *Beet soil-borne virus* (BSBV, family *Virgaviridae*). This is the first report of an *R* gene providing resistance across different plant virus families.

Introduction

Growing sugar beets and beet sucrose production is highly dependent on an effective control of the viral soil-borne disease rhizomania, mainly caused by *Beet necrotic yellow vein virus* (BNYVV). Rhizomania is the most devastating disease in sugar beet due to its worldwide occurrence in growing areas, the long persistence of the soil-borne protist vector, *Polymyxae betae* Keskin. This disease causes severe sugar yield losses of up to 80% (McGrann et al., 2009; Peltier et al., 2008). After the first report of the disease in Italy 1952, the causative agent of the disease was detected from infected sugar beet plants in Japan a few years later (Tamada and Baba, 1973). Infection with BNYVV results in a reduced taproot weight, massive proliferation of lateral rootlets and a brownish discoloration of the vascular system. Virus infection is mainly restricted to the root system, as canopy symptoms, like necrotic leaf veins and yellowing, can only be rarely observed in infected field plants (reviewed in Peltier et al. 2008).

BNYVV belongs to the genus *Benyvirus* and consists of a multipartite plus-sense single-stranded RNA genome. RNA1 contains one open reading frame (ORF) encoding a replicase protein that harbors motifs for methyltransferase, helicase, papain-like protease and RNA-dependent RNA polymerase (Bouzoubaa et al., 1987). The first ORF on RNA2 encodes the coat-protein (CP) with its minor read-through domain (CP-RT) responsible for encapsidation and vector transmission (Tamada and Kusume, 1991). The next three overlapping ORFs form the triple-gene block (TGB) and the proteins are responsible for cell-to cell movement (Gilmer et al., 1992; Verchot-Lubicz et al., 2010). The 3´proximal located ORF

encodes the viral suppressor of RNA silencing (VSR) P14 (Chiba et al., 2013; Dunoyer et al., 2002). RNA3 encodes the P25 protein required for virus pathogenicity, long-distance movement and symptom development in *B. vulgaris* (Koenig et al., 1991; Lauber et al., 1998; Tamada et al., 1989). The P31, translated from RNA4, is responsible for efficient vector transmission and suppression of gene silencing (Rahim et al., 2007; Tamada and Abe, 1989). There are three different subgroups of BNYVV which can only be differentiated according to sequence polymorphism of the different RNA components and phylogenetic relationships (Koenig et al., 1995; Koenig et al., 1997, Koenig and Lennefors, 2000, Kruse et al., 1994).
Since the first occurrence of BNYVV and the rapid spread of the disease, the pressure for development of resistant varieties was extremely high as until today no alternative control mechanism exists. The most important resistance genes controlling BNYVV are *Rz1* and *Rz2*. The first detected dominant resistance gene *Rz1* was introduced in cultivars in the 1980´s (Scholten and Lange, 2000). A second dominant resistance gene, named *Rz2*, was identified in a *Beta vulgaris* L. ssp. *maritima* population in Kalundborg, Denmark, conferring even a higher resistance level (Acosta-Leal et al., 2010; Scholten et al., 1999). The first *Rz1* resistance breaking strains were described by Liu et al. (2005) which can only be controlled by a combined usage of sugar beet varieties possessing both, *Rz1* and *Rz2*, genes (Bornemann et al., 2015; Bornemann and Varrelmann, 2011; Pferdmenges and Varrelmann, 2009). The genetic background of *Rz1* has not been identified yet, but it is assumed that the pathogenicity factor P25 represents the avirulence gene, as mutations within this protein could be linked to resistance breaking (Schirmer et al., 2005). In 2017, the gene encoding *Rz2* was identified as a coiled-coil-nucleotide-binding site-leucine-rich repeat (CC-NB-LRR) protein (Capistrano-Gossmann et al., 2017), representing a major class within resistance genes (*R* genes). *R* genes confer a dominant resistance by an active recognition of pathogens due to pathogen-derived effectors, namely avirulence determinants (*Avr*). As reaction of the pathogen recognition, a hypersensitive response (HR) in form of localized cell-death at the infection site, occurs (Jones and Dangl, 2006). Dominant *R* genes can be classified into two groups, the major class encoding a nucleotide-binding and leucine rich repeat domain (NLR) containing proteins and all others having different architecture (De Ronde et al., 2014). Furthermore, the NLR *R* genes can be divided according to their functional domain at the N-terminal part of the protein, either encoding a coiled-coil (CC) or a Toll and interleukin-1 receptor (TIR) domain (Moffett, 2009; Pan et al., 2000). Both domains are supposed to be involved in recognition of the *Avr* determinant (Rairdan et al., 2008). The specificity of *R* proteins is determined by the LRR domain displaying the most variable region and therefore assumed to be mainly responsible for recognition of the target proteins (Moffett, 2009). The central domain of *R* proteins is characterized by a nucleotide-binding site and an ARC (Apaf-1, R proteins, CED-4) domain, which is responsible for hydrolyzing ATP. These domains are known to be involved in

cell death signaling also in animals and nematodes (Leipe et al., 2004). As indicated by the review of De Ronde et al. (2014), nearly 20 dominant antiviral *R* genes with this architecture were already cloned and the functional diversity of viral *Avr* determinants shows that the ability to induce resistance can be uncoupled from the function in the viral infection cycle.

The recent discovery of the gene encoding *Rz2* enabled us to investigate the resistance mechanism in the present study. First, we confirmed that *Rz1* and *Rz2* represent different BNYVV resistance genes by using a resistance test with homozygous breeding lines and an artificial infection method. Using the heterologous host and model plant *Nicotiana benthamiana* (*N. benthamiana*), it was possible to trigger a resistance response when *Rz2* and BNYVV RNA1-4 were co-expressed in leaf tissue. Moreover, we demonstrate with this plant system that BNYVV TGB1 represents the *Avr* determinant of *Rz2*. Besides BNYVV TGB1, two distinct TGB1 protein variants from related and unrelated viruses also triggered cell death in the presence of *Rz2*. Thus, this study provides evidence on a sugar beet dominant resistance gene conferring resistance against plant viruses derived from two different families.

Results

Sugar beet resistance test

Two different BNYVV resistant sugar beet lines, homozygous for *Rz1* or *Rz2* as well as two susceptible lines were tested in a resistance test investigating the ability of BNYVV to infect genotypes with different genetic background. For inoculation, systemically infected leaf material from artificially infected *B. macrocarpa* plants was used by means of vortex inoculation of 7-day old seedlings according to Bornemann and Varrelmann (2011) and modified by Liebe et al. (2020). Furthermore, the ability of *Rz2* to mediate resistance against the closely related *Beet soil-borne mosaic virus* (BSBMV) was proven. Seven-day old seedlings of each genotype (12 replicates) were inoculated, planted in sterile soil and grown for six weeks before virus detection was conducted by means of DAS-ELISA (Table 1). The results revealed no detectable virus multiplication in case of BNYVV infection in lateral roots of both resistant lines but a clear propagation in both susceptible lines. In case of BSBMV, considerable amounts of virions could be detected in *Rz1* and in both susceptible lines but not in the *Rz2* line. This indicates, that *Rz2*, in addition to BNYVV, does confer resistance against BSBMV, whereas *Rz1* mediated resistance is restricted to BNYVV.

TABLE 1 Results of BNYVV and BSBMV resistance test with different sugar beet breeding lines after mechanical inoculation. Mean absorbance values (A_{405}) determined by DAS-ELISA in lateral roots of BNYVV and BSBMV infected and healthy plants of homozygous breeding lines harboring different resistance genes. Significant differences between treatments are indicated (*, p =< 0.002).

	BNYVV		BSBMV	
Genotype	Mean A_{405}	SD	Mean A_{405}	SD
Rz1Rz1+rz2rz2	-0.087	0.0832	0.41*	0.22
rz1rz1+Rz2Rz2	-0.111*	0.037	-0.161*	0.043
rz1rz1+rz2rz2 line 1	0.21*	0.08	0.37*	0.23
rz1rz1+rz2rz2 line 2	0.52*	0.13	0.62*	0.31

Tissue specific expression of *Rz2*

In the study of Liebe et al., 2020, *Rz1* tissue specific resistance was revealed by using an mRFP fluorescently labeled BNYVV clone (BNYVV-mRFP) (Laufer et al. 2018). Following vortex inoculation, no fluorescent signal was detectable in roots of the resistant genotype, indicating a root specific *Rz1* expression. In contrast, following mechanical leaf inoculation local lesion occurred but the virus was unable to move systemically from leaves into roots of *Rz1* resistant plants (Liebe et al., 2020). Therefore, we conducted a similar experiment with genotypes being either susceptible or harboring homozygous *Rz2* or a combination of *Rz1* and *Rz2* resistance genes. Mechanical inoculation was performed with plant sap from BNYVV-mRFP systemically infected *B. macrocarpa* leaf material as described below. After 8 days, local virus symptoms in form of yellow spots were visible on the inoculated leaves of all genotypes in similar appearance and virus replication was detected by means of epifluorescence microscopy (data not shown). At 13 dpi, enlargement of the lesions in all genotypes was observed, but a systemic infection was not detectable in any genotype (Figure 1(a)).

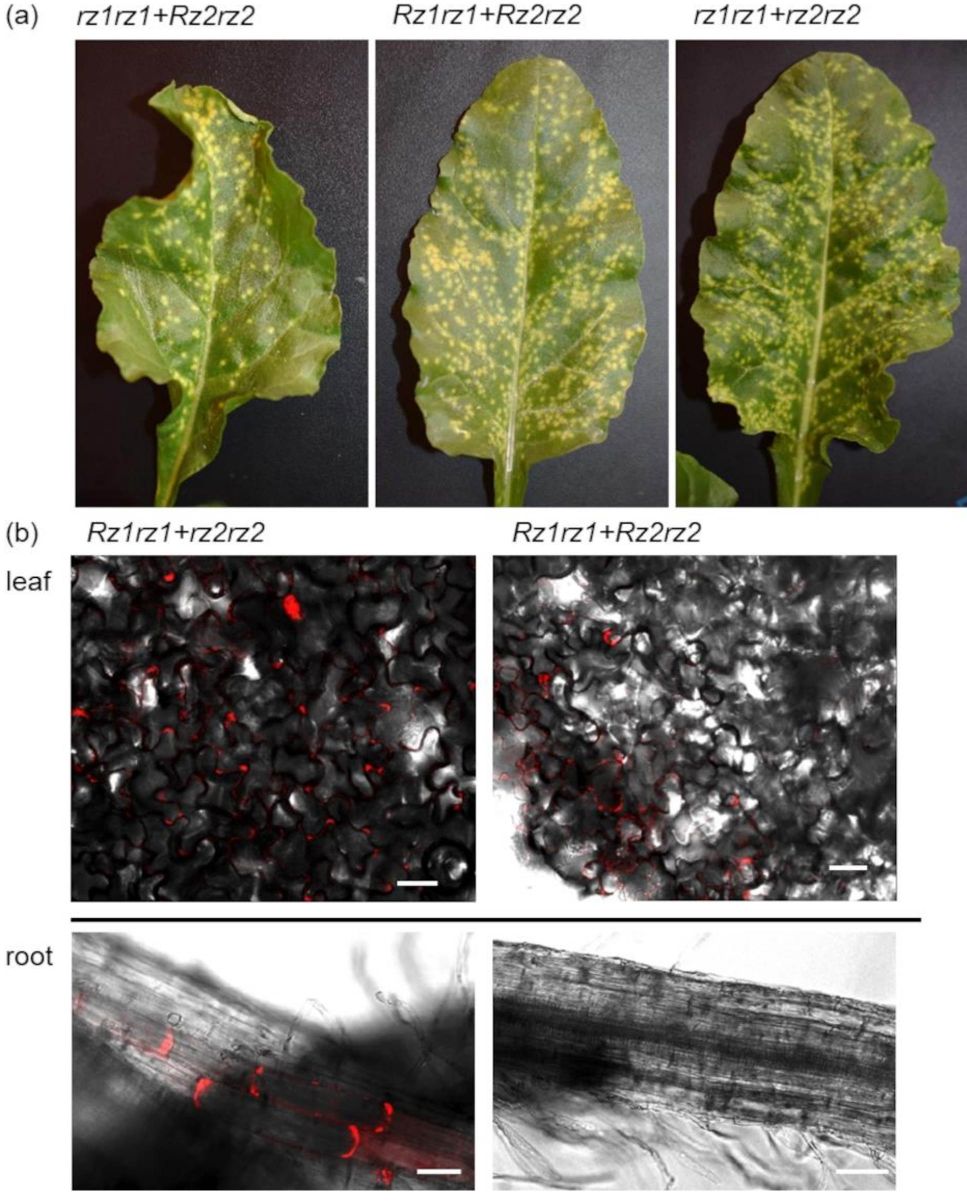


FIGURE 1 Leaf symptom (a) and systemic infection development (b) in sugar beet plants with different resistance genes levels after BNYVV inoculation. (a) Development of local lesions in sugar beet leaves after mechanical BNYVV inoculation at 13 days postinoculation (dpi). Plants with different resistance genes (homozygous *Rz2* (*rz1rz1/Rz2Rz2*), heterozygous (*Rz1rz1/Rz2rz2*) or susceptible (*rz1rz1/rz2rz2*)) were used for inoculation. Mechanical inoculation was performed with sap produced from systemically infected leaves of *Beta macrocarpa* agroinfiltrated with BNYVV-mRFP cDNA clone. (b) Systemic infection of roots and locally restricted infection of leaf tissue after vortex inoculation of sugar beet plants harboring different rhizomania resistance genes: either only *Rz1* (*Rz1rz1/rz2rz2*) or *Rz1* in combination with *Rz2* (*Rz1rz1/Rz2rz2*). Vortex inoculation was performed with systemic BNYVV-mRFP infected *B. macrocarpa* leaf material of 7-day-old seedlings. Confocal imaging was performed at 28 dpi. White bars indicate a scale of 50 µm.

Besides mechanical leaf inoculation, mechanical inoculation of seedlings of different genotypes was performed via vortexing with plant sap infected with BNYVV-mRFP and movement of the virus was monitored. In comparison to a natural infection, which occurs only in root tissue, leaf tissue is additionally inoculated using vortex inoculation. For infection, a variety harboring heterozygous *Rz1* and *Rz2* and a susceptible one was used. Confocal microscopy of newly emerging leaf tissue showed fluorescence signals in both genotypes (Figure 1(b)). This is in accordance with the mechanical inoculation of leaf material, showing local lesion on all genotypes, even in presence of both BNYVV resistance genes. In lateral roots of the susceptible genotype, fluorescent signals were detected at 28 dpi, indicative for the virus replication in all tissues and local virus movement. In contrast, no fluorescence signal was detected in the resistant line, neither at 28 dpi nor at a later time-point (up to 60 dpi). These two different inoculation experiments indicated that in leaves a tissue specific resistance independent of *Rz1* or *Rz2* is active. This allowed multiplication of BNYVV but inhibited systemic spread of the virus. This resistance seems to be also present in susceptible plants, as no virus systemic movement was observed after mechanical leaf inoculation. Furthermore, after vortex inoculation of young seedlings, which includes inoculation of leaf tissue, the systemic spread of the virus was inhibited, indicating a time independent manner of this resistance type.

To provide further evidence for root specific resistance mechanism by *Rz2,* tissue specific expression of *Rz2* was measured by quantitative Reverse-Transcriptase PCR (qRT-PCR). For this purpose, sugar beet seedlings of the homozygous *Rz2* breeding line and the susceptible line 1 were planted in naturally infected BNYVV A-type soil. Then, leaf and root materials were harvested at different time-points after infection. Primers were designed for specific amplification of *Rz2* mRNA. The relative expression in root material to leaf tissue was calculated in healthy (black bars) and infected plants (white bars) (Figure 2). The expression of *Rz2* in roots in comparison to leaves was significantly higher at most of the tested time-points. Before the seedlings were planted in infected soil (0 dpi), a roughly 100-times higher expression in roots compared to leaves was detected. After inoculation (60 dpi), the root specific expression increased by a factor of roughly 900. The different expression level of *Rz2* in root and leaf tissue was observed in healthy as well as in infected tissue. An expression increase over time of *Rz2* could be detected in both tissues and treatments, but this induction was lower in infected samples. At each time point after infection, a higher *Rz2* expression was detected in infected tissue with a ratio between 1.5 and 10, respectively.

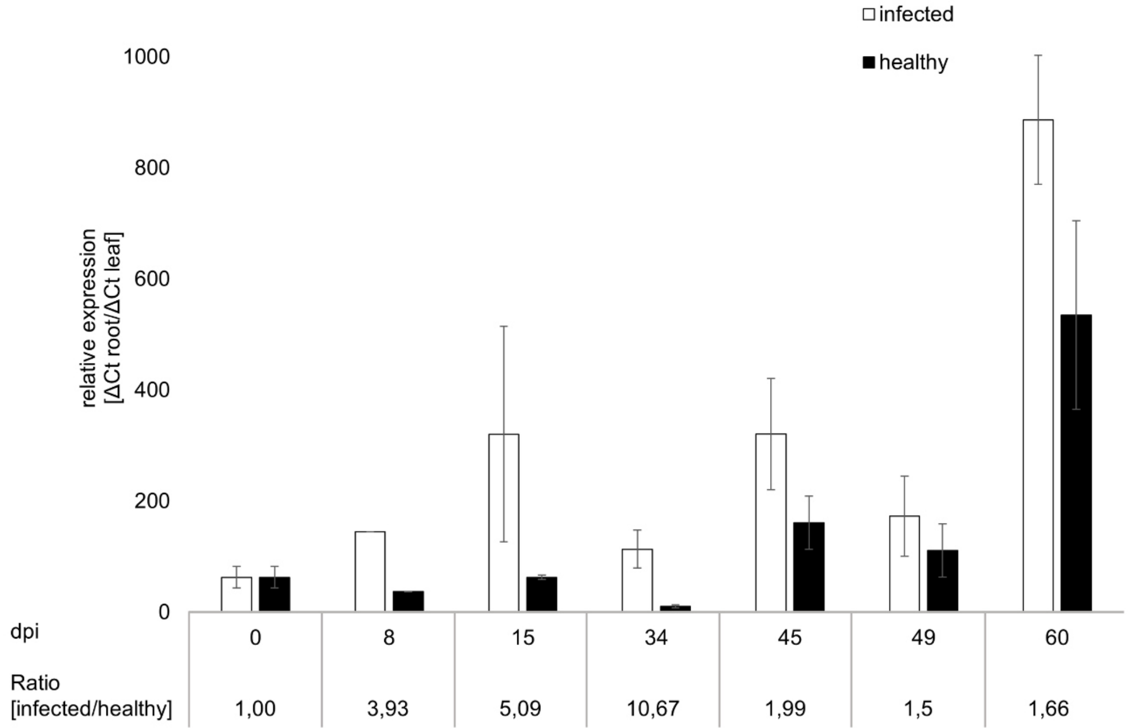


FIGURE 2 mRNA expression level of *Rz2* in sugar beet root tissue relative to leaf tissue. Homozygous sugar beet plants harboring *Rz2* (*rz1/Rz2*) were planted in naturally infected soil or heat sterilised soil (healthy control) and roots and leaves were sampled at different time-points after replanting into different soils. *Rz2* expression was normalised with *GAPD-H* and *EEF1b2* housekeeping genes for each timepoint. Bars indicated the different expression in root tissue compared to leaf tissue. Ratio [infected:healthy] indicated the difference of *Rz2* expression from infected versus healthy plants at each timepoint. Error bars indicate standard derivation and significant differences between root and leaf tissue were calculated for the individual ΔC_t values at each timepoints and are indicated (one-way analysis of variance followed by HSD Tukey's *t* test, ***p* > 0.05).

Transient co-expression of BNYVV and *Rz2* in *N. benthamiana* induce cell death

As expression analysis in sugar beet indicated a root specific expression of *Rz2*, further analyses by transient agrobacterium mediated expression in the host plant was not feasible. As shown by several studies using agrobacterium mediated inoculation (agroinoculation), *R* genes will be expressed and can recognise the corresponding *Avr* determinant resulting in a similar response comparable to the original host in presence of essential co-factors (Bendahmane et al., 2000; Hallwass et al., 2014; Peiró et al., 2014; Tran et al., 2015). Additionally, agroinoculation of BNYVV cDNA clone and transient expression in sugar beet roots is extremely inefficient (Laufer et al., 2018a).

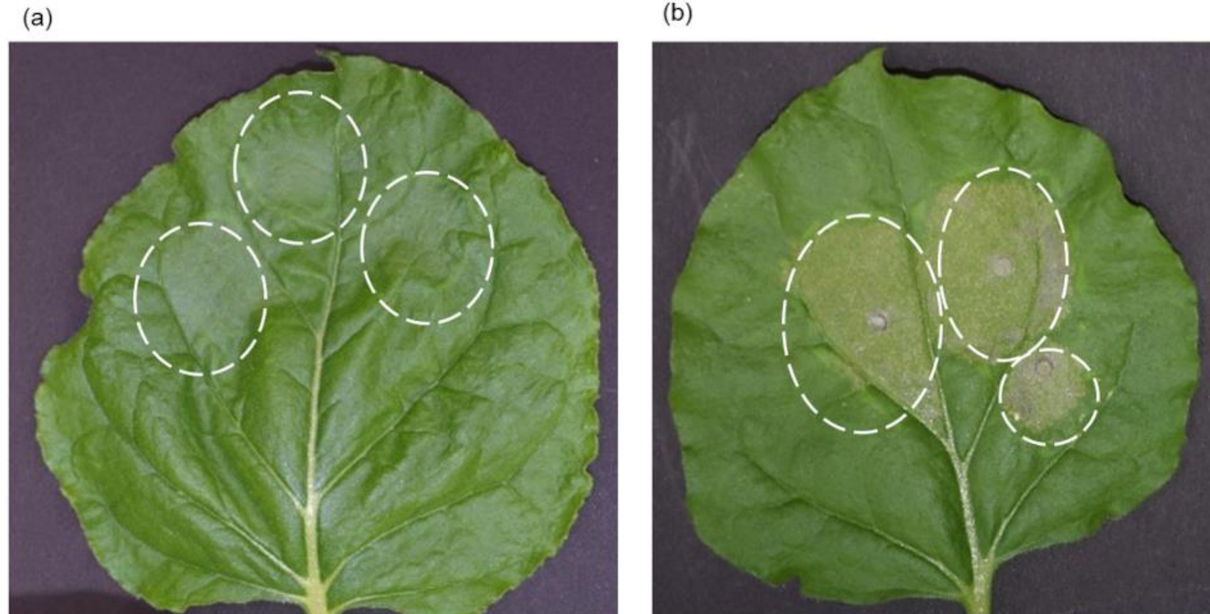


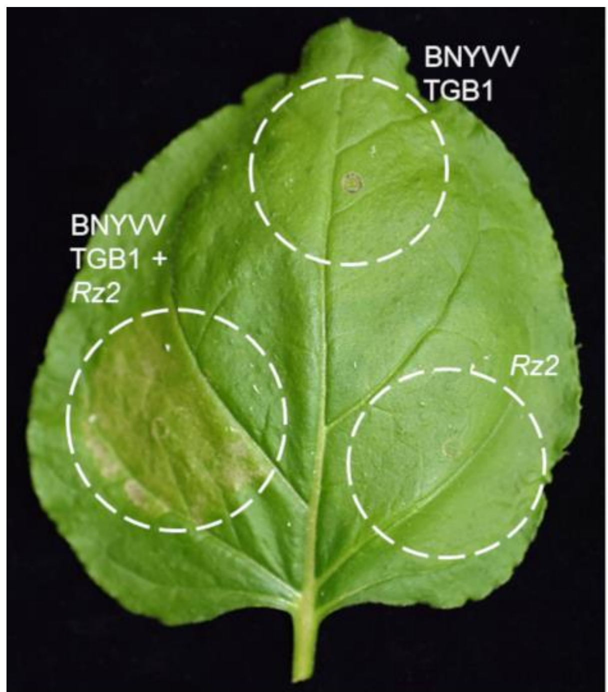
FIGURE 3 Coexpression of BNYVV and *Rz2* elicits hypersensitive response-like necrosis in *Nicotiana benthamiana* tissue after *Agrobacterium*-mediated expression of (a) mRFP labelled BNYVV cDNA clone and (b) mRFP labelled BNYVV cDNA clone and *Rz2*. Symptom recording was performed at 5 days postinoculation.

N. benthamiana represents an experimental host plant for BNYVV, showing yellowing and necrosis of leaves after agroinoculation with an infectious clone. To investigate the ability of *Rz2* to confer resistance and to limit the spread of the virus in *N. benthamiana*, the *Rz2* ORF was cloned under control of the constitutive CaMV 35S promotor in a binary vector. Transient expression was achieved by *N. benthamiana* leaf tissue infiltration with *Agrobacterium tumefaciens* cells harboring the protein of interest. In parallel BNYVV-mRFP (including RNA1-4) was agroinoculated to analyse autoactivation of HR or local plant resistance response (Laufer et al., 2018a). Agroinoculation of BNYVV RNA1-4 resulted in local lesions in the infiltrated patches and systemic infection. In contrast, local cell death was visible after 5 dpi when BNYVV was co-expressed with *Rz2* (Figure 3) and no systemic infection was detected. By infiltration of *Rz2* alone (Figure 4), no visible symptoms in form of local cell death were detected. HR-like cell death was also confirmed by fluorescence microscopy, indicated by a strong auto-fluorescence of dying cells using the GFP filter (Koga et al., 1988) (data not shown). In addition, infiltrated leaves were stained with 3,3´-diaminobenzidine (DAB). DAB staining allows a discrimination between dying and viable cells by production of a brownish H_2O_2 precipitate, which is produced during cell death. To confirm that the *Rz2* protein is responsible for the resistance reaction, a non-translatable version of *Rz2* was also co-expressed with BNYVV RNA1-4 (data not shown). No cell death occurred in the infiltrated leaf patches, showing that *Rz2* protein is required to induce the resistance reaction.

TGB1 derived from BNYVV displays the *Avr* determinant recognized by *Rz2*

As shown in the previous experiment, the sugar beet resistance gene *Rz2* mediates resistance when transiently expressed in the heterologous plant host *N. benthamiana*. A resistance phenotype in form of local cell death could be detected, meaning that the biochemical cascade that led to an HR-like resistance response is present in *N. benthamiana* and can be initiated by BNYVV after *Rz2* recognition. To investigate the elicitor activity of encoded genes by BNYVV, all individual viral ORFs were cloned under 35*S* promotor and applied for transient co-expression with *Rz2*. Infiltration of the individual genes without *Rz2* did not result in an HR in any case (data not shown). A resistance reaction in form of an HR reaction was only detected by co-expression of *Rz2* and the movement-protein triple gene block protein 1 (TGB1) gene (Figure 4). For control, cell death was monitored by the absence of fluorescence signals in tissues co-expressing 35*S*-dsRed as marker in all variants (data not shown). This allowed a discrimination of living tissue, showing a strong red fluorescence, and dead tissue detected by a strong autofluorescence visible in the GFP filter (Koga et al. 1988). HR was verified by DAB staining as a brownish precipitation of H_2O_2 (Figure 4). A non-translatable version of TGB1 did not produce local cell death in the infiltrated tissues (data not shown). For protein expression analyses, proteins of interest, namely *Rz2* and TGB1 variant were fused to a human influenza hemagglutinin (HA) tag and expression was verified by Western blot (Figure S1). The C-terminal HA tag did not interfere with the resistance reaction as the initiation of cell death after BNYVV TGB1 and *Rz2* co-expression was still observed (data not shown).

It is known that some TGB1 variants possess RNA silencing suppressor (VSR) activity in addition to their cell-to cell movement function. This has been reported for the P25 from *Potato virus X* and the TGB1 from *Anthernanthera mosaic virus* as well as *Potato virus M* (Lim et al. 2010; Senshu et al. 2011; Voinnet et al. 2000). Further, the PVX derived P25 also elicits an HR response in presence of the resistance gene *Nb* (Malcuit et al. 1999). To exclude the ability of HR elicitation by co-expressed VSRs, we co-infiltrated the BNYVV VSR P14 encoded by RNA2, and a second deviating VSR derived from *Tomato bushy stunt virus*, P19. In both treatments, no HR was detected after 4 dpi, excluding the ability of VSR to lead to non-specific HR (Figure S2).



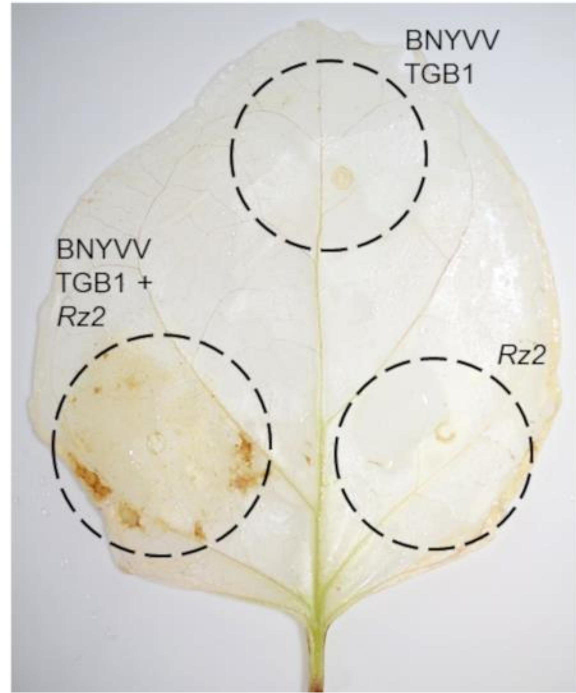


FIGURE 4 Coexpression of BNYVV TGB1 and *Rz2* elicits hypersensitive response-like symptoms in *Nicotiana benthamiana* leaf tissue after *Agrobacterium*-mediated expression. Leaves were infiltrated with binary plasmids harbouring *TGB1* from BNYVV and *Rz2* in combination and individually (3 days postinoculation). Cell death was validated by the brownish precipitate after 3,3′-diaminobenzidine (DAB) staining of detached leaves.

TGB1 from both BSBMV (*Benyviridae*) and BSBV (*Virgaviridae*) are recognized by *Rz2*

The resistance test in naturally infested soil indicated that *Rz2* additionally recognizes and targets the closely related BSBMV (Table 1) which is also transmitted by *P. betae* and part of the rhizomania disease in some parts of the world (Workneh et al. 2003). These results raised the question whether *Rz2* also recognizes TGB1 derived from BSBMV. To test this hypothesis, the BSBMV TGB1 ORF was cloned and used for transient co-expression with *Rz2* in *N. benthamiana* leaf tissue. In accordance with BNYVV TGB1 recognition and cell death initiation by *Rz2*, BSBMV TGB1 showed a resistance response in form of HR when co-expressed with *Rz2*. Cell death was verified by DAB staining and fluorescence microscopy (Figure 5). Furthermore, protein expression was confirmed by Western blot, after C-terminal fusion of BSBMV TGB1 to an HA-tag (Figure S1). As reported above, translational HA-tag fusion had no influence on the resistance mechanism.

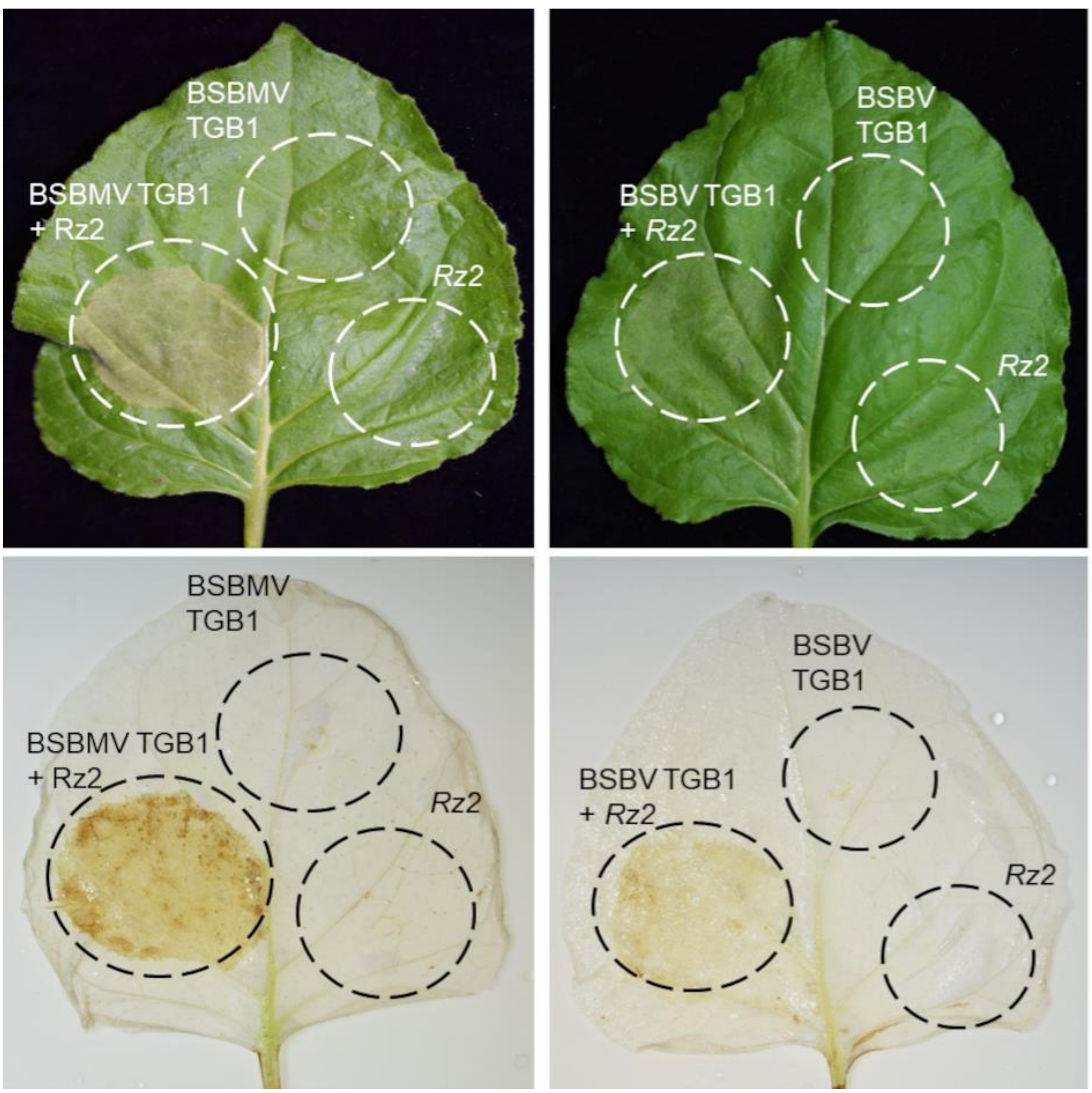


FIGURE 5 TGB1 variants from distinct plant virus families elicit hypersensitive response-like necrosis on *Nicotiana benthamiana* leaf tissue after agroinoculation in combination with *Rz2*. Leaves were inoculated with binary plasmids harboring TGB1 variants from beet soil-borne mosaic virus (BSBMV) and beet soil-borne virus (BSBV) in combination with and without resistance gene *Rz2*. Cell death was validated by the brownish precipitate visible after 3,3'-diaminobenzidine (DAB) staining of detached leaves. Symptoms were recorded at 2 days postinoculation.

Since it could be demonstrated that Rz2 confers resistance to the TGB1 protein from BSBMV with only 74% homology to BNYVV TGB1, a third sugar beet infecting virus, Beet soil-borne virus (BSBV), belonging to the genus Pomovirus within the family Virgaviridae, was included in the experiments. BSBV possesses a TGB1 protein that shares around 22% homology to BNYVV TGB1. BSBV is transmitted by the same vector and occurs in mixed infections with BNYVV (Meunier et al., 2003). Foliar symptoms on leaf tissue but no symptom

development on root tissue after BSBV infection on sugar beet plants were recently reported by Mahillon *et al.* 2021. Whether BSBV interferes with symptom expression in mixed infection with BNYVV is unknown (Biancardi and Tamada, 2016). To test the ability of *Rz2* to mediate resistance towards a broad spectrum of TGB1 variants, sugar beet plants were analyzed for their BNYVV and BSBV content by means of virus specific ELISA after planting seedlings in naturally BNYVV P-type and BSBV infected soil (Table 2). Using this type of naturally infected soil, we verified the ability of BNYVV P-type virus strains to evade *Rz1* resistance, represented by high ELISA values in the genotypes containing homozygous *Rz1.* Furthermore, no BSBV virus content could be detected in *Rz2* resistant plants, validating the ability of *Rz2* to control another virus belonging to the genus *Pomovirus*. Finally, the TGB1 from BSBV was used for co-expression experiments with *Rz2*. Co-expression of BSBV TGB1 and *Rz2* indicated a HR reaction, which was verified by DAB staining (Figure 5). BSBV TGB1 expression was analyzed by Western blot of C-terminal fusion of an HA-tag (Figure S1).

TABLE 2 Results of BNYVV and BSBV resistance test with different sugar beet breeding lines planted in naturally infected soil. Absorbance values (A405) determined by DAS-ELISA on lateral roots of BNYVV and BSBV infected and healthy plants of homozygous breeding lines harboring different resistance genes. Significant difference between infected and healthy plants are indicated (*, p =< 0.002).

	BNYVV		BSBV	
Genotype	Mean A_{405}	SD	Mean A_{405}	SD
Rz1Rz1+rz2rz2	0.43*	0.17	0.94*	0.71
rz1rz1+Rz2Rz2	0.00*	0.01	-0.12*	0.01
rz1rz1+rz2rz2 line 1	0.37*	0.19	0.41*	0.12
rz1rz1+rz2rz2 line 2	0.37*	0.20	0,38*	0.13

Sequence alignment of the different TGB1 proteins shows that BNYVV and BSBMV proteins share 74.6% homology, whereas BNYVV TGB1 and BSBV TGB1 only share 22.4%. The TGB1 of plant viruses contains a NTPase/helicase sequence domain, belonging to the helicase superfamily I and consisting of seven motifs (Koonin and Dolja, 1993; Morozov and Solovyev, 2003). Sequence alignment of the TGB1 variants from BNYVV, BSBMV and BSBV indicates a high homology in all seven conserved motifs across the different species (Figure 6). A highly conserved motif within motif I (GKS/T tripeptide) can be found in all variants.

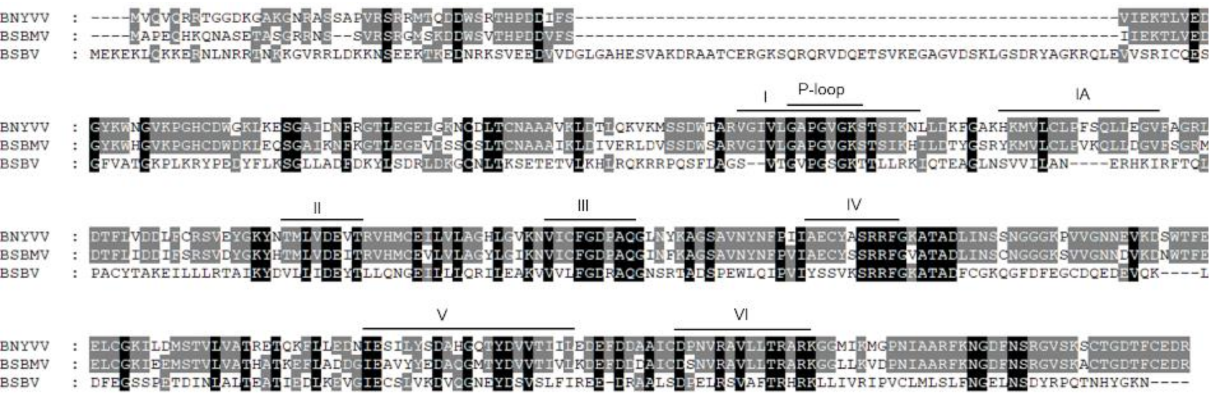


FIGURE 6 Multiple sequence alignment of amino acid sequence of TGB1 variants from beet necrotic yellow vein virus (BNYVV, AQT03618.1), beet soilborne mosaic virus (BSBMV, APZ76016.1)) and beet soilborne virus (BSBV, NP_612625.1). Amino acid conserved in two (three) sequences are highlighted in gray (black). Motifs I-IV of RNA helicase superfamily 1, including a P-Loop, according to Koonin et al. 1993, are indicated.

Discussion

Rz2 mediates resistance in a tissue specific manner

The present research was conducted to characterize the sugar beet antiviral resistance gene, *Rz2*. A resistance test with BNYVV and BSBMV clearly demonstrated that *Rz1* does not control BSBMV and only targets BNYVV while *Rz2* targets both viruses. This gave evidence that *Rz1* and *Rz2* represent different genes, which is consistent to previous genetic mapping work (Barzen et al., 1997; Scholten et al., 1999; Scholten and Lange, 2000). These results are further supported by the finding that BSBV is controlled by *Rz2* but not by *Rz1*. Finally, it highlights that *Rz2* confers resistance across different plant virus families.

Former studies of Liebe et al. (2020) and Chiba et al. (2008) concluded that *Rz1* mediates a root specific resistance. No virus was detected in lateral roots after mechanical inoculation of whole seedlings, whereas leaf tissue developed local lesions after mechanical inoculation. The present study obtained comparable results in sugar beet genotypes harboring *Rz2*. No virus was detectable in roots after vortex inoculation, but local lesions occurred following mechanical leaf tissue inoculation, comparable to local lesion after infection of susceptible plants (Liebe et al. 2020). Local lesions represent a symptom that is associated with resistance in *Chenopodiacae* (Canto and Palukaitis, 1999; Loebenstein, 2009) which is independent on the known BNYVV resistance genes (*Rz1* and *Rz2*). Furthermore, this type of resistance seems to be restricted to leaf tissue, as in resistant genotypes no virus could be detected in roots, whereas after leaf inoculation lesions on leaf tissue developed. With these results, there is a distinct indication, that the virus can multiply and spread in leaflets, but the virus is unable to cause a systemic infection. Taken together, this study emphasizes the characteristics of both

known BNYVV resistance genes to confer resistance only in root tissue. These results are former verified by measuring transcript levels of *Rz2* in leaf and root tissue in naturally infected and heathy plants. In this test, the relative expression level of *Rz2* in root tissue was remarkably higher compared to the leaf tissue, indicating a tissue specific expression in roots. Considering that BNYVV is vectored by the soil-borne pathogen *P. betae*, the root specific expression of *Rz2* seems to be plausible as it takes place at the site of vector infection. These results are in accordance with the observation of systemically infected plants in fields indicated by yellow necrotic veins. These symptoms occur very rarely, indicative for a resistance host response in aboveground tissue that unsuccessfully or incompletely attempts to limit the spread. The reason for the infrequent movement to aboveground tissue where obviously a deviating mechanism limits the spread, is unknown. Tissue specific expression of NLRs is reported in other studies and seems to be related to the tissue where infection occurs (Lai and Eulgem, 2018; Munch et al., 2017) to avoid metabolic disadvantages.

Although there are some studies available, addressing NLR expression in response to pathogen stimuli, only one study investigated the transcription level of an antiviral *R* gene in its native host. Levy et al. (2004) analysed the transcript level of *Tobacco mosaic virus* (TMV) resistance gene (*N* gene) before and after TMV inoculation. A constitutive *N* gene expression was detected in non-inoculated plants, which was also observed in the current study. Constitutive expression of *R* genes was also observed in other studies and appears plausible as receptors need to be present during initiation of infection in order to be effective (Joshi and Nayak, 2011; Schornack et al., 2005; van der Biezen and Jones, 1998). It was shown that, after inoculation, the *N* gene expression was increased to a 165-fold higher level and the elevated mRNA level remained constant for at least 10 days (Levy et al., 2004). In the case of *Rz2*, an increase of expression was also observed with the highest expression level at 60 days after infection. In contrast to the other studies with the *N* gene, where only a single inoculation was reported, in the present study a continuous infection occurs when plants are grown in infected soil. This is due to the multiple *P. betae* zoospore mediated infection cycles. This can be an explanation for the steady increase of *Rz2* mRNA level over time, whereas the mRNA level of *N* gene decreases after 10 days.

Agrobacterium mediated expression of Rz2 in combination with BNYVV leads to HR in the heterologous host plant N. benthamiana

An efficient transient expression method in plant research is often achieved by agrobacterium mediated expression of the gene of interest. In case of sugar beet, agroinoculation on leaf tissue with BNYVV cDNA clone cannot be applied, as local lesions are formed and considering the tissue specific expression of *Rz2*, investigations on the natural host is rather complicated. *R* genes and corresponding *Avr* determinants have been used successfully for heterologous expression and HR induction within the same plant family as demonstrated in several cases

(Abbink et al., 1998, Tomita et al., 2011; Tran et al., 2015, Bendahmane, 1999). The present study, however, shows that an *R* gene derived from the plant family *Amaranthaceae* can be functionally transferred to a plant species belonging to a different clade. The present study demonstrates that all downstream signalling components are present in the heterologous plant and no additional proteins from the host plant sugar beet are required for proper functionality of *Rz2*. Although interfamily transient expression of *R* genes as well as the use of a heterologous promotor like 35*S* from *Cauliflower mosaic virus* (CaMV) can result in autoimmune reaction (Bendahmane et al., 2002, Joshi and Nayak, 2011, Takahashi et al., 2012, Oliveira et al., 2016), this was not observed in terms of *Rz2* expression under the CaMV 35*S* control in *N. benthamiana*. Even no autoinduction was observed when VSRs were co-expressed.

TGB1 proteins from different plant virus families display *Avr* determinants towards *Rz2*

Using a heterologous plant system, TGB1 from BNYVV was identified as the *Rz2* corresponding *Avr* determinant. The TGB1 protein is encoded on RNA2 and expression is achieved by subgenomic RNA at an early stage of infection (Morozov and Solovyev, 2003). There are two additional TGB1 known to represent an *Avr* determinant towards a plant resistance gene, the TGB1 derived from *Barley stripe mosaic virus* (BSMV) (Lee et al., 2012) and the P25 derived from *Potato virus X* (PVX). BSMV TGB1 is recognised by a CC-NBS-LRR coding *R* gene product, called *Barley stripe resistance 1* (*Bsr1*) (Cui et al., 2012). Moreover, Lee et al. (2012) analysed a resistance breaking strain of BSMV and identified only three amino acid displaying variability. These amino acid changes occur in the helicase region of the protein (positions 390, 392 and 404) and substitution of two amino acids resulted in virus mutants able to overcome *Bsr1* (Lee et al., 2012). Whether these amino acids also play a crucial role in the *Rz2*-TGB1 recognition, requires further analyses. The second TGB1 representing an *Avr* determinant is the P25 from PVX that is the elicitor of the resistance gene *Nb* being unidentified yet (Malcuit et al. 1999). The P25 proteins additionally represents the PVX VSR protein with movement activity (Voinnet et al. 2000). It is known that TGB1 variants from potexviruses show different properties compared to TGB1 belonging to benyviruses or pomoviruses, as described by Verchot-Lubicz et al. (2010). Important for the present study is the ability to increase or influence the *Rz2* induced HR by VSR activity, which was not detected when applying BNYVV P14 and TBSV P19 in the assays. This indicates that the HR reaction observed in this study by co-expression of TGB1 variants and *Rz2* is not influenced by VSR activity.

In a second approach, TGB1 variants from two other plant viruses were tested for their capacity to trigger an HR response. Thereby, the TGB1 from BSBMV and BSBV were recognized and resulted in a resistance response. The ability of *Rz2* to additionally confer resistance to BSBMV and BSBV was further verified by a plant resistance test. It is known that in field soils inducing

rhizomania, in addition to BNYVV, frequently BSBMV and BSBV virus isolates can also be detected in roots.

Other reported *R* genes are known to confer resistance against a broad spectrum of virus species belonging to the same plant virus family. For example, both the *Phaseolus vulgaris* resistance gene I and *Pvr4* from pepper mediate resistance against several *Potyvirus* species (Cadle-Davidson and Jahn, 2006; Janzac et al. 2009). Similarly, the *L*-gene locus from pepper, the *Tm-2* and *Tm-2*^{2} genes from tomato and the resistance gene *Krish* from sorghum act against several *Tobamovirus* species (Moury and Verdin, 2012; Seifers et al. 2012. More the *Rx1* gene recognizes and defends several members only within the genus *Potexvirus* (Baurès et al. 2008;). In the study of Baurès et al. (2008) it was shown that *Rx1* is able to recognise not only the CP derived from *Potato virus X* (PVX) but as well the more distinct CPs encoded from *White clover mosaic virus* (WClMV), *Narcissus mosaic virus* (NMV) and *Cymbidium mosaic virus* (CymMV) showing only 39, 33 and 35% sequence homology compared to the PVX CP, respectively. In the current study, the *Avr* determinants are derived from different plant virus families, which to our knowledge was not reported before. This recognition triggers an HR-like response, even when the homology of the proteins is only 22%. In the study of Baurès et al. (2008) it was also reported that the region responsible for elicitation shows only distant homology. It was assumed that the recognition is more likely due to structural dimensions rather than the specific amino acid sequence composition (Baurès et al., 2008).

Sequence alignment of the TGB1 variants recognized by *Rz2* revealed the most conserved amino acids within the motifs of the RNA helicase superfamily I. However, the large number of conserved amino acids makes it hardly impossible to predict the region of recognition. Taken together, the assumption that the recognition by *Rz2* is triggered by a structural motif might be the most probable explanation. This hypothesis is supported by the distant phylogenetic relationship between the virus species supported by the TGB1 sequence and phylogenetic analyses performed by Adams et al. (2009). According to the gene-for gene hypothesis it was formerly expected that one *R* protein is specifically recognising a single *Avr* determinant. With the data provided, we suggest that recognition of more than one virus species might be more the rule than an exception. A possible hypothesis for this might be the selection of *Rz2* to function against all three viruses as they occur in mixed infection or the selection of *Rz2* against one of the three viruses but conferring resistance by accident to the others. In case of the present study, where the recognition is dependent on the movement-protein of the virus, this motif might be evolutionarily conserved and essential for efficient host colonisation, preventing or at least delaying the selection of variants in the population with resistance breaking abilities. Further, this might be also a hypothesis for the development of a resistance, recognising a protein present in a variety of plant viruses and being especially important for virus completion

of the infection cycle. It might be interesting to investigate the ability of *Rz2* to confer resistance not only to sugar beet infecting viruses, when the recognition is based on a highly conserved motif of TGB1 present in a variety of plant viruses. Although studies exist, reporting about elevated virus contents in sugar beet genotypes carrying both resistance genes (Galein et al., 2018), TGB1 variants leading to resistance breaking were not yet identified. Population analyses by deep-sequencing of TGB1 from suspicious soils might identify resistance breaking virus strains and will help to characterise the mode of action between the resistance gene *Rz2* and the corresponding *Avr* determinant TGB1 in more detail.

Material and Methods

Virus, plant and soil materials

Beta vulgaris ssp. *vulgaris* homozygous resistant (*Rz1Rz1+rz2rz2* and *rz1rz1+Rz2Rz2*) and susceptible (*rz1rz1+rz2rz2*) breeding lines were applied for the resistance tests. The two susceptible lines differentiate in their genetic constitution according to Capistrano-Gossmann et al. (2017), susceptible line 1 containing a transposon insertion within the *Rz2* ORF and susceptible line 2 containing a premature stop codon. For monitoring virus spread after vortex inoculation a susceptible (KWS03) genotype and heterozygous (Rz1rz1+Rz2/rz2) KWS Angelina were used. These genotypes were former used for mechanical rub inoculation additionally to the homozygous *Rz2* breeding line. For production of virus inoculum, *Beta vulgaris* ssp. *macrocarpa* Guss. was used. *N. benthamiana* wildtype plants were applied for co-expression experiments. All plants were grown and maintained under greenhouse conditions (24 °C/14 h 18 °C/10 h), unless otherwise stated. For agroinoculation the BNYVV A-type and BSBMV full-length infectious clones were used (Laufer et al., 2018b).
Origin of soils used in the study are as follows. For expression analysis, plants were planted in soil possessing BNYVV A-type soil, origin Thurnhof, Straubing, Germany (Pferdmenges et al., 2009). For BSBV resistance test, soil derived from Pithiviers, France was used carrying BNYVV P-type.

Cloning of the viral genes in binary plant expression vector

For generation of constructs for agroinoculation of individual viral gene and *Rz2*, the full-length clone plasmids of BNYVV RNA1, RNA2, RNA3, RNA4 and total root cDNA from *Rz2* sugar beet were used as template and the binary vector pDIVA (Acc. No. KX665539). To ensure high protein expression an optimal ribosomal binding site was added and the ribozyme sequence was removed. Following sequence accessions served as a basis for primer design for BNYVV KX665536, KX665537, KX665538 and MF476800 and patent EP3011037 for the resistance gene *Rz2*, BSBMV cDNA clone and BSBMV TGB1 sequence based on KX352033, KX352170, KX352171 and KX352034 and TGB1 of BSBV (NC_003519.1), respectively (Laufer et al., 2018b; Törjèk et al., 2014). PCR was performed using Phusion Flash High-Fidelity PCR Master

Mix (Thermo Fisher Scientific, Waltham, Massachusetts) following manufacturer´s instructions and primer listed in Table S1 (Eurofins Genomics, Hamburg, Germany). Cloning was performed using the isothermal one step Gibson Assembly, followed by transforming recombinant plasmids into chemical competent *E. coli* DH5α cells (Gibson et al., 2009) and verification by Sanger sequencing. For generation of non-translatable genes, two TGA stop-codons were inserted directly after the start codon of each ORF by PCR-mutagenesis. The coding sequence of the Human influenza hemagglutinin (HA) tag consisting of YPYDVPDYA was inserted as C-terminal translational fusion to the target protein for Western blot analysis. TSBV P19 was kindly provided by Edgar Maiss (University of Hannover) (Zilian and Maiss, 2011).

Sequence alignment and protein structure prediction

Sequence homology of the different TGB1 variants was determined using ClustalOmega online service, MultAlin online software was used for multiple sequence alignment and visualization was done by GeneDoc. Accession numbers were as followed: BNYVV (AQT03618.1), BSBMV (APZ76016.1) and BSBV (NP_612625.1).

Protein structure prediction was performed with the BNYVV TGB1. The online software InterPro was used to predict functional domains (Mitchell et al., 2019).

Plant infection

Mechanical virus inoculation of sugar beet seedlings (vortex inoculation) and mechanical rub inoculation of different sugar beet genotypes was performed using a sap from *B. macrocarpa* leaves systemically infected with BNYVV and BSBMV, respectively, as described by Bornemann and Varrelmann (2011) and modified after Liebe et al. (2020).

For transient co-expression experiments, recombinant 35*S* expression vectors with viral genes or *Rz2* were electroporated into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) strain C58C1 and conducted as described by Voinnet et al. (1998). *R. radiobacter* suspensions were adjusted to OD_{600} of 0.5. In addition to viral genes and *Rz2*, a fluorescent marker gene under 35*S*-control (35*S*-dsRED) was used to confirm cell vitality by transient protein expression and facilitate detection of HR. To ensure equal agrobacteria cells density in each variant, *R. radiobacter* cultures carrying different plasmids were mixed in equal amounts. Four to five-week-old *N. benthamiana* plants were used for inoculation. Experiments contained three replicates and were repeated at least twice.

Detection of cell death

Hypersensitive cell death reaction was detected by staining the infiltrated parenchymatic leaf tissue with 3,3´-diaminobenzidine (DAB) according to Thordal-Christensen et al. (1997) with minor modifications. Infiltrated leaves were detached and vacuum-infiltrated with 0,1% DAB (0,003% Silvet) in PBS puffer and incubated for 2-3 hrs at room temperature. Then leaves

were boiled at 96 °C in 96% Ethanol until complete chlorophyll bleaching. Documentation was done using Nikon (Nikon DX, AFS mikro Nikkor 40 mm) on a white light transilluminator table. Discrimination between viable and dying cells was further analyzed by marker gene expressed fluorescence (DsRed). As a consequence of cell death, the cell showed an autofluorescence explained by the release of cell compartments in terms of dying. This can be visualized in the GFP filter (Koga et al., 1988). Virus replication was detected using the mRFP labeled BNYVV clone (Laufer et al., 2018a). Fluorescence was detected with epifluorescence microscope (Leica DMR) using specific DsRed filters (Emitter HQ 620/60, Beamsplitter Q 585 LP, Exciter HQ 565/30) and GFP filter (Emitter D 510/540, Beamsplitter 425 DCLP, and Exciter D 395/40). Photographs were taken using a Leica DFC camera, DFC300 FX. Further, virus mediated fluorescence was analyzed using Leica TSC SP2 confocal laser scanning microscope. mRFP fluorescence was excited at 561 nm and emitted at 580-590 nm. Images were processed with LAS-AF software version 2.6.3.8173.

Immunodetection of viral coat-protein and HA-tagged proteins *in planta*

Western blot analysis was conducted using anti-HA (monoclonal Anti-HA high affinity from rat, Sigma 1:500, Sigma Aldrich, St. Louis, Missouri) and mouse-anti rabbit antibodies (Anti-Rat IgG-AP 1:10000, Sigma Aldrich). Protein extraction was produced as described by Thiel and Varrelmann (2009) and total protein extracts were separated by 8% SDS-PAGE, followed by electroblotting on a nitrocellulose membrane Protran BA85 (Whatman® Schleicher & Schuelle®, London, UK). Signal detection was performed by the colorimetric NBT-BCIP method and documented on a white light transilluminator.

Virus replication was measured in lateral root tissue by means of ELISA as described by Pferdmenges and Varrelmann (2009). For detection of BNYVV a DAS-ELISA kit (DSMZ, Braunschweig, Germany, AS-0737) was used. Dilution of samples was increased to 1:200, the remaining was performed following manufacturer's instructions. BSBV was detected by means of TAS-ELISA (DSMZ, AS-0576) following manufacturer's instructions. Incubation time for antibodies was 4 hrs, final incubation time for staining was 90 min for both. BSBMV was detected by DAS-ELISA with a primary antibody provided by H.Y. Liu (USDA, Salinas California 4 hrs incubation) at 1/1.000 dilution and Alkaline Phosphatase secondary antibody (Goat anti-Rabbit-AP, Sigma A3687) at 1/1.000 dilution for 4 hrs incubation. Final staining was performed for 60 min. OD_{405} values measured were subtracted by blanks, absorptions of healthy control. Samples were considered positive if they exceeded the mean plus three standard deviations of the healthy controls. Statistically independence of the DAS ELISA values was calculated for each genotype between treatment (healthy and infected) using unpaired t-Test by SigmaPlot14.

RT-qPCR analysis

Rz2 mRNA was quantified in total RNA extracts by means of reverse transcriptase quantitative PCR (RT-qPCR) as described by Fernando Gil et al. (2020). Primers were designed to discriminate between the susceptible line and *Rz2* resistant line. Sugar beet *elongation factor 1 beta* (EEF1B2, NM_001303081.2) and *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH, XM_010679634.2) were used as reference genes. Amplification was performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, California) using the iTaq Universal SYBR Supermix (BioRad) and primer listed in Table S2 (Eurofins Genomics). For each time point five biological replicates were tested. Data normalization and relative expression value were calculated using the $2^{-\Delta\Delta ct}$ method after Livak and Schmittgen (2001). Statistically independence of the data was carried out using R®-software using Tukey HSD test. The statistical independence between root tissue versus leaf tissue was calculated for the individual Δct values at each timepoints using Tukey HSD test by R®-studio.

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Data Availability Statement

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

Conflict of interest

All authors declare no conflicts of interest.

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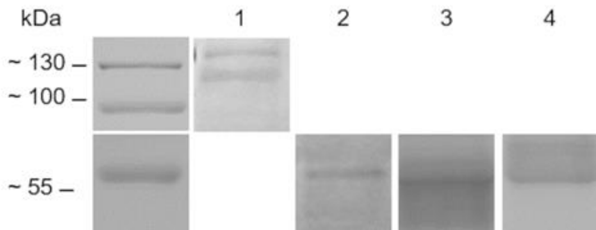

FIGURE S1 Western blot analysis of HA tagged proteins after agroinoculation of *N. benthamiana* leaves with Rz2 (lane 1, expected protein size 123 kDa), BNYVV TGB1 (lane 2, expected size 43 kDa), BSBMV (lane 3, expected size 43 kDa) and BSBV TGB1 (lane 4, expected size 47,5 kDa) sample of infiltrated patch was taken at 5 dpi and total protein extraction was used for blotting.

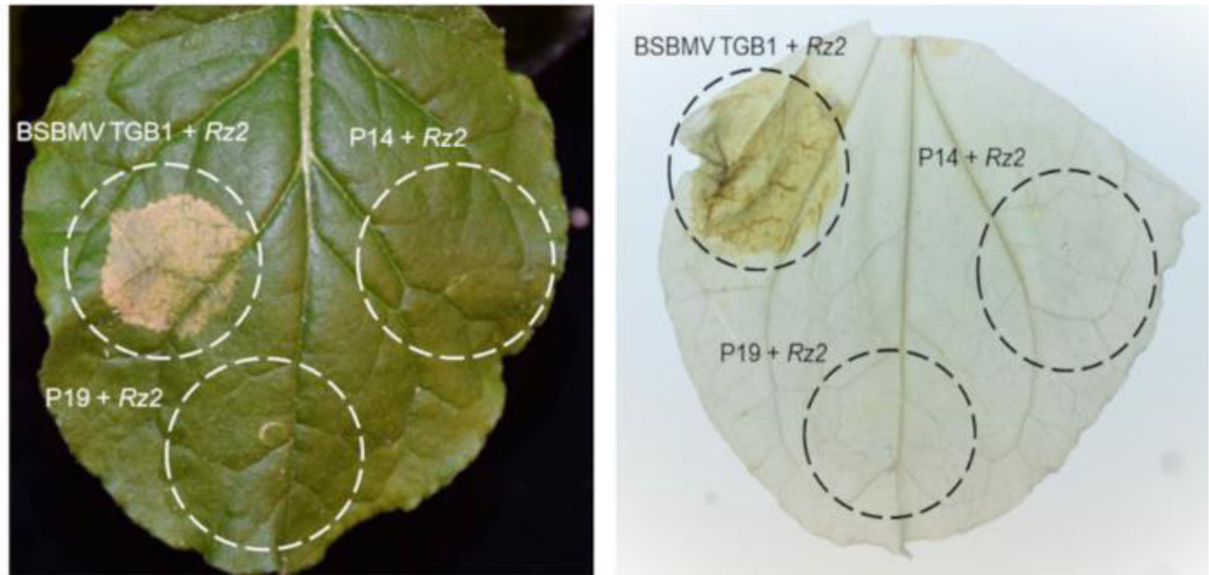

FIGURE S2 HR reaction is not elicited by co-expression of BNYVV P14 (VSR protein of BNYVV) or P19 (VSR protein of Tomato bushy stunt virus). Infiltration of resistance protein Rz2 and 35*S* driven BNYVV P14 or P19 does not result in HR reaction compared to coexpression of BSBMV TGB1. Symptoms were recorded at 4 dpi and DAB staining was performed to validate cell death.

TABLE S1 List of primer used for cloning in binary pDIVA vector. Underlined sequences represent the nucleotides overlapping to the vector.

Primer name	Primer sequence 5´>3´
pDIVA_as	TGTTATATCTCCTAATCGATCCTC
pDIVA_s	TTCTCCAGAATAATGTGTGAGTAGT
RNA3-p25 as	CATTATTCTGGAGAATCAACCATCATCATCAACACCGTCA
RNA3-p25 s	ATCGATTAGGAGATATAACAATGGGTGATATATTAGGCGC
RNA1 as	TCACACATTATTCTGGAGAATCAAAACTTTTTCATATCGA
RNA1 s	ATCGATTAGGAGATATAACAATGGCAGATTCGTTCGGTTT
RNA2-CP as	TCACACATTATTCTGGAGAATCATTGTCCGGGTGGACTGG
RNA2-CP s	ATCGATTAGGAGATATAACAATGTCGAGTGAAGGTAGATA
RNA2-CP-RT as	ACACATTATTCTGGAGAATCATCCGGCGGGAGCGGACGGG
RNA2-CP-RT-s	ATCGATTAGGAGATATAACAATGTCGAGTGAAGGTAGATA
RNA2-TGB2 as	CATTATTCTGGAGAATCAACCACAAGCACCATTACAAATA
RNA2-TGB2 s	ATCGATTAGGAGATATAACAATGTCTAGGGAAATAACCGC
RNA2-TGB1 s	ATCGATTAGGAGATATAACAATGGTCCAAGTACAGCGTAG
RNA2-TGB1 as	CATTATTCTGGAGAATCATCTATCTTCGCAAAAAGTATCT
RNA2-TGB3 as	CATTATTCTGGAGAATCATCTATGACACCAAAACCAAACT
RNA2-TGB3 s	ATCGATTAGGAGATATAACAATGGTGCTTGTGGTTAAAGT
RNA2-VSR-P14 as	CATTATTCTGGAGAATCACACCTCAGGATCGACAATAACA
RNA2-VSR-P14 s	ATCGATTAGGAGATATAACAATGAGTATGGGGATGGTAGA
RNA4-P31 s	ATCGATTAGGAGATATAACAATGGCTGATGGAGAGATATG
RNA4-P31-TGA as	TCACACATTATTCTGGAGAATCAATCGTGATAAAAGACAA
Rz2 as	CATTATTCTGGAGAACTATTCATACGGCAACACTAAAAAC
Rz2 s	ATCGATTAGGAGATATAACAATGGATGTTGTAGGCACTGC
BSBMV TGB1 s	ATCGATTAGGAGATATAACAATGGCGCCAGAACAGCATAA
BSBMV TGB1 as	TCACACATTATTCTGGAGAATCATCTATCCTCGCAAAAGG
BSBV-TGB1 s	AGAGGATCGATTAGGAGATATAACAATGGAAAAGGAGAAGTTGCA
BSBV-TGB1 as	ACTACTCACACATTATTCTGGAGAATCATTAGTTCTTACCATAAT

TABLE S2 List of primers used for RT-qPCR of *Rz2.*

Primer name	Primer sequence 5´>3´
qP-Rz2 s	CAGCAGCAATACACAAGTCCA
qP-Rz2 as	TGATGAATGTAATGGAGCATAGAAATT
BvEF2_E_1+	AGCTGCGAAAATGGTGAAGT
BvEF2_E_1-	AGCGTTGATTTCCCGTGATC
BvGAPDH+	CACCACCGATTACATGACATACA
BvGAPDH-	GGATCTCCTCTGGGTTCCTG

CHAPTER 4: Manuscript II

Interfamily transfer of the sugar beet CC-NB-LRR R gene *Rz2* results in generation of resistance in *Nicotiana benthamiana* plants and confers varying resistance phenotypes when challenged with *Beet necrotic yellow vein virus*

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Author contribution:
Despite initially plant transformation VW conducted all experimental research, analyzed and visualized the data and composed the first draft.

Summary

The generation of transgenic plants is a valuable approach to study disease in more detail if the host plant cannot be used for investigation due to experimental difficulties. In plant resistance research, functional transfer of R genes has only been reported in a few cases in which the target plant subsequently showed resistance to the pathogen without displaying abnormalities or autoimmunity reaction. This is of particular importance for sugar beet since the crop is reluctant to transformation with Rhizobium radiobacter. In the present study, the antiviral R gene, Rz2, originating from sugar beet, conferring resistance against the devastating root infecting Beet necrotic yellow vein virus (BNYVV), was used to perform Agrobacterium tumefaciens mediated leaf disc transformation of the experimental host Nicotiana benthamiana under control of the constitutive Cauliflower mosaic virus (CaMV) 35S promotor. After generation of T3 seeds, homozygous lines were obtained and selected to confer resistance towards BNYVV and preventing systemic infection. Two phenotypes of resistance were identified following mechanical BNYVV leaf inoculation. One transgenic line displayed an extreme resistance (ER) phenotype while the second line showed a hypersensitive response (HR). Expression analysis in the two lines indicates a twofold higher transcript level of Rz2 that might be responsible for the development of the two different resistance phenotypes. The transgenic plants can be used to study downstream signaling following R gene mediated pathogen recognition in more detail and provide information of conserved signaling partners in distinct plant families

Introduction

The plant immune system consists of several layers of defense mechanisms, helping the plant to prevent infection with fungi, bacteria, insects, nematodes and viruses. In the first layer of the innate immune system, pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PPRs) which are located on the cell surface. The recognition of PAMPs by PRRs leads to PAMP-triggered immunity (PTI) and pathogen invasion can be stopped. Another defense pathway is activated, when individual effectors from the pathogen are recognized by disease resistance (R) proteins leading to effector-triggered immunity (ETI). R proteins are specialized proteins that can either directly or indirectly sense effectors segregated by the pathogen, also referred to as *avirulence* determinants (*avr* determinants). ETI usually activates a hypersensitive response (HR) reaction in form of necrotic local lesions which restricts the pathogen attack and systemic spread is inhibited [1,2]. For certain *R* genes, an extreme resistance (ER) has been described that prevents pathogen accumulation at the site of inoculation without any phenotype [3]. In case of viruses, infection is achieved by mechanical inoculation through wounds in the cell surface or by vectors like insects, nematodes, and sometimes fungal cells. Upon infection, viruses are replicating and expressing their proteins intracellularly, which can be recognized by R proteins and virus replication and

movement can be stopped. Most of the known R proteins can be assigned to the class of nucleotide-binding (NB) and leucine rich repeat (LRR) (NLR) proteins. According to their N-terminal domain, *R* proteins can be further classified into two different groups, encoding either a coiled-coil (CNL) or a Toll and interleukin-1 receptor (TNL) domain [4]. Specificity of *R* genes is determined by the LRR-domain at the C-terminal part which is also reflected by the highest variability within all known *R* genes [3,5]. The nucleotide-binding domain in the core of the resistance protein consists of different motifs found as well in cell death signaling in animals and nematodes [6]. In addition to *R* gene mediated anti-viral resistance, RNA silencing serves as a defense mechanism against plant viruses [3]. Triggered by double stranded (ds) RNA, the virus genome will be degraded, and the viral spread is inhibited [5]. Although, viral defense happens mainly in the cytoplasm, recent studies reported about receptor-like kinases located at the cell surface and sensing signals at the extracellular space, being involved in antiviral mechanisms, reviewed in Macho und Lozano-Duran (2019) and Teixeira et al. (2019) [7,8].

Successful functional transfer of *R* genes into a distinct plant family has been reported for about 20 *R* genes, [9,10], with receptor-like proteins (RLP) being the most promising ones. RLPs are localized on the cell surface and most of them lack an intracellular signaling domain, they interact with kinases in the cellular space. Kinases like SUPRESSOR OF BRASSINOSTERIOD INSENSITIVE-1 (SOBIR1) and BRASSINOSTERIOD INSENSITIVE-1 ASSOCIATED KINASE-1 (BAK1) can be found throughout the plant kingdom and are involved in resistance reaction mainly against fungi [11]. The fact that RLPs possess a transmembrane domain and recognize the pathogen on the surface of the cell and the wide distribution of interaction proteins, might be the most reasonable explanation for successful functional transfer of RLPs between plant species. Next to RLPs, NLRs that work in pairs have been functionally transferred between plant species. The TNL pair consisting of “Resistance to *Ralstonia solanacearum*” (RRS1) and “Resistance to *Pseudomonas syringae 4*” (RPS4) proteins were transferred from *Arapidopsis thaliana* (*A. thaliana*) into the model plant *N. benthamiana* as well as into tomato and cucumber [12]. Although several dominant *R* genes involved in virus resistance have been identified, cloned and further characterized, the generation of resistant plants by transfer of *R* genes into a heterologous plant species is limited [5,10]. While the transfer of antiviral *R* genes within the same family into stable transformed plants was reported in several cases, for example for *Rx* [13] or *Sw-5b* [14], interfamily transfer has only be reported twice [15,16]. The first interfamilial transfer of an antiviral *R* gene was reported by Takahashi et al. (2012) [16] who transferred the *A. thaliana RCY1* CNL protein that confers HR resistance against *Cucumber mosaic virus* (CMV), functionally into *N. benthamiana*. In the study of Okano et al. (2020) [15] the non NLR-type resistance type JACALIN-TYPE LECTIN REQUIRED FOR POTEXVIRUS RESISTANCE1 (JAX1) protein, inhibiting potexvirus infection in *A. thaliana*, was functionally transferred to *N. benthamiana*

and the economically important crop tomato, both belonging to the family *Solanaceae.* Even when the classical sign of resistance in form of HR was not observed in the resistance reaction mediated by JAX1, restriction of *Pepino mosaic virus* (PepMV) systemic movement was observed in the transgenic plants.

Sugar beet infection with the soil-borne *Beet necrotic yellow vein virus* (BNYVV), belonging to the family *Benyviridae*, causes a tremendous yield loss up to 80% if not controlled by host resistance. Responsible for the reduction in the sugar yield loss is the size reduction and deformation of the tap roots caused by excessive growth of lateral roots. Systemic infection in form of necrotic and yellowing veins on leaf tissue, however, can only be seldom observed in the field (reviewed in [17]). BNYVV can be found in nearly all sugar beet cultivation areas world-wide and is transmitted by the soil-borne protist *Polymyxa betae* Keskin [17]. BNYVV consists of a multipartite genome with four or five single-stranded, (+)ssRNA components, encoding proteins for replication, transmission, coat-protein, cell to cell movement, suppressor of RNA silencing and the pathogenicity factor P25 [17].

Effective control of this soil-borne disease can only be achieved by using resistant cultivars, carrying either one of the two known the dominant resistance genes *Rz1* or *Rz2* alone or in combination. While the genetic background of *Rz1* is still unknown, the genetic identity and the protein structure of *Rz2* was identified in 2017 by Capistrano-Gossmann et al [18]. *Rz2* encodes for an NLR protein with a CC-domain at the N-terminus. Sugar beets carrying *Rz2* show a stronger resistance towards BNYVV infection compared to *Rz1* plants, as no virus content can be measured in rootlets exposed to viruliferous zoospores [19,20]. In naturally infected sugar beets, BNYVV is mainly restricted to the roots as well as *Rz2* expression which complicates the study of a resistance phenotype [21]. To identify the BNYVV *avr* determinant we therefore applied *Agrobacterium* mediated co-expression of virus genes with *Rz2* in leaf tissue of the experimental host *N. benthamiana* under control of CaMV 35S promotor [22], resulting in the identification of the RNA2 encoded triple gene block protein 1 (TGB1) as *avr* determinant [21]. Co-expression with *Rz2* resulted in rapid leaf tissue necrosis induction. In the current study we aimed to generate a plant system, allowing investigation on the resistance mode induced by *Rz2* and to circumvent the difficulties of sugar beet infection by BNYVV. Transgenic *N. benthamiana* plants expressing *Rz2* under constitutive 35S promotor were obtained by *Agrobacterium* leaf disk transformation and led to the identification of two different types of resistance reaction, connected to the level of transgene expression, following BNYVV inoculation. With the transfer of the resistance gene *Rz2* into the model plant *N. benthamiana* we are now able to study in more detail the resistance mechanisms induced by *Rz2* and identify plant proteins, necessary for plant resistance and seemingly conserved over clades.

Material and Methods

Plant material and growth conditions

N. benthamiana wild-type plants were used for transformation. *Rz2* was isolated from a homozygous sugar beet breeding line (Bvz2) (SESvanderHave). Transformed plants were cultivated under controlled greenhouse conditions (24°C/14 h 18°C/10 h).

Plasmid construction

The binary vector pBIN19 [23] was used as vector for plant transformation. The vector possesses a neomycin phosphotransferase II gene (NPTII) under the control of a nopaline synthase (nos) promotor and terminator. For insert generation, the plasmid pDIVA-Rz2, described by [21] was used which contained the *Rz2* ORF under control of *Cauliflower mosaic virus* (CaMV) derived enhanced 35*S* promotor and terminator. Based on this plasmid, primers were generated to amplify the *Rz2* expression cassette, including the promotor and terminator sequence and adding flanking restriction sites *Kpn*I and *Xba*I (Thermo Fisher Scientific, Waltham, MA, US) for cloning into pBIN19. Sequence for primer design are based on Törjèk *et al.* 2014. The resulting plasmid was named pBIN19-Bv*Rz2*. Generated plasmids were transformed into *E. coli* DH5α followed by plasmid isolation and insert verification by sequencing. pBIN19-Bv*Rz2* was transformed into *A. tumefaciens* C58C1 by electroporation and used for *N. benthamiana* leaf disk transformation.

Leaf disk transformation

In vitro plants were produced from surface-sterilized wild-type *N. benthamiana* seeds and leaf disks of around 1 cm^2 were cut. Disks were incubated for 48 h in agrobacteria suspension, carrying pBIN19-BvRz2. Plant transformation and regeneration was performed after Horsch et al. 1985 [25]. After shoot development of the transgenic plants, plantlets were transferred into sterile soil and kept under greenhouse conditions until replanting was possible.

Plant inoculation

Plant inoculation with a BNYVV infectious cDNA clone consisting of RNA1-4 [26] was performed using *A. tumefaciens* mediated leaf inoculation (agroinoculation) according to 27 [27]. Agrobacterium suspension was adjusted to an OD_{600} to 0.5 in all experiments. Four-to five-week-old *N. benthamiana* plants were used for inoculation with a syringe without a needle, in three replicates and experiments were repeated at least twice. For leaf patch infiltration, plasmids containing full-length infectious cDNA of BNYVV RNA 1-4 and the mRFP labelled RNA2 cDNA clone of BNYVV (BNYVV-mRFP) [28] were used. Transient expression of the BNYVV *avr* determinant TGB1 or the fluorescent marker protein dsRed was achieved by leaf patch infiltration with agrobacteria carrying binary vectors with TGB1 or dsRed under CaMV 35S control [21]. For control, wild-type *N. benthamiana* plants were inoculated with BNYVV

TGB1 and *Rz2* alone and in combination, as well as with BNYVV-mRFP to follow systemic plant infection.

BNYVV inoculation by mechanical rub inoculation was performed with sap from systemically infected leaf tissue of agroinoculated *N. benthamiana* diluted in phosphate buffer (10mM Na_2SO_3, pH 7.0). For agroinoculation, the mRFP labeled infectious clone was used. Plants were rubbed gently on the leaf surface and symptom recording was performed at 4 dpi and 2 weeks after inoculation.

To discriminate between viable and dying cells, *A. tumefaciens* cultures containing a binary plasmid encoding 35*S* driven dsRed 21 [21] were co-infiltrated in transient expression experiment. Dying cells are displaying decompartmentalization which leads to autofluorescence that can be visualized via epifluorescence microscopy using the GFP filter (Emitter D 510/540, 497 Beamsplitter 425 DCLP, and Exciter D 395/40) [29]. Discrimination between viable and dead cells was achieved by examination of plant leaf sections using DsRed filter (Emitter HQ 620/60, 496 Beamsplitter Q 585 LP, Exciter HQ 565/30) and GFP filter.

Plant regeneration and selection

Transformed and regenerated *N. benthamiana* T_0 plants were checked for *Rz2* integration with PCR from total DNA extracts using primer specific for *Rz2* amplification (N.b_Rz2_s (TATCCTTCGCAAGACCCTTC) and N.b_Rz2_as (AAGGGGGTTGGAATCAGAAA) resulting in a PCR product of 416 bp. Therefore, total DNA from each transgenic line was extracted using NucleoSpin DNA extraction kit (Macherey-Nagel, Düren, Germany) and PCR was performed with Phusion High-Fidelity PCR Mastermix (Thermo Fisher Scientific), using 63.5 °C annealing and 10 s elongation. Resistance towards BNYVV infection was assessed by agroinfiltration of BNYVV cDNA clones RNA1-4 or transient expression of BNYVV TGB1. Resistant lines were selected and propagated until T_3 by selfing. Homozygosity of T_3 seeds was determined by a segregation test [30]. Seeds were surface sterilized, and germination was monitored by plating seeds on Murashige & Skoog agar containing 400 mg/l kanamycin. The herbicidal effect of kanamycin causes the plant to turn white after the formation of cotyledons and inhibits further plant development.

Rz2 integration and expression in T_3 lines was verified by specific RT-PCR on total RNA extracted with RNeasy kit (Qiagen, Hilden, Germany). DNase treatment of 1 µg RNA was performed, following RT-PCR with RevertAid Reverse Transcriptase (Thermo Fisher, Scientific) and Random Hexamer Primer. PCR was done using Phusion High-Fidelity Master Mix (Thermo Fisher, Scientific) and primer specific for *Rz2* amplification as followed, Rz2_s ATGGATGTTGTAGGCACTGCG and Rz2_as CTATTCATACGGCAACACTAA. Primer annealing temperature was 58.7°C and elongation 50 sec, resulting in a PCR product with the complete *Rz2* ORF (3492 bp). Furthermore, *Rz2* expression was analyzed using RT-qPCR from total RNA extracts followed by DNase digestion and cDNA transcription as previously

described. RT-qPCR was performed with following primers specific for *Rz2* (qP-Rz2_s: CAGCAGCAATACACAAGTCCA and qP-Rz2_as: TGATGAATGTAATGGAGCATAGAAATT) and housekeeping gene 60S primer (60S-s: AAGGATGCCGTGAAGAAGATGT, 60S_as: GCATCGTAGTCAGGAGTCAACC) [31]. Amplification was performed using the CFX96 TouchTM Real-Time PCR Detection System 520 (BioRad, Hercules, California) using iTaq Universal SYBR Supermix (BioRad). Analysis for relative expression of *Rz2* in the wild-type and transgenic lines was done using the formula $2^{-\Delta ct}$ for each genotype [32].

Results

Generation of *Rz2* transgenic *N. benthamiana* plants

For generation of 35S-*Rz2* transgenic *N. benthamiana* plants by leaf-disk transformation, *A. tumefaciens* harbouring pBIN19-BvRz2 plasmid, expressing NPTII gene under the control of a nopaline synthase (nos) and *Rz2* under control of a CaMV 35S promotor and terminator between left (LB) and right border (RB) was used. In total, 59 T_0 plants were regenerated. All plants were tested positive for integration of *Rz2* via specific PCR. Transgenic plants showed no developmental abnormalities, indicating no detrimental effect of *Rz2* on plant development and growth.

Plant selection

To evaluate symptom phenotypes in different transgenic T_1 lines, 20 lines were randomly selected and 15 plants from each line (L1-L20) were inoculated with BNYVV cDNA clone (RNA1-4) by *Agrobacterium* mediated infiltration.

It was expected that *Rz2* should be constitutively expressed in all organs including leaves in contrast to its source plant *B. vulgaris*. Infection of wild-type *N. benthamiana* using agroinoculation with BNYVV cDNA clones resulted in yellowing of the infiltrated patches at 5 dpi, which developed into slight necrosis after eight days (Figure 1A). Newly emerging leaves became necrotic two weeks after infection (Figure 1B). By applying the mRFP labelled BNYVV cDNA clone, virus expressed mRFP red fluorescence could be visualized with epifluorescence microscopy in the infiltrated patch at four dpi as well as systemically in newly emerging leaves at 14 dpi (data not shown). BNYVV-mRFP symptoms did not differ from those produced by the unmodified recombinant virus as already reported by 26 [26]. When *Rz2* was co-expressed with BNYVV cDNA clone or the *avr* determinant in *N. benthamiana* leaf tissue, yellowing of the leaf patch occurred at 4 dpi, which became necrotic (8 dpi) (Figure 1A). In transgenic plants, two different phenotypes were observed. Several plants reacted similar to wild-type *N. benthamiana* plants that allowed systemic infection with strong necrosis on emerging leaves. The remaining plants displayed no systemic viral symptoms at 20 dpi. No homozygous line was detected after symptom monitoring, as both phenotypes occurred in all transgenic lines. To better visualize virus propagation and movement, the BNYVV-mRFP cDNA clone

was applied for inoculation of five plants of each of the 20 transgenic T_1 lines. In wild-type *N. benthamiana* plants, applied as control, the virus movement in newly emerging leaves was confirmed by red fluorescence detection similar to transgenic lines that did display a susceptibility phenotype. No fluorescence was observed in transgenic plants that showed no typical systemic BNYVV symptoms.

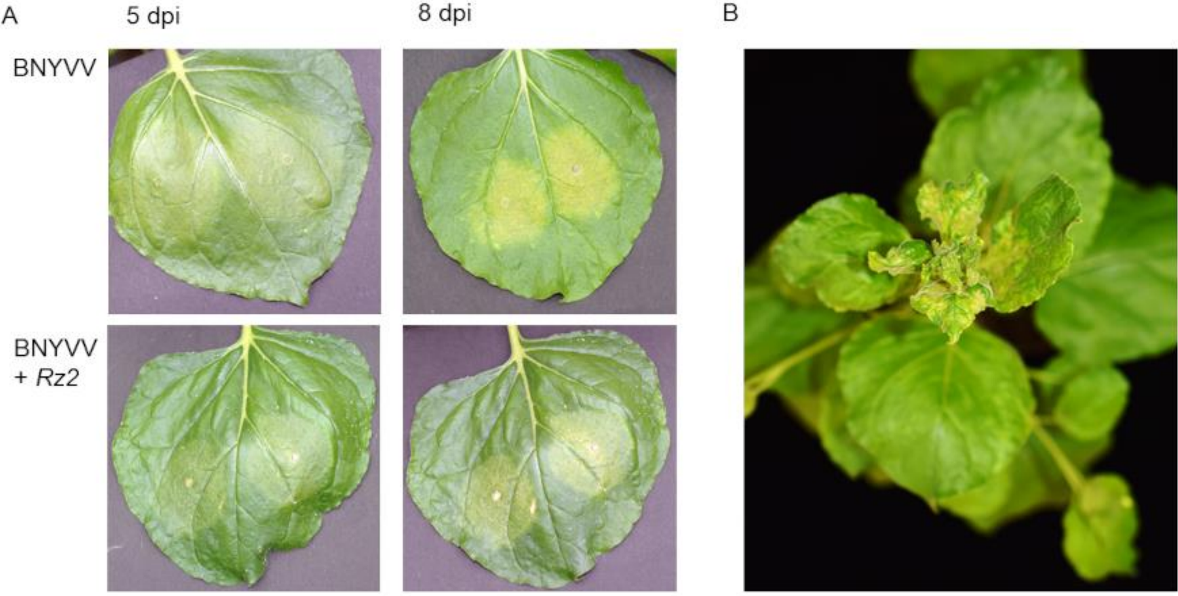

FIGURE 1 Symptom development on wild-type *N. benthamiana* plants inoculated with BNYVV-mRFP cDNA clone.

(A) Local symptom development after leaf agroinfiltration of BNYVV cDNA clone and in combination with 35*S* driven Rz2 at 5 dpi and 8 dpi.

(B) Systemic symptom development of wild-type *N. benthamiana* plants at 21 dpi.

After virus symptom recording, 10 plants showing no virus symptoms and no virus replication mediated fluorescence, were selected to produce seeds of the T_2 generation by selfing. Resistance test towards BNYVV infection was repeated as described for T_1 selection with BNYVV cDNA clone with and without mRFP labelling. A total of 10 individual plants of each line were applied in each resistance test. After symptom recording, again no *Rz2* homozygous transgenic line could be identified, as in all lines phenotypic variation following BNYVV inoculation was observed. In total, seven plants were selected showing no systemic infection and no virus-mediated fluorescence after BNYVV challenging (L11.6.5, L15.12.8, L15.2.3, L11.1.2, L15.2.5, L15.3.1, L11.6.8). From these seven plants, seeds were produced by selfing to generate the T_3 generation.

Evaluation of BNYVV resistance in transgenic T_3 *N. benthamiana* plants

The homozygosity state of T_3 transgenic lines was analyzed using kanamycin selection test in all seven lines generated. Segregation of the trait can be identified by a mixture of green and white plants after development of the first leaf pair. This test confirmed integration of NPTII in

a homozygous state in transgenic lines 11.1.2, 15.2.5 and 15.3.1 (Table 1). Lines 11.1.2. and 15.3.1 displayed homogenous phenotype and were considered to be homozygous. Line 15.2.5 also displayed homozygosity, but seemingly reacted susceptible and was excluded for further analysis as well as all lines displaying heterozygosity. Wild-type *N. benthamiana* seedlings applied as control homogenously displayed kanamycin susceptibility.

The presence of *Rz2* in homozygous transgenic lines 11.1.2 and 15.3.1 was verified by RT-PCR from total plant RNA extracts using primers specific for the complete *Rz2* ORF (Figure 2). For PCR control, the original plasmid (described by 21 [21]) was used. No PCR signal was obtained when wild-type plant extracts was used as template, as well in all controls without Reverse Transcriptase.

Next, both transgenic T_3 lines 11.1.2 and 15.3.1 were evaluated for BNYVV resistance by challenging them by means of agroinoculation with BNYVV. A total of 10 individual plants per transgenic line were inoculated. As control wild-type *N. benthamiana* plants were used for BNYVV inoculation and transient expression of *Rz2* (Figure 1). Yellowing of the infiltrated patches was observed in the transgenic lines challenged with BNYVV-mRFP at 5 dpi (Figure 3). Wild-type *N. benthamiana* plants reacted to agroinoculation with BNYVV cDNA clone (RNA1-4) with first systemic virus symptoms at about 14 dpi in form of mosaic patterns and necrotic lesions on newly emerging leaf tissue (Figure 1). Transgenic lines 11.1.2 and 15.3.1 in comparison developed no systemic virus symptoms 14 dpi after infection and symptom monitoring was ended six weeks after inoculation (Figure 3).

TABLE 1 Segregation analysis of selected transgenic lines (T_3) by kanamycin selection. In total 50 seeds per lines were plated in MS medium agar containing kanamycin. Plants that carry the transgene developed normally, while plants that do not have the transgene turned white. The ration between white and green plant shoots are indicated in percent (%). Only lines which displayed 100% kanamycin resistance or susceptibility are anticipated as homozygous

	WT	11.1.2	11.6.5	11.6.8	15.2.3	15.2.5	15.2.8	15.3.1
Kanamycin resistant plants (in %)	0	100	70	86	2	0	5	100
homozygous	yes	yes	no	no	no	yes	no	yes

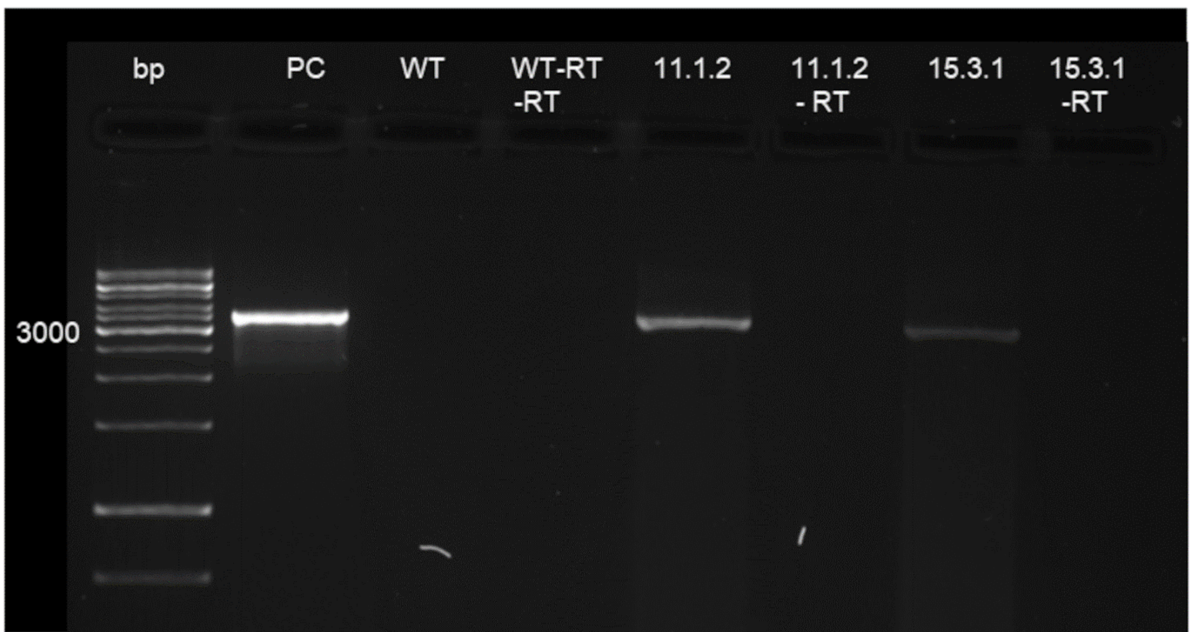

FIGURE 2 RT-PCR verification for transgene integration. Verification of *Rz2* (3492 bp) expression in *N. benthamiana* transformed with *Rz2* using RT-PCR (product size 3492 bp). To exclude DNA contamination in PCR control samples without Reverse Transcriptase (-RT) were included. Positive control contained cloned *Rz2* DNA as template (lane 1). As marker GeneRuler 1 kb DNA Ladder was used. PC – positive control, WT – wild-type *N. benthamiana* plant, 11.1.2 - transgenic line 11.1.2, 15.3.1 - transgenic line 15.3.1.

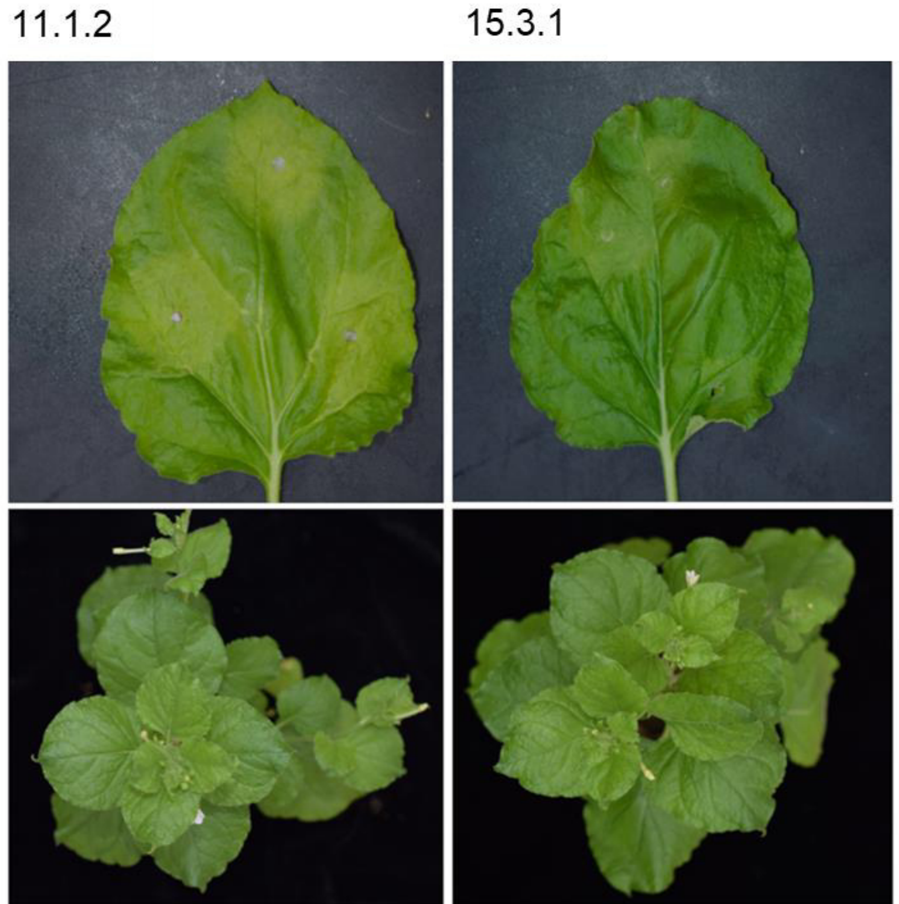

FIGURE 3 Absence of symptom development on *N. benthamiana* transgenic plants 11.1.2 and 15.3.1. Local symptom expression (upper panel) on *N. benthamiana* leaves after agroinfiltration of BNYVV-mRFP cDNA at 5 dpi. Symptom development (lower panel) after agroinoculation of BNYVV-mRFP cDNA at 21 dpi.

For further verification of the functional transfer of *Rz2* into *N. benthamiana*, transgenic lines 11.1.2 and 15.3.1 were used for transient expression by means of agrobacterium mediated patch infiltration with the known *avr* determinant BNYVV TGB1. Five plants of each line were used and infiltrated at least three times. In the transgenic line 11.1.2 all individual plants infiltrated showed a strong HR response already at two dpi, indicating a resistance reaction (Figure 4). Plants of line 15.3.1 showed a slightly delayed HR response resulting in a complete cell death after five days in the infiltrated patch. In wild-type plants agrobacteria mediated expression of *Rz2* and TGB1 resulted in a comparable phenotype three days after infiltration, while expression of the individual proteins did not result in any detectable cell death reaction.

FIGURE 4 Agrobacterium mediated expression of the *avr* determinants results in cell death in transgenic plants. Avirulence determinant TGB1 from BNYVV triggers cell death in presence of transiently expressed or transgenic Rz2. Infiltration of wild-type *N. benthamiana* plants with transiently expressed Rz2 (A), BNYVV TGB1 (B) or co-expression of BNYVV TGB1 and Rz2 (C). Agrobacterium mediated transient expression of BNYVV TGB1 in transgenic *N. benthamiana* lines 11.1.2 (D) and 15.3.1 (E) results in cell death at 3 dpi.

In a second experiment, 15 plants per transgenic line 11.1.2 and 15.3.1 were mechanically inoculated with plant sap from *N. benthamiana* leaf tissue systemically infected with BNYVV-mRFP. Local infection with BNYVV was monitored by epifluorescence microscopy of inoculated leaf tissue and examination of infection foci. A total of three leaves were analyzed per genotype at four dpi (Figure 5). In wild-type *N. benthamiana* plants used as control in average 135,5 infection foci per leaf were detected. In leaves of the line 15.3.1 a mean of 57,3 foci were counted and plants of the transgenic line 11.1.2 showed no infection foci at all in all leaves examined.

Yellow spots, indicative for BNYVV infection, occurred on inoculated leaves of wild-type *N. benthamiana* after 5 dpi; systemic symptoms on new emerging leaves were detected at 11 dpi (Figure 5). On inoculated leaves of line 11.1.2 no yellow spots could be detected and consistently no symptoms of systemic infection on new emerging leaves occurred in all 15 plants inoculated (final timepoint of monitoring six weeks after inoculation). Small infection foci in form of yellow spots were also visible on inoculated leaves of line 15.3.1, indicative for virus replication in this area. Virus replication was verified by fluorescence microscopy (mRFP fluorescence) but at the same time, cell death was detected by a strong autofluorescence signal in the GFP filter (Figure 6). In contrast, in parallel inoculated wild-type *N. benthamiana* leaf tissue, only mRFP expression was detected, indicative for virus replication and genome expression (Figure 6). Although at the onset of infection, virus multiplication was detected in inoculated leaves of line 15.3.1, no systemic infection was observed. This result indicates, that in line 15.3.1 at the beginning of infection, the virus can replicate and move until the resistance mechanism is activated and stops further virus spread, whereas in line 11.1.2 the virus seems to be stopped already in the initially infected cell, as no virus mediated mRFP fluorescence could be detected.

A

	WT	11.1.2	15.3.1
number of infection foci per leaf, 5 dpi	135,3	0	57,3
number of plants systemically infected after mechanical inoculation, 20 dpi	15/15	0/15	0/15

B

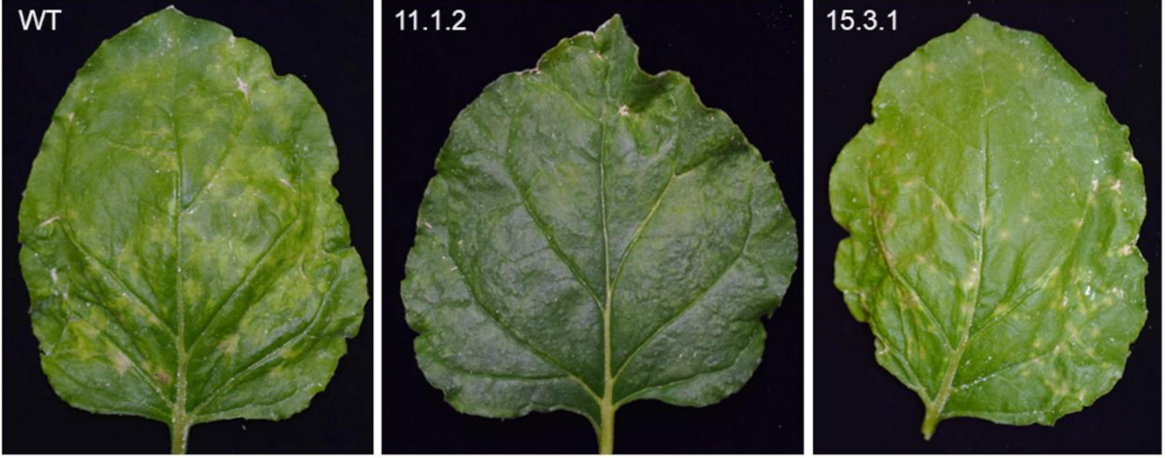

FIGURE 5 BNYVV resistance analysis in *N. benthamiana* plants transformed with *Rz2* and wild-type plants as control BNYVV resistance analysis in *N. benthamiana* plants transformed with *Rz2* and wild-type plants as control. Plants were rub inoculated with sap from systemically infected *N. benthamiana* plants.

(A) Symptom monitoring of control plants and transgenic lines. The number of infection foci counted on three leaves 5 dpi by fluorescent microscopy. The number of systemically infected plants was counted after mechanical inoculation after 20 days. In total, 15 plants were inoculated.

Symptom development on mechanically inoculated leaves of wild-type and transgenic *N. benthamiana* lines at 11 dpi

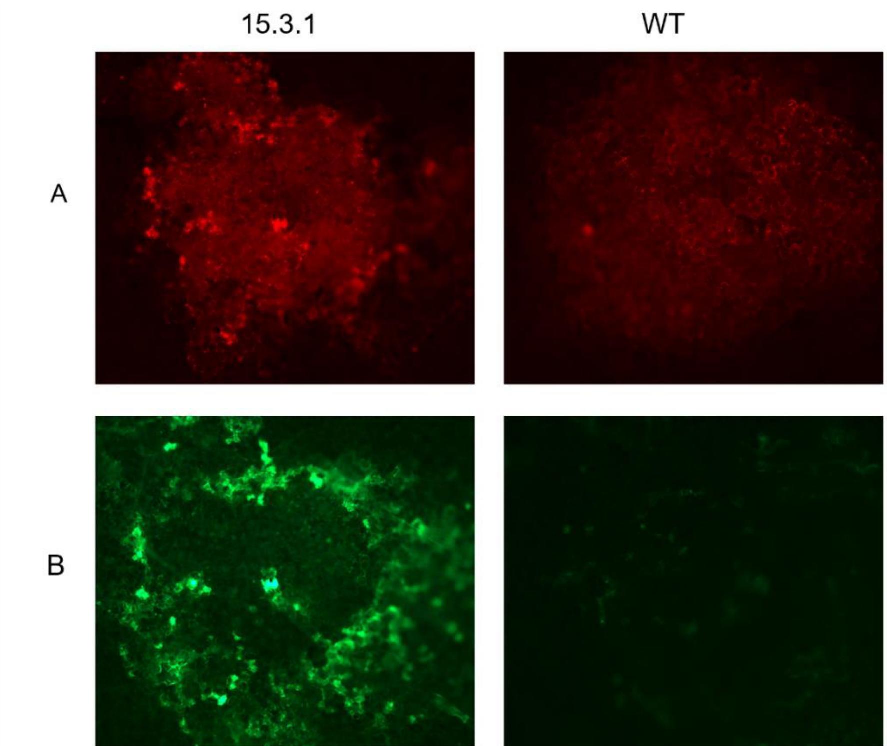

FIGURE 6 Epifluorescence imaging of N. benthamiana leaf samples after mechanical inoculation of BNYVV-mRFP. Wild-type *N. benthamiana* plant and transgenic line 15.3.1 were investigated at 5 dpi, no fluorescence signal was detected in transgenic line 11.1.2 (data not shown). Red florescence indicated virus replication and genome expression(A), whereas green fluorescence indicated cell death (B). Images were taken from the same position with dsRed and GFP filter, respectively.

To find evidence whether different resistance phenotypes observed might be related to the transgene expression level, *Rz2* expression was analyzed by RT-qPCR from total RNA extracts from healthy leaf tissue. In Figure 7 relative expression of *Rz2* in comparison to the wild-type plant is displayed. These data led to the statement, that in transgenic line 11.1.2 the expression of *Rz2* was twofold higher as in transgenic line 15.3.1. In the wild-type plant no *Rz2* transcript was measured by RT-qPCR.

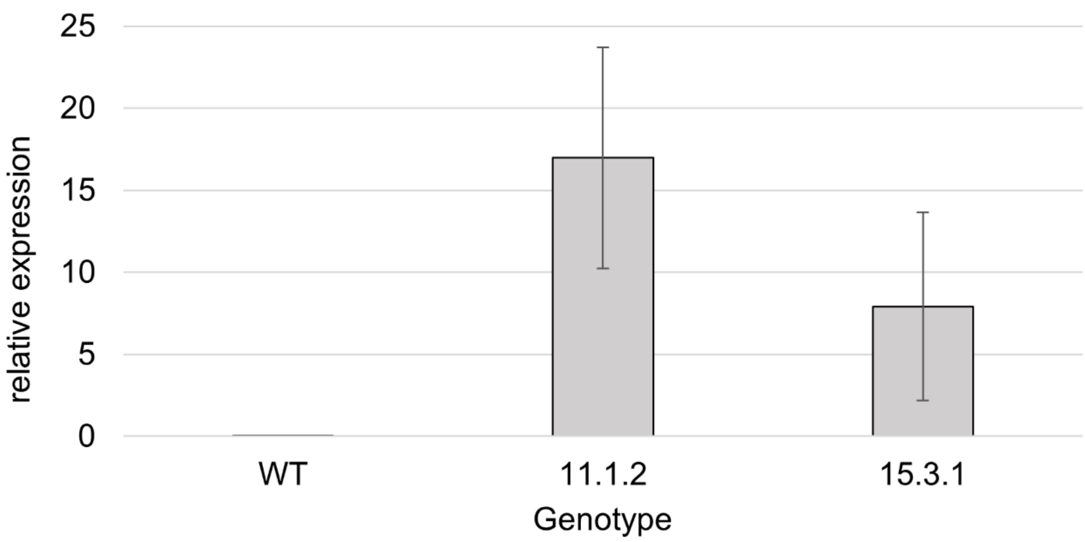

FIGURE 7 Transcript level detection of *Rz2* by RT-qPCR in wild-type *N. benthamiana* and transgenic lines 11.1.2 and 15.3.1. qRT-PCT was performed with *Rz2* specific primer. Normalization of transcripts was performed by amplification of the housekeeping gene 60*S* and calculation of $2^{-\Delta ct}$ for relative expression of the transgene.

Discussion

Plant diseases are a major threat for food supply and with climate change and increasing globalization, control of pests is a major concern worldwide. By transferring plant resistance genes between distantly related plant species that are attacked by the same pathogen species, the genetic resistance resources would increase tremendously. In the present study we have shown that it is possible to functionally transfer the *Beta vulgaris* antiviral *R* gene *Rz2* to the heterologous plant species *N. benthamiana*. As the natural *B. vulgaris* promotor is unknown and *Rz2* expression is mainly restricted to the roots [21] the constitutive CaMV 35*S* was applied to direct *Rz2* expression to all tissues. We verified the ability of *Rz2* to recognize the BNYVV encoded *avr* determinant, the TGB1 protein, resulting in an HR response of transgenic leaf tissue following transient expression in agroinfiltrated leaf patches. Further, in the transgenic plants no systemic virus infection was detected in contrast to wild-type plants after BNYVV infection (agroinoculation with cDNA clone or mechanical virus inoculation). Comparable to our previous findings, the transgene *Rz2* expression resulted in a similar local phenotype compared to the transient expression if challenged with transient *avr* expression or BNYVV full-length clone inoculation by agroinoculation.

To the best of our knowledge, this is the second report of a functional interfamily transfer of an antiviral plant *R* gene belonging to the NLR class [16]. The first *R* gene (*RCY1)* reported, was transferred from *A. thaliana* to the experimental host *N. benthamiana*. Interestingly, the authors reported about dose-dependent auto-defense reaction in *N. benthamiana* following transient expression as well as dwarfism in one transgenic line showing high *RCY1* expression. In the

current study, differences in the phenotype or auto-activation of defense responses in form of cell death were not observed at any time in any line. Although a recent study by Okano et al. (2020) [15] reported the transfer of an elevated resistance level towards PoMV after transfer of an *A. thaliana* gene into tomato, JAX1 does not represent a classical *R* gene as it belongs to the jacalin-lectin type genes and confers resistance to several potexviruses without exhibiting an HR reaction [33]. Resistance by JAX1 is mediated by targeting the replicase and interfering viral replication, but independent of additional host factors [34]. According to Okano et al. (2020) [15] this is the most likely reason for the functional interfamilial transfer.

Besides *RCY1* and *JAX1*, several antiviral *R* genes were transferred between plant families, belonging to RLPs or R proteins working in pairs [10]. Common for most of the R proteins successfully transferred is the fact, that evolutionary conserved proteins are known to be involved in the resistance mechanism as demonstrated for the functionality of the resistance gene *N* against TMV [35,36], *Rx* against PVX [37] and only recently Ry_{sto} mediated resistance against *Potato virus Y* (PVY) [38]. Furthermore, common proteins involved in resistance mediated by *R* genes possessing a TIR domain were reported in several studies [39,40]. As *Rz2* belongs to the class of CNLs, the question arises whether similar conserved pathways exist in CNL mediated resistance which is hypothesized by the current study and former by Maekawa et al. (2012) [41], Takahashi et al. (2012) [16] as well as Tameling und Baulcombe (2007) [42].

Generating resistant plants by gene transfer over host family borders is most often restricted due to unwanted reactions like growth inhibition or missing function in the target plant species [43], also referred to as “restricted taxonomic functionality” [44]. One reason for lacking resistance can be family specific downstream signaling pathways, missing interaction partners like co-receptors, baits or decoys, which are absent in the target species [3]. Furthermore, the undirected integration of the transgene mediated by *A. tumefaciens* transformation, positional effects of the transgene and the number of integrations (copy number) as well as the promotor used to control protein expression [45]. can explain varying transgene effects.

The most important factor influencing the transgenic protein functionality is the expression level of the transgene, which is regulated by several factors, like integration site, number of transgene integration, promotor used as well as transcriptional and posttranscriptional modifications. In the current study, two distinct resistance phenotypes were observed, HR in transgenic line 15.3.1 and ER in line 11.1.2. In case of *Rz2* mediated resistance against BNYVV, it is only known that following natural infection of sugar beet plants, no virus accumulation can be detected in roots [21] and that *Rz2* expression is mainly restricted to root tissue.

The integration site of the transgene an with this the genetic background plays an important role for the functionality of the transgene as stated by Baurès et al. (2008) [46]. The transfer of

the antiviral *R* gene *Rx2* conferring ER towards PVX in the source plant potato, results in HR local cell death in transgenic *N. benthamiana* [47]. It seems that the genetic background plays an important role in the specificity of recognition and the mechanisms of resistance as stated before, leading to different mode of resistance compared to the original source plant [13]. Furthermore, the promotor used to control protein expression is important as shown by Bendahmane et al. (2000) [47]. Transgenic plants expressing *Rx2* directed by the natural promotor resulted in HR while plants expressing *Rx2* controlled by the constitutive CaMV 35S promotor resulted in ER when challenged with PVX. In the current study the unrelated constitutive promotor CaMV 35S promotor was used to ensure protein expression in all transgenic tissues, but still two different phenotypes were observed, meaning that the promotor itself is not the main influence on the occurrence of different phenotypes but rather the integration site. Furthermore, *Rz2* expression analysis demonstrated, that in transgenic line 11.1.2 an *Rz2* mRNA level twice as high as in line 15.3.1 was detected. Our observations are comparable with the study of the resistance gene *RCY1*, mediating resistance against the *Cucumber mosaic virus* (CMV). Different resistance modes were detected after transferring *RCY1,* under control of its natural promotor, from its host *A. thaliana* in susceptible *A. thaliana* plants. Transcription analysis of *RCY1* indicated a 100-fold higher expression in the genotype seemingly to mediated ER towards CMV infection, whereas the transcription was significantly lower in transgenic plants resulting in HR after infection [48]. These results are further confirmed by a study of *HRT* mediated resistance against *Tobacco crinkle virus*. When *HRT* is transformed in susceptible *A. thaliana,* plants showing ER reveal a three to fivefold higher *HRT* mRNA content, compared to genotypes with low *HRT* transcript level exhibiting HR [49]. These findings support the assumption that in case of *Rz2* the phenotype of the resistance, ER or HR, is dependent on the expression level of *Rz2*. Besides the genetic background and the promotor used, the number of transgene integration is influencing the expression level. *A. tumefaciens* driven leaf disk transformation is an undirected transformation technique. This can lead to an integration of the transgene in a region with higher or lower transcriptional activity, leading to different expression levels in the transgenic plants (epigenetic position effects) [50]. Besides epigenetic effects elevated expression level can also occur by multiple insertions of the transgene into the plant genome although *Agrobacterium* mediated transformation is known to result in a low-copy number of the transgene (reviewed in 51 [51]. Furthermore, posttranscriptional gene regulation, like polyadenylation, can influence protein functionality.

Besides the observation that the transcription level of the resistance gene is the main reason for the occurrence of different resistance phenotypes, studies by Bendahmane et al. (1999) [52] using *Rx* and its *avr* of PVX and in a recent study by Grech-Baran et al. (2019) [38] and the PVY resistance gene $Ry_{sto,}$ demonstrated that the quantitative levels of the resistance

protein and the *avr* are important for the outcome of the resistance phenotype. In case of the *Rz2* and TGB1, this was not investigated in the present study.

The presented results highlight the complexity to draw conclusion of resistance characteristics using transgenic plants and how several factors influence the outcome of a resistance mechanism. To verify the ability of *Rz2* to generate an extreme resistance, generating protoplasts from root cells can help to validate the mode of resistance *Rz2* is mediating as ER is acting on single cell level [53,54].

Taken together, this study demonstrates, that in general a transfer of resistance gene can result in a resistant plant variety encoding a CNL, but it needs some prerequisites as stated in the review by Schultink und Steinbrenner (2021) [55]. Knowing that *Rz2* confers resistance to distinct plant virus families by recognizing the movement protein TGB1 (Wetzel et al. 2021) and the ability to transfer Rz2 between plant families, this resistance gene *Rz2* remains an interesting tool to study open questions in terms of plant resistance in future.

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CHAPTER 5: Manuscript III

Interaction studies between the resistance protein Rz2 and the avirulence determinant *TGB1*

Autor´s Original.

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Author contribution:
VW conducted all experiments, analyzed, and visualized the data and composed the first draft.

Introduction

The plant immune system is dependent on a functional interaction between plant sensors recognizing the invading pest and the pathogen itself. The first layer of plant defense relies on pathogen-associated molecular patterns (PAMPs) which are sensed by pattern recognition receptors (PRRs) located on the surface of the cell. Most often, this basal defense is triggered by bacteria, fungi, and nematodes, as they secrete similar effectors or cell-components (for example chitin by insects, flagellin by bacteria) which are sensed by these receptors. After recognition, a defense reaction to stop pathogen invasion is activated, the so-called pathogen-triggered-immunity (PTI). In viral based PTI only double-stranded RNA was identified to trigger resistance responses (Niehl *et al.* 2016). To evade defense, pathogen release effector proteins counteracting PTI which can also be the target of recognition. The second layer of defense called effector-triggered immunity (ETI) is driven by recognition of these effectors in the intercellular lumen. ETI is manly dependent on *R* genes encoding nucleotide-binding site (NB) leucine-rich repeat (LRR) (NB-LRR, short NLR) proteins. These proteins (R proteins) are thought to be the key host factor in ETI resistance and as a consequence of pathogen recognition, cell death at the site of infection, a hypersensitive response (HR), stops the colonization of the host plant. In case of virus derived defense, virus release proteins necessary for virus genome replication or to help the virus to colonize the host, which are sensed by NLRs in direct or indirect interaction. Direct interaction between a plant resistance protein (R protein*)* and its corresponding *avr* determinant was reported for *Sw-5b* and NSm derived from *Tomato spotted wilt virus* (TSWV) (Brommonschenkel *et al.* 2000; Hallwass *et al.* 2014; Peiró *et al.* 2014). Sw-5b binds to a 21 amino acid sequence of NSm with the NB-ARC part and additionally with an integrated domain in the N-terminal part, observed in many *Solanaceae* plants (Li *et al.* 2019; Zhu *et al.* 2017). In comparison, indirect interaction with an additional protein was verified in terms of the resistance gene *N* derived from tobacco against *Tobacco mosaic virus* (TMV) (Burch-Smith *et al.* 2007). The *avr* determinant derived from TMV displays the p50 protein. Interaction between p50 and the N RECEPTOR INTERACTION PROTEIN 1 (NRIP1), a sulfurtransferase located at the chloroplast, resulted in a relocalization of the complex from the cytoplasm and the nucleus, where the N protein interacts with the complex and resistance is activated (Caplan *et al.* 2008). Indirect recognition is based on the biochemical changes of additional host proteins, the guard or decoy proteins (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). While guard proteins are also essential in host function, decoy proteins are only involved in plant immunity (van der Hoorn and Kamoun, 2008). Posttranslational modification, positional changes, cleavage products or even degradation of guardee or decoys can be sensed by R proteins leading to resistance activation (van der Hoorn and Kamoun, 2008). Indirect recognition by R proteins was observed for *Rx* and *Potato virus X* in potato (Farnham and Baulcombe, 2006),

for *RCY1* and *Cauliflower mosaic virus* in *A. thaliana* (Takahashi *et al.* 2012) and for *HRT* recognition of *Turnip crinkle virus* (TCV) also in *A. thaliana* (Kang *et al.* 2008).

In the following experiments, different interaction studies based on protein-protein interaction are described and were performed to investigate the interaction between the *B. vulgaris* resistance protein Rz2 and the *avr* determinant encoded by the TGB1 from BNYVV.

Material and Methods

Yeast two-hybrid

The Yeast two-hybrid (YTH) system is based on the interaction of two proteins and the activation of a reporter gene in the eukaryotic host *Saccharomyces cerevisiae*. YTH is relying on the principle that many transcription activators are build up by two physically interacting domains which are essential for its proper function. Most of them consist of a DNA-binding domain and a transcriptional activation domain. In case of the experimental system YTH, both domains, the DNA binding domain represented by the LexA domain, and the transcriptional activation domain (B42) are separated and can be fused to the proteins of interest (POI). Upon interaction of the POIs the reporter genes are activated. Two reporter genes, LEU2 and GFP, are used in the current study to verify interaction. Activating the reporter gene LEU2 enables yeast cells to grow on selective media missing leucine while the expression of GFP results in fluorescent cells when excited with UV light in the dark. In the current test, the Grow'n'Glow YTH system Mo Bi Tec (Goettingen, Germany) was used together with the yeast strain EGY48. While the reporter gene for GFP (pGNG1, supplied by manufacturer) is encoded on an additional plasmid, LEU2 reporter gene is integrated in the yeast chromosome. Both genes of interest, encoding for *Rz2* and *TGB1*, were cloned in the bait (pEG202, containing the LexA) and the prey (pJG4-5, containing B42) plasmid.

The original vectors were supplied by the manufacturer and treated as indicated. To generate plasmids containing either the open reading frame (ORF) for *TGB1* or *Rz2*, primers were designed to generate the indicated overlaps for restriction sites at the extreme ends of the ORFs. Cloning of *Rz2* into pEG202 vector was performed by attaching restriction sites for *Bam*HI and *Sal*I to the 5'- and 3'-terminal ends of the *Rz2* ORF using PCR. *TGB1* sequence was prolonged with restriction sites for *Eco*RI and *Xho*I for cloning in both target vectors. PCR reaction was performed using Phusion Flash High-Fidelity Polymerase (Thermo Fisher Scientific). After PCR amplification, PCR products were processed with FastDigest® restriction enzymes (Thermo Fisher Scientific) according to manufacturer's instructions. Target vectors (pEG202 and pJG4-5) were treated with the same enzymes as inserts followed by ligation of insert and vector with T4 DNA Ligase (Thermo Fisher Scientific) over night at room temperature. To generate pJG4-5-Rz2, vector pJG4-5 was digested with *Eco*RI and *Xho*I. PCR was performed with *Rz2* ORF as template and primer were designed to generate an overlap

of 20 nt to the vector to perform cloning by Gibson assembly (Gibson *et al.* 2009). New generated plasmids were transformed in chemical competent *E. coli* DH5α for amplification followed by isolation and sequencing before transforming into yeast cells after Gietz and Woods (2002) and modified as described by Thiel and Varrelmann (2009). Therefore, the reporter plasmid containing GFP, pGNG1, and either pEG202-Rz2 or pEG202-TGB1 were transformed into competent yeast cells EGY48, while pJG4-5-TGB1 and pJG4-5-Rz2, respectively, were transformed into yeast strain RFY206. Following yeast mating, interaction between Rz2 and TGB1 as well as autoactivation of the single proteins was studied by plating on selective media and UV light excitation. Dropout base (DOB) media was supplemented with carbon source and amino acid complementation according to Origene DupLEX-A user's manual (Rockville, Maryland, US) either with agar (DOBA) or without (DOB). To grow yeast cells after transformation, growth media with glucose as carbon source was used, missing histidine and tryptophan. Colonies were grown on selective agar containing galactose/raffinose as carbon source and missing uracil, histidine, tryptophan and leucin to test autoactivation as well as interaction. Transcription autoactivation of the reporter genes by Rz2 or TGB1 was tested by mating yeast cells containing the respective gene and the complementary plasmid without insert. After growing colonies on growth media, at least three colonies were selected, and yeast suspension was diluted with $_{dd}H_2O$ up to 10^{-3} and dropped on selective media. Plates were incubated at least for 4 days and recorded. GFP fluorescence was validated using a hand-held long-wave ultraviolet (UV) lamp (Black Ray model B 100 AP, 100 W; UV Products Upland, CA, U.S.A.) and photographs were taken using Nikon camera (Nikon DX, AFS mikro Nikkor 40 mm).

Bimolecular fluorescence complementation assay

To study the interaction of TGB1 and Rz2 *in planta*, the Bimolecular fluorescence complementation assay (BiFC) was used. (Jach *et al.* 2006). The basic idea of this assay is that by physical interaction of two proteins, which are fused to the C-terminal or N-terminal part of monomeric red fluorescent protein (mRFP), the two parts will reconstitute, and a red fluorescence can be detected using fluorescence microscopy. In the following assay, *Rz2* and *TGB1* ORFs were fused to one mRFP part and infiltrated in *N. benthamiana* leaf tissue by *Agrobacterium* mediated infiltration (agroinfiltration) according to Voinnet *et al.* (1998). For the current experiments, plasmids were supplied by Edgar Maiss, University Hannover (Zilian and Maiss, 2011a, 2011b).

All plasmids were generated by Gibson assembly (Gibson *et al.* 2009) and Phusion Flash High-Fidelity polymerase (Thermo Fisher Scientific) according to manufacturer's instructions. Primer design based on the sequence for resistance gene *Rz2* from *Beta vulgaris* published by (Törjèk *et al.* 2014) and *TGB1* sequence published by Laufer *et al.* (2018b). For Gibson assembly, primers were designed to produce a homologous overlap to the insert and vector. Vectors were

amplified with primer 1-8 in Table S1 according to orientation. In the current experiment, the N-terminal part of mRFP was fused to *Rz2* at both extreme ends, whereas the BNYVV *TGB1* was tagged with the C-terminal end of mRFP to both ends of *TGB1*. Following Gibson Assembly, plasmids were verified by sequencing and transformed into *Agrobacterium tumefaciens* C58C1 by electroporation. Both orientations were tested in all possible combinations and autoactivation of Rz2 or TGB1 was tested by coexpression of *A. tumefaciens* cells transformed with the complementary plasmid without fusion protein. As positive control, the Plum pox virus (PPV) coat protein (CP), which is self-interacting and as negative control a deletion mutant of PPV-CP were used (Zilian and Maiss, 2011b).

Co-localization

By labelling two possible interacting proteins with different fluorescent proteins, the cellular distribution of both proteins can be determined and possible chances in distribution after interaction can be visualized. In case of the current study, the fluorescent dyes smRSGFP and mRFP were used for labelling. The resistance protein Rz2 was fused to GFP at the C-terminal and the N-terminal end of the ORF, whereas the same was performed for the *avr* determinant TGB1 and mRFP.

For the generation of translational fusion proteins of Rz2 or TGB1 the fluorochromes smRSGFP (Davis and Vierstra, 1998) or mRFP (Campbell *et al.* 2002) were used and the binary vector pCB301, possessing CaMV 35*S* promotor and terminator (Xiang *et al.* 1999). For cloning, Gibson assembly was used for assembling inserts and vector. Primer were elongated to produce an overlap to the vector sequence of 20 nt and amplification was performed by PCR using Phusion Flash High-Fidelity Polymerase (Thermo Fisher Scientific). *smRSGFP* and *mRFP* were amplified from BSBMV-smRSGFP and BNYVV-mRFP full length clones former produced by Laufer *et al.* (2018a). Integration of the fluorochrome was proven by sequencing and used for further cloning, resulting in plasmids pCB-GFP-Rz2 and pCB-Rz2-GFP for GFP labelling and pCB-mRFP-TGB1 and pCB-TGB1-mRFP. Primer sequence used to generate PCR products for Gibson assembly are listed in Table S1. Following Gibson Assembly, plasmids were verified by sequencing and transformed into *A. tumefaciens* C58C1 to be used for agroinfiltration. *In planta* interaction study of Rz2 and TGB1 were performed by agroinfiltration of four-to five-week-old *N. benthamiana* leaf tissue and development of fluorescence was monitored at different time points. As in one combination cell death developed within a short period, time series experiments were also conducted. In the first step only plasmids carrying Rz2-smRSGFP was agroinfiltrated, and expression was monitored by GFP expression, followed by infiltration of plasmids containing TGB1-mRFP.

Agrobacterium mediated infiltration

In planta interaction study of Rz2 and TGB1 were performed by agroinfiltration of four-to five-week-old *N. benthamiana* leaves and development of fluorescence was monitored two to

seven days post inoculation. *A. tumefaciens* bacteria cultures (strain C58C1) harboring different plasmids were grown over night and OD_{600} was adjusted to 0.5. For coexpression of different proteins of interests under control of 35*S*, bacteria cultures carrying different plasmids were mixed in equal amounts before infiltration. Infiltration of plant tissue was performed as described by Voinnet *et al.* (1998). Experiments contained three replicates and were at least repeated three times. To ensure protein expression, a virus silencing suppressor derived from *Tomato bushy stunt virus* (P19) was coinfiltrated in BiFC experiment (kindly supplied by Edgar Maiss).

Fluorescence microscopy

Fluorescence was monitored by a fluorescence microscope (Leica DMR) using specific mRFP filter (Emitter HQ 620/60, Beamsplitter Q 585LP, Exciter HQ 565/30) or GFP filter (Emitter D 510/540, Beamsplitter 425 DCLP, ExciterD 395/40). Photographs were taken using a LeicaDFC camera, DFC300 FX. Confocal imaging of GFP- and mRFP-expressing leaf tissues was performed using a Leica TCS SP5 confocal imaging system with excitation and emission wavelengths of 488 and 510 to 515 nm for GFP and 514 to 561 and 600 to 630 nm for mRFP. images were processed with LAS-AF software version 2.6.3.8173.

Results

YTH

After cloning and mating, dilution series up to 10^{-3} (lowest dilution shown in Figure 1) were dropped on selective media to study the interaction of Rz2 and TGB1 as well as autoactivation of single proteins (Fig 1). For control of the assay, the provided positive and negative controls were transformed besides *Rz2* and *TGB1*. The positive control, pJG4-5-LTA and pEG202-p53 resulted in colony forming cells and GFP expression was detected, whereas in the negative control almost no cell growth was observed as well as no GFP emission.

	Growth control DOBA glu (-H, -W)	**Autoactivation** DOBA gal/raf (-H, -L, -U, -W)		**Interaction** DOBA gal/raf (-H, -L, -U, -W)	
		Bright light	UV light	Bright light	UV light
pJG4-5 (pEG202)					
pJG4-5-LTA (pEG202-p53)					
pJG4-5-Rz2 (pEG202-TGB1)					
pEG202-TGB1 (pJG4-5-Rz2)					
pJG4-5-TGB1 (pEG202-Rz2)					
pEG202-Rz2 (pJG4-5-TGB1)					

FIGURE 1 Yeast two-hybrid study to test interaction between resistance gene *Rz2* and the *avr* determinant BNYVV *TGB1*. Growth of yeast cells transformed with plasmids containing sequences either for *Rz2* or *TGB1* as well as positive (pJG4-5-LTA with pEG202-p53) and negative control (empty vectors pJG4-5 and pEG202) was tested on dropout base agar (DOBA) supplemented with glucose (glu) and missing histidine (-H) and tryptophan (-W). Autoactivation of Rz2 and BNYVV TGB1 was tested on selective media (DOBA supplemented with galactose and raffinose and missing histidine (-H), uracil (-U), leucine (-L) and tryptophan (-W)) and analyzed in bright light and UV light as well as interaction of the corresponding combination (mating partner indicated in brackets). 10^{-1} dilution is shown.

Autoactivation was analyzed for pEG202-*Rz2* and pJG4-5-Rz2. On these plates, small colonies were detected but no GFP expression. In comparison to autoactivation of TGB1, which resulted in colony growth and GFP expression when cloned in pEG202 and pJG4-5 and mated with empty corresponding vector. This autoactivation was detected in both yeast strains (transformed in EGY48 (pEG202-TGB1) or RFY206 (pJG4-5-Rz2)), indicating a strong transcription activation ability of TGB1. GFP expression of grown colonies were also detected by mating pEG202-Rz2 with pJG4-5-TGB1 and vice versa, indicating either an interaction between Rz2 and BNYVV TGB1 or autoactivation by TGB1 resulted in transcription of reporter genes.

Bimolecular fluorescence complementation assay

In total, agroinfiltration of BiFC vectors into *N. benthamiana* leaves was performed five times. Four days after infiltration, an mRFP signal was detected in the *N. benthamiana* leaf tissue by

epifluorescence microscopy using PPV-CP control, whereas no fluorescence signal was detected in the negative control (Figure 2). Only in one variant, when Rz2 was fused to the N-terminal part of mRFP at its C-terminal end (Rz2-mRFPN) and the C-terminal part of mRFP fused to the N-terminal end of TGB1 (mRFPC-TGB1) resulted in a reproducible fluorescence in two of the five repetitions (Figure 2).

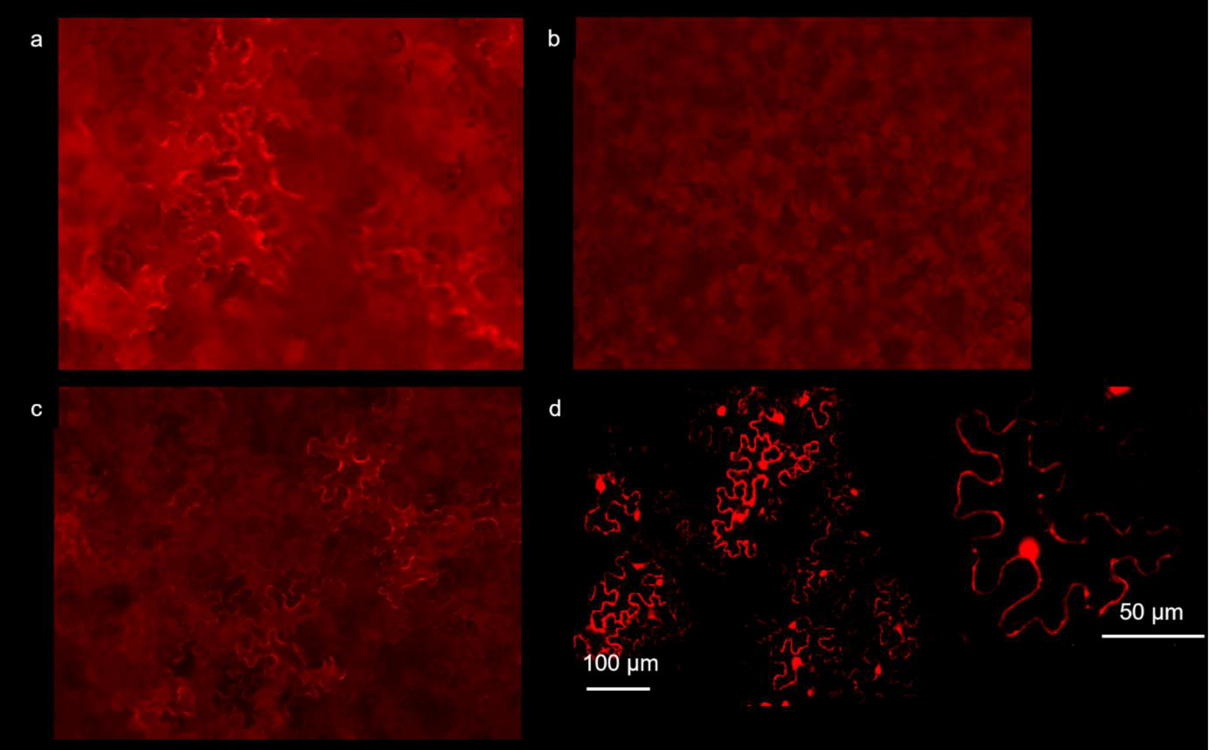

FIGURE 2 Results of Bimolecular fluorescence complementation (BiFC) of mRFP-fusion to Rz2 and TGB1. Visualization following transient *A. tumefaciens*-mediated expression in *N. benthamiana* leaves was monitored by epifluorescence microscopy (a, b, c, 10x magnification) or confocal laser scanning microscopy (d). a: positive control pCB-mRFPN-PPV-CP and pCB-mRFPC-PPV-CP at 5 dpi (Zilian and Maiss, 2011b); b: negative control pCB-mRFPN-CP-F3 + pCB-mRFPC-CP-F3 at 5 dpi (Zilian and Maiss, 2011b); c: Rz2-mRFPN+mRFPC-TGB1 3 dpi; d: Rz2-mRFPN+mRFPC-TGB1 at 4 dpi.

Up to three days after infiltration a fluorescent signal was detected, indicating a direct interaction between Rz2 and TGB1. Even when the development of a fluorescent signal indicated a direct interaction, no local cell death was observed in this protein variants in comparison to wild-type proteins without fluorescence tag.

Co-localization

Labelling of Rz2 and TGB1 resulted in an expressed fluorescence after infiltration into *N. benthamiana* leaves. Both proteins were detected after tagging (Figure 3). In case of Rz2 the C-terminal labelling resulted in a strong fluorescence distribution, which is mainly visible at

the cytoplasm and cell wall. Tagging Rz2 at the N-terminal end (smRSGFP-Rz2) showed only single cells emitting fluorescence.

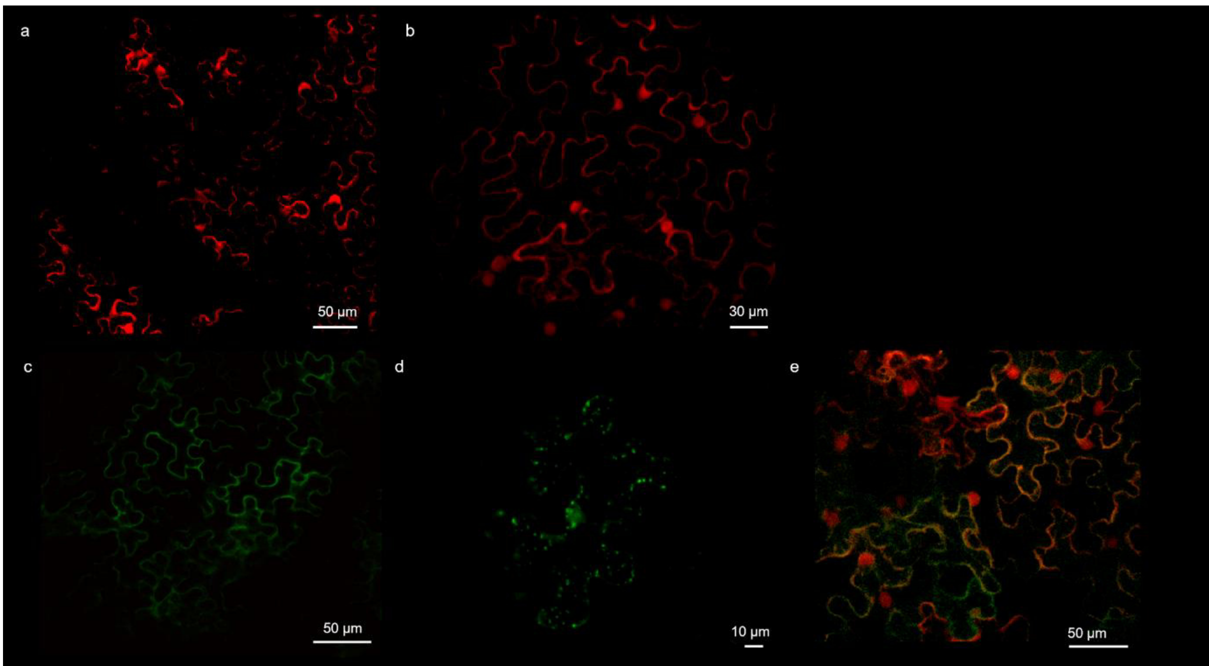

FIGURE 3 Subcellular localization following transient *A. tumefaciens* mediated expression of *Beta vulgaris Rz2* and BNYVV *TGB1* fused to *smRSGFP* and *mRFP*, respectively. Images were taken by confocal laser scanning microscopy with specific filters at 3 days after infiltration of epidermal leaf tissue of (a) TGB1-mRFP (b) mRFP-TGB1 (c) Rz2-smRSGFP (d) smRSGFP-Rz2 (e) colocalization of Rz2-smRSGFP + mRFP-TGB1.

As the fluorescence occurred rather dot-like and not homogenous throughout the cell, this indicates a different localization probably within different organelles. This suggested that tagging Rz2 at both extreme ends affected its subcellular localization. Due to this, variant smRSGFP-Rz2 was excluded from later experiments and only Rz2-smRSGFP was used in coexpression studies. Tagging of TGB1 resulted in both orientations in a visible signal. The fluorescent signal suggested a localization of TGB1 in both variants in the cytoplasm and the nucleus. In the combination Rz2-smRSGFP and mRFP-TGB1, subcellular localization of both proteins was observed. Both proteins were located in the same cell compartments as in single infection (Rz2 in cytoplasm and TGB1 in the cytoplasm and nucleus). Appearing yellow fluorescence indicated the presence of both proteins in the same cell, but the fact that no cell death occurred implies a functional impairment. In contrast, the combination between Rz2-smRSGFP together with TGB1-mRFP resulted in the expected reaction in form of an HR response (Figure 4).

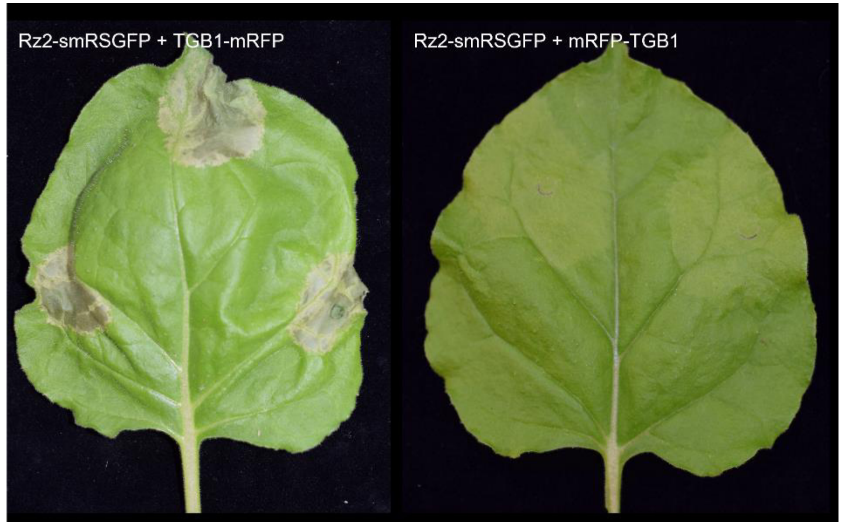

FIGURE 4 HR development after coexpression of Rz2 and TGB1 is dependent on the orientation of the fusion fluorescent protein. Coexpression of Rz2-smRSGFP and TGB1-mRFP results in a strong HR after 3 dpi, whereas coexpression of Rz2-smRSGFP and mRFP-TGB1 showed no resistance response.

Cell death occurred before cellular distribution of Rz2 and TGB1 were detected. In comparison, to protein coexpression without fluorescent tag, cell death appeared in a much shorter timeframe. Therefore, time series of infiltration were performed. Agroinfiltration of Rz2-smRSGFP was performed and TGB1-mRFP was infiltrated after 24 hrs, 30 hrs, and 34 hrs, respectively. In all cases, no TGB1-mRFP1 expression in form of detectable fluorescence was observed. But still, cell death developed in all cases. These results made it impossible to determine the cellular co-localization of both proteins in the same cell to answer the question whether localization will change when bot proteins are present.

Discussion

Plant resistance relies on the recognition of the pathogen followed by a downstream signaling and restriction of the pathogen. Direct recognition of the pathogen relies on a physical interaction between the effector and the R protein, whereas indirect recognition involves changes in host proteins caused by pathogenic effectors (Jones and Dangl, 2006; Moffett, 2009; van der Hoorn and Kamoun, 2008). Direct interaction of R proteins was verified in case of the flax *L* locus and *M* resistance genes (Catanzariti *et al.* 2010; Dodds *et al.* 2006) , the rice *Pi-ta* and the allelic variants from Pik against *Magnaporthe grisea* (Jia *et al.* 2000; Kanzaki *et al.* 2012; Zhai *et al.* 2014), RRS1 and RPP1 from *A. thaliana* (Deslandes *et al.* 2003; Krasileva *et al.* 2010), *Sr35* from wheat against the fungal pathogen *Puccinia graminis* f. sp. (Salcedo *et al.* 2017) and *Sw-5b* against TSWV (Li *et al.* 2019; Zhu *et al.* 2017). But for most pathosystems, indirect recognition is more likely. Indirect recognition is mediated by additional proteins acting as guardees or decoys. Both protein variants interact with the effector protein

and as a result modification of the guardee/decoy is sensed by the NLRs leading to a resistance mechanism (Sun *et al.* 2020; van der Hoorn and Kamoun, 2008). In the review of Sun *et al.* (2020) properties of different NLR-interacting proteins are described, and the authors classify the interacting partner into seven major classes. Interestingly to note is that one NLR can interact with several proteins and vice versa, demonstrating the versatility of NLRs in pathogen defense and how important fine-tuning of this system is (Sun *et al.* 2020).

The *in vivo* YTH system was previously reported to investigate interaction between a plant R protein and its corresponding *avr* determinant (Catanzariti *et al.* 2010; Deslandes *et al.* 2003; Dodds *et al.* 2006; Jia *et al.* 2000; Kanzaki *et al.* 2012). One major drawback of YTH is the generation of false-positive results by proteins that activate transcription of the reporter gene in the absence of the interaction partner. Autoactivation was observed when TGB1 was fused to the transcription activation domain as well DNA binding domain. Because of this, YTH was not suitable to investigate protein-protein interaction in this case. A functional domain of TGB1 is a viral RNA helicase which is involved in RNA replication (Koonin and Dolja, 1993). Furthermore, TGB1 is known to be involved in cell-to-cell movement by binding viral genomic RNA (Makarov *et al.* 2009). *In silico* analysis (https://www.med.muni.cz/9aaTAD/, (Piskacek *et al.* 2016), data not shown) of BNYVV TGB1 indicated a transcription activation domain (YDVVTIILE, amino acid 316 -324, based on acc. number KX665537.1). This domain is missing in TGB1 derived from *Barley stripe mosaic virus* (BSMV) and *Potato mop-top virus*, which were not resulting in an autoactivation when used in YTH (Cowan *et al.* 2002; Lim *et al.* 2008). If this domain is responsible for autoactivation or the viral RNA helicase variant from BNYVV itself needs further investigation.

Interaction between the fungal pathogen *Puccinia graminis* f. sp*. tritici* and in particular its avirulence gene *AvrSr35* and the plant resistance gene *Sr35* was investigated by using the *in planta* system BiFC (Salcedo *et al.* 2017). This model system, compared to YTH, has one major advantage since the interaction can be studied under physiological conditions and no heterologous host (yeast) is required to study protein-protein interaction (Zilian and Maiss, 2011a). Moreover, BiFC displays a very sensitive method to study protein-protein interaction in real time in living plant cells. One drawback of this technique is that the tagging of the POI might influence the interaction and mobility of the formed complex which can influence its biological function (Miller *et al.* 2015). In the current study, direct interaction of the two proteins was detected by reconstituted fluorescence using BiFC when Rz2 was tagged with the N-terminal part of mRFP at the C-terminal end and the C-terminal part of mRFP at the N-terminal part of TGB1. The fluorescent signal was visualized in the cell nucleus and the cytoplasm of the cell. However, the biological property of the formed complex seemed to be disrupted since after infiltration no HR was detected in the infiltrated leaf tissue.

By using different fluorochromes to highlight POI it is possible to visualize the localization of both proteins alone, in presence of the other proteins, and possible re-localization. In the present study, labeling Rz2 with GFP and TGB1 with mRFP permitted to analyze the localization of both proteins in the cell. In case of Rz2, labeling the N-terminal part of the protein, smRSGFP-Rz2, resulted in single cell fluorescence, while tagging the C-terminal part resulted in green fluorescence throughout the infiltrated area. Results indicated the localization of Rz2 in the cytoplasm. Cellular localization of distinct R proteins was determined before and summarized by Wang and Balint-Kurti (2015). Remarkable is, that most R proteins were localized in cytoplasm and/or the nucleus which is in accordance with the present study. Tagging of TGB1 with the monomeric red fluorescent protein (mRFP) was possible in both orientations. In the study of Li *et al.* (2018) BSMV TGB1 was mainly located to the cytoplasm, but also accumulated in the nucleus. These distributions, independent of the integration site of mRFP, was also observed in the current work. As observed in coexpression experiments with Rz2 and TGB1 without translational fusions, coexpression of Rz2-GFP and TGB1-mRFP resulted in an HR response (Wetzel *et al.* 2021). This reaction occurred in a very short time frame after *Rz2* and *TGB1* expression that it was not possible to observe *TGB1* driven expression of mRFP. Furthermore, it was not possible to determine direct interaction of Rz2 and TGB1 and in which cell compartments interaction occurred. Cell death was not detected when TGB1 was tagged at the N-terminal part. This observation is in accordance with the result of BiFC, where the reconstituted mRFP using the N-terminal fused TGB1 was visible after coexpresison but no HR occurred. Even, when recognition of TGB1 is still possible (BiFC fluorescence), the functionality of the complex (Rz2-TGB1) seems to be disrupted (no HR). As this is not occurring when TGB1 is tagged at the N-terminal part, the C-terminal tagging is assumed to be responsible for this observation, leading to non-valid results.

A well-studied example of an R protein interaction is Rx1 with the *avr* PVX CP. The inactive state of Rx1 is achieved by intramolecular interactions between the N-terminal coiled-coil domain and the nucleotide-binding domain of the resistance protein (Moffett *et al.* 2002; Rairdan *et al.* 2008; Slootweg *et al.* 2013). In absence of PVX, Rx1 interacts with the Ran GTPase-activation protein 2 (RanGTP2) (Sacco *et al.* 2007; Tameling and Baulcombe, 2007). In presence of PVX, RanGTP2 is association with PVX CP leading to a disruption of the intramolecular interaction of *Rx1* and the exchange of ADP to ATP and Rx1 is activated (Sacco *et al.* 2007; Tameling and Baulcombe, 2007). To elicit a resistance response, Rx1 must be present in the cell cytoplasm as well as in nucleus as shown by Slootweg *et al.* (2010). It also has been shown that activated *Rx1* is interacting with transcription factors leading to DNA binding and defense gene expression is enabled (Fenyk *et al.* 2015; Townsend *et al.* 2018). If similar pathways are activated in case of Rz2 and BNYVV TGB1 remains under question and the applied experiments have not contributed to a more detailed knowledge. However, using

the whole fluorochromes for protein labeling, the subcellular localization of the proteins was possible. Moreover, the initiation of HR, as observed for untagged proteins, was still functional, although it occurred rapidly. This leads to the assumption, that recognition and downstream signaling in case of Rz2 and TGB1 happens fast. In total, no method used in the present study was suitable to answer the question whether the resistance protein Rz2 and BNYVV TGB1 interact directly or indirectly. However, it could be shown by co-localization experiments that labeling of Rz2 and TGB1 resulted in cell death, indicating the functionality of the interaction. In future, the localization of the single proteins can be analyzed by coexpression of cell-markers (Nelson *et al.* 2007) and fluorescent labeled proteins.

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TABLE S1 List of primer used for indicated experiments.

Primer name	Primer sequence 5´>3´
Yeast two-hybrid	
pJG4-5-Rz2_s	GATTATGCCTCTCCCGAATTCATGGATGTTGTAGGCACTGCGC
pJG4-5-Rz2_as	GTCCAAAGCTTCTCGAGTCGGCCGCTATTCATACGGCAACAC
RNA2-TGB1-EcoRI s	ATCGAATTCATGGTCCAAGTACAGCGTA
RNA2-TGB1-XhoI as	TGACTCGAGTCATCTATCTTCGCAAAAA
pEG202-Rz2-BamHIs	ACCGGATCCATGGATGTTGTAGGCACTG
pEG202-Rz2-SalI as	ACTGTCGACTCATTCATACGGCAACACT
Bimolecular fluorescence complementation assay	
pCB:GOI-mRFPC_s	GGAGGTGGATCTGGTGGAGGTA
pCB:GOI-mRFPC_as	TGTTATATCTCCTTCGAAGATCT
pCB:mRFPC-GOI_s	TAAGGATCGATCCTCTAGAGTCC
pCB:mRFPC-GOI_as	GTCGACTTTGGATCCACCTCCAC
pCB-mRFPN-GOI_s	TAGGTCCGCAAAAATCACCAG
pCB-mRFPN-GOI_as	TCCACCTCCACCAGATCCACCTC
pCB-GOI-mRFPN_as	TGTTATATCTCCTTCGAAGATCT
pCB-GOI-mRFPN_s	GGAGGTGGATCTGGTGGAGGTA
pCB:BN-mRFPC_s	GATCTTCGAAGGAGATATAACAATGGTCCAAGTACAGCGTAGAACG
pCB:BN-mRFPC_as	AGTACCTCCACCAGATCCACCTCCTCTATCTTCGCAAAAAGTATC
pCB:mRFPC-BN_s	TGGAGGTGGATCCAAAGTCGACATGGTCCAAGTACAGCGTAGAAC
pCB:mRFPC-BN_as	GCGGACTCTAGAGGATCGATCCTTATCTATCTTCGCAAAAAGTATC
Rz2-mRFPN_s	GATCTTCGAAGGAGATATAACAATGGATGTTGTAGGCACTGCG
Rz2-mRFPN_as	CCTCCACCAGATCCACCTCCTTCATACGGCAACACTAAAAAC
mRFPN-Rz2_s	GGTGGATCTGGTGGAGGTGGAATGGATGTTGTAGGCACTGCG

Co-localization	
mRFP-GOI_s:	CATACGATGTTCCAGATTACGCTATGGCCTCCTCCGAGGACGTC
mRFP-GOI_as	CCTCCACCAGATCCACCTCCGGCGCCGGTGGAGTGGCGGCCC
GOI-mRFP_s	CATACGATGTTCCAGATTACGCTATGGCCTCCTCCGAGGACGTC
GOI-mRFP_as	GCGGACTCTAGAGGATCGATCCTTAGGCGCCGGTGGAGTGGCGGCC
GOI-GFP_s	ACGATGTTCCAGATTACGCTATGAGTAAAGGAGAAGAACT
GOI-GFP_as	GCGGACTCTAGAGGATCGATCCTTATTTGTATAGTTCATCCATGC
GFP-GOI_s	CCCATACGATGTTCCAGATTACGCTAGTAAAGGAGAAGAACTTTT
GFP-GOI_as	ATCCACCTCCACCAGATCCACCTCCTTTGTATAGTTCATCCATGC
GFP-Rz2_s:	GGAGGTGGATCTGGTGGAGGTATGGATGTTGTAGGCACTGCGC
GFP-Rz2_as	CTGGTGATTTTTGCGGACTCATTCATACGGCAACACTAAAAAC
Rz2-GFP_s	GATCTTCGAAGGAGATATAACAATGGATGTTGTAGGCACTGCG
Rz2-GFP_as	GTACCTCCACCAGATCCACCTCCTTCATACGGCAACACTAAAAAC
mRFP-TGB1_s	GGTGGATCTGGTGGAGGTGGATCCATGGTCCAAGTACAGCGTAG
mRFP-TGB1_as	GGACTCTAGAGGATCGATCCTTATCTATCTTCGCAAAAAGTATC
TGB1-mRFP_s	GATCTTCGAAGGAGATATAACAATGGTCCAAGTACAGCGTAGAACG
TGB1-mRFP_as	AGTACCTCCACCAGATCCACCTCCTCTATCTTCGCAAAAAGTATC

CHAPTER 6: Manuscript IV

In search of resistance-breaking variants of the avirulence determinant against Rz2

Author`s Original.

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Keywords**:** *Beet necrotic yellow vein virus*, R protein, resistance-breaking, NGS

Author contribution:
VW conducted all experiments, analyzed, and visualized the data and composed the first draft.

Introduction

An effective and durable pathogen resistance in crop plants is a long-term objective for farmers as well as breeding companies. Based on the gene-for gene hypothesis by Jones and Dangl (2006), where a single plant resistance gene (*R* gene) recognizes a single pathogen avirulence (*avr*) determinant resulting in a resistance reaction, the durability of this resistance can hardly be predicted. Resistance, where a single *R* gene is responsible for the resistance mechanisms, is called qualitative resistance. Durability is defined as an effective control over a long period of time over a pathogen in its favorable conditions. Consequently, loss of durability is achieved by the occurrence of a pathogen variant which can no longer be recognized by the *R* gene product (resistance protein). Point mutation within the *avr* determinant, deletions of a whole gene or even gene expression modifications enable the pathogen to evade *R* gene mediated resistance. While some *R* genes, like *Rz2* used in the present study, appear to achieve resistance alone, others work in pairs or require additional factors to recognize and restrict pathogen colonialization. As stated by Brown (2015) the durability of a single-gene resistance can be overcome quickly by the pathogen and can last less than a decade. Reasons for this are large pathogen population size, high mutations rates in short generation times and high selection pressure by using genetically identical host plants (McDonald and Linde, 2002). For most of the pathogens, resistance-breaking variants are associated with fitness penalties. Stated by McDonald and Linde (2002), resistance-breaking is mainly dependent on the evolutionary potential of the pathogen rather the nature of the resistance gene. While the durability for qualitative resistance is expected to be low, the durability of quantitative resistance, where the resistance mechanism is based on multiple genes with minor effects, is thought to be more effective as the pathogen needs to adapt to several resistance mechanisms. Quantitative resistance genes are often mapped on genomic regions, quantitative trait loci (QTL), inheriting several additive effects to increase the resistance level. Reasons for a more durable resistance mediated by quantitative resistance are the fact that the pathogen needs to break several mechanisms to evade resistance, compared to resistance mediated by an *R* gene. But still, some *R* gene-mediated resistance are known to be durable, for example the *Pvr4* gene against *Potato virus Y* (PVY) in pepper being used in commercial varieties for almost 30 years (Janzac *et al.* 2010). *In vitro* generation of resistance-breaking variants in the *avr* determinant, the RNA-dependent RNA polymerase NIb, seems to result in a loss of recognition (Janzac *et al.* 2010). But passaging the resistance-breaking strain in susceptible plants indicate a high fitness cost of the virus (Janzac *et al.* 2010). The only way for the virus to be able to survive and compete with other viral strains, was by mutation of the former changed mutation back to the “original” sequence (Janzac *et al.* 2010). Although viruses are known to have a high mutation rate due to missing control mechanisms, the complex genome structure, often with overlapping ORFs and multifunctional proteins, mutations can

lead either to lethal effects or resistance breaking (Harrison, 2002). Moreover, it is assumed that compared to other pathogens, breaking resistance in virus disease is observed seldom, as stated by Harrison (1981) and Janzac *et al.* (2010).

Most *R* genes encode nucleotide binding and leucine-rich-repeat (NLR) proteins either possessing a Toll-Interleukin-1-receptor (TIR) or a coiled-coil (CC) domain at their N-terminal end, but other proteins responsible for resistance are also known, reviewed in Ronde *et al.* (2014). The resistance gene *Rz2*, used in the present study, was identified as an NLR protein, conferring resistance to the devastating sugar beet infecting virus *Beet necrotic yellow vein virus.* Being a member of the NLR´s, *Rz2* encodes a coil-coiled-nucleotide binding and leucine-rich repeat domain (CC-NB-LRR) protein (Capistrano-Gossmann *et al.* 2017). Up to date, two different dominant, monogenic resistance genes are used to control BNYVV in sugar beet cultivation, *Rz1* and *Rz2*. As *Rz1* has been introduced into cultivars in the 1990´s, the first resistance-breaking BNYVV strains were identified in the early new century (Liu *et al.* 2005). Meanwhile, a resistance gene, *Rz2*, has been identified as a monogenic dominant resistance gene leading to even higher resistance in infected plants (Peltier *et al.* 2008). The genetic background of *Rz1* has not been identified, but as resistance-breaking strains show a high variability in the P25 proteins of BNYVV, the corresponding avirulence gene is known (Acosta-Leal *et al.* 2008; Acosta-Leal *et al.* 2010; Chiba *et al.* 2011; Koenig *et al.* 2009; Liu and Lewellen, 2007; Schirmer *et al.* 2005). BNYVV isolates containing a fifth RNA (P- and J-types) were reported to overcome the *Rz1* resistance (Bornemann and Varrelmann, 2013). In former works of the current study, the *avr* determinant towards *Rz2* was identified as the triple gene block protein 1 (TGB1) located on RNA2 of the virus by using a transient coexpression of virus encoded proteins and *Rz2* (Wetzel *et al.* 2021). In field experiments with varieties possessing different resistance levels towards BNYVV, the evolution of virus diversity was investigated over the growing season (Galein *et al.* 2018). The study indicated that in regions where the BNYVV P-type is prevalent, virus content was detected in plants carrying *Rz1* and *Rz2* in combination (Galein *et al.* 2018). Moreover, two reports at the American Society of Sugar Beet Technologist in 2019 (ASSBT) indicate the occurrence of resistance-breaking strains towards *Rz2* in North America (American Society of Sugar Beet Technologists, 2019). One reported about elevated ELISA values in plants expressing *Rz1* and *Rz2* and the authors were planning to evaluate RNAseq to identify possible mutation. And a second report indicated single mutation leading to an amino acid change at position 326 from glutamate to lysine (Q326K). Although in both reports resistance breaking of *Rz2* is indicated, the results were not validated, and neither has it been tested whether virus strains exist evading *Rz2* resistance.

In the present study, different sequencing approaches were used to identify resistance-breaking strains of BNYVV towards *Rz2*. Several point mutations found were introduced into

the TGB1-expression vector to study possible reaction in coexpression experiments with isolated *Rz2* in *N. benthamiana* leaf tissue by transient *Agrobacterium*-mediated infiltration (agroinfiltration) (Wetzel *et al.* 2021). In total five SNP were tested for their ability to cause cell death in presence of *Rz2*.

Material and Methods

Virus, soil and plant material

An infectious full-length cDNA clone of BNYVV (RNA1-4) was used to perform *Agrobacterium tumefaciens* mediated inoculation (agroinoculation) (Laufer *et al.* 2018). For agroinoculation, the experimental host *Nicotiana benthamiana* (*N. benthamiana*) was used. Homozygous sugar beet breeding lines, containing homozygous *Rz1* (*Rz1/Rz1/rz2rz2*) or *Rz2* (*rz1/rz1/Rz2Rz2*) were obtained from SESvanderHave for resistance test and for isolation of viral RNA to amplify *TGB1*. As susceptible variety, KWS03 (KWS, Einbeck, Germany) was used. Following soils were used in the current study, BNYVV P-type infected soil obtained from Pithiviers, France (Pferdmenges *et al.* 2009) and soil from Bondaroy 2014, Belgium obtained from SESvanderHave (Galein *et al.* 2018).

RNA extraction and cDNA synthesis

Root tissue of plants used for all experiments were harvested at 42 dpi. Total RNA was extracted using NucleoSpin RNA plant kit (Macherey-Nagel, Düren, Germany) and cDNA was produced by reverse transcription using Oligo(dT) primer (Thermo Fisher Scientific, Waltham, Massachusetts, US) and RevertAid H minus reverse transcriptase (Thermo Fisher Scientific). PCR of cDNA was performed with Phusion Flash High-Fidelity Polymerase (Thermo Fisher Scientific) using primers indicated in each experiment (Table S1).

Plant infection

Different methods to infect sugar beet plants were used in the study. First, naturally infected soil was used. Moreover, plants used for amplicon sequencing (only susceptible varieties) were infected by mechanical BNYVV cDNA clone infection using the method described by Liebe *et al.* (2019). For each experiment, 12 plants of each genotype were used.

Amplicon sequencing

Investigation of the *TGB1* variability in plants grown in naturally infected soil, originated from Pithivier region, was performed by amplicon sequencing. Plants were grown for 42 dpi, root material was harvested and virus infection was measured by DAS-ELISA. Samples used for sequencing were selected according to their virus level. After RNA extraction and cDNA synthesis as described above, PCR products for deep sequencing were produced. As nucleotide size is limited in this method due to quality score, the *TGB1* open reading frame (ORF) was divided in three amplicons of 421 nt, 447 nt and 460 nt in size, respectively. In the

first PCR, the universal tag was added to the sequence of the PCR product of interest (indicated in Table S1). After clean-up using NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel), individual tags for identification of the amplicons were attached by PCR (primers are given in Table S1) using the same primer in forward direction for all samples and sample individual primers for reverse direction. To exclude *TGB1* variations occurring in susceptible plants, next to plants harboring *Rz1* or *Rz2*, susceptible plants were also analyzed. To exclude artificial mutation occurring during experimental procedure, TGB1 expression vector used for cell death experiments was used as internal control and treated the same (Wetzel *et al.* 2021). Amplicon sequencing was performed by CeBitec (Bielefeld, Germany) using a MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) performing MiSeq System (Illumina) in paired-end mode (2 x 600 bp) followed by an in-house processing described by Liebe *et al.* (2016). Processed sequences were mapped to BNYVV A-type *TGB1* sequence (accession number AQT03618.1) using bowtie 2 (v2.1.0) (Langmead and Salzberg, 2012) and analyzed for SNPs using ReadXplorer platform 2.2.3.
In total four susceptible, four plants harboring homozygous *Rz1* and one plant harboring homozygous *Rz2* were used for sequencing. To detect *TGB1* variability in plants possessing *Rz1*, P-type infested soil was used to grow plants possessing *Rz1* and used for *TGB1* amplicon sequencing. As control, a susceptible variety (KWS03) was used.

Sequencing

DNA sequencing of PCR products or plasmids was either performed by Microsynth AG (Balgach, Swiss) or Eurofins Genomics (Ebersberg, Germany) as indicated in the results. The required volume and quantities were preparade according to suppliers' requests. Analysis of the sequencing was performed using SnapGene®5.0.8 (Insightful Science, San Diego, Ca, USA). Electropherograms of sequencing results were compared to the published sequence of BNYVV A-type *TGB1* sequence (Acc. AQT03618.1).

SNP integration in TGB1 expression vector and BNYVV cDNA full length clone

After identification of possible resistance-breaking mutations by sequencing, mutations were introduced into the TGB1 expression vector (vector described in Wetzel *et al.* (2021)). Primers were designed to perform PCR mutagenesis with the corresponding mutation as indicated in Table S1. Primers were designed to integrate the SNP and amplifying the remaining vector backbone. Subsequently, PCR products were joined by ligation to obtain a plasmid. PCR was performed with Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific) followed by ligation (T4 DNA Ligase, Thermo Fisher Scientific). The generated plasmids were transformed into *E. coli* DH5α for multiplication followed by sequencing to verify mutation integration. Selected plasmids were transformed into *A. tumefaciens* C58C1 and used for coexpression experiments as described by Wetzel *et al.* (2021)

Cell death detection

To investigate the ability of TGB1 mutants to elicit cell death or to avoid recognition by Rz2, coexpression was performed in the experimental host *N. benthamiana*. As described in (Wetzel *et al.* 2021), plasmids containing *Rz2*, *TGB1* and a fluorescence marker gene *dsRed* (Campbell *et al.* 2002) were co-infiltrated into leaf tissue in different combinations. For infiltration, *A. tumefaciens* suspensions (OD_{600} = 0.5) containing the plasmids of interest were used. After infiltration, plants were kept in dark for 24 hours and maintained under greenhouse conditions for monitoring of resistance response for seven days. Cell death was detected by using epifluorescence microscopy of the infiltrated patches. Since the plasmid containing florescence marker *dsRed* was co-infiltrated, living cells can be identified by strong red fluorescence. In contrast, if cell death occurs this can be visualized by GFP autofluorescence of the cell (Koga *et al.* 1988). In the current work the epifluorescence microscope (Leica DMR) was used with specific filters for DsRed (Emitter HQ 620/60, Beamsplitter Q 585 LP, Exciter HQ 565/30) and GFP (Emitter D 510/540, Beamsplitter 425 DCLP, and Exciter D 395/40). Photographs were taken using a Leica DFC camera, DFC300 FX.

DAS-ELISA

Plants were harvested after 42 days and lateral roots were taken and processed as described by Pferdmenges *et al.* (2009). BNYVV detection was performed by DAS-ELISA with BNYVV antibody (DSMZ Braunschweig, Germany, AS-0737). Visualization was performed with p-nitrophenyl phosphate. The mean of A_{405} values of the healthy controls plus three times of standard deviation was calculated and subtracted from sample values. Samples were considered positive if they exceeded the mean.

Results

Amplicon sequencing

The cultivate sgar beet plants possessing *Rz2* in a homozygous state, naturally infected soil obtaines from a region with already existing BNYVV virus strains able to overcome *Rz1* mediated resistance was used. The soil was former used in the study by Galein *et al* 2018 indicating elevated BNYVV contents in resistant varieties. After growing of sugar beet plants with different resistance levels in BNYVV infected soils, DAS-ELISA with BNYVV antibodies indicated no virus infection in plants carrying homozygous *Rz2* (Table 1).

But still, it was possible to amplify BNYVV *TGB1* using RNA from one sample. In the susceptible variety, infected by mechanical inoculation with BNYVV A-type cDNA clone, no mutation leading to an amino acid change was detectable (Table 2). In the variety possessing *Rz1* in total six mutation occurred, with one being non-synonymous, leading to a change of arginine to tyrosine at amino acid position 18.

All mutations were found in all samples that were sequenced. Mutations identified by sequencing were traced back to a BNYVV P-type isolate found in Pithiviers (accession number HM117903.1). In the plant containing homozygous *Rz2* these mutations were found as well. Further, one additional mutation leading to an exchange in the amino acid sequence at position 697 (proline to serine change (P697S)) was detected in the sample derived from the plants carrying homozygous *Rz2.*

TABLE 1 DAS-ELISA values of sugar beet plants with different BNYVV resistance levels (*Rz1Rz1/rz2rz2*, *rz1rz1/Rz2Rz2*, or *rz1rz1/rz2rz2*). *Rz1* and *Rz2* resistance plants were grown in naturally infected soil (BNYVV P-type). Susceptible line (KWS03) was artificially infected by vortex inoculation with BNYVV cDNA full-length clone. 12 plants per genotype were grown for 42 days and tested by DAS-ELISA. Samples used for NGS are highlighted (*).

Number of plants	Absorption at A405		
	Rz1Rz1/rz2rz2 Naturally infected P-type soil	*rz1rz1/Rz2Rz2* Naturally infected P-type soil	*rz1rz1/rz2rz2* BNYVV vortex inoculation
1	0,65*	0,02	0,33*
2	0,64*	0,01	0,66*
3	0,45*	0,01	0,00
4	0,39	0,01	-0,10
5	0,73*	0,01	0,22
6	0,41	0,01	0,00
7	0,35	0,02	0,47*
8	0,47	0,02	0,00
9	0,22	0,17*	0,28
10	0,38	0,03	0,00
11	0,38	0,02	0,40*
12	0,14	0,02	0,38

TABLE 2 Results of NGS amplicon sequencing of BNYVV *TGB1* isolated of sugar beet plants harboring different resistance levels. Mutations were found in all susceptible and in the resistance (*Rz1Rz1/rz2rz2 and rz1rz1/Rz2Rz2*) plants. One mutation (bold) was only found in resistance plant harboring *Rz2*.

nucleotide position	mutation	reference A-type	reference B-type	reference P-type	reads A	reads C	reads G	reads T	amino acid change
52	T	C	C	T	12	1554	4	215492	Arg 18 Tyr
53	A	G	G	A	215696	5	1350	23	
456	T	C	T	T	2	2857	0	218477	no
588	T	C	T	T	24	23298	54	198021	no
697	**T**	**C**	**C**	**C**	**81**	**156145**	**308**	**210781**	**Pro 233 Ser**
1056	G	A	G	G	9416	16	204279	75	no
1071	T	C	T	T	32	8962	177	204602	no

Sequencing by Sanger

Soils, originating from Bondaroy, France, which, according to Galein *et al.* (2018), found to have elevated BNYVV levels in plants harboring *Rz1* and *Rz2*, were used for cultivation of 12 homozygous *Rz2* plants and six susceptible plants (KWS03). After 42 days, lateral roots were harvested and BNYVV content was determined by DAS-ELISA (Figure 1).

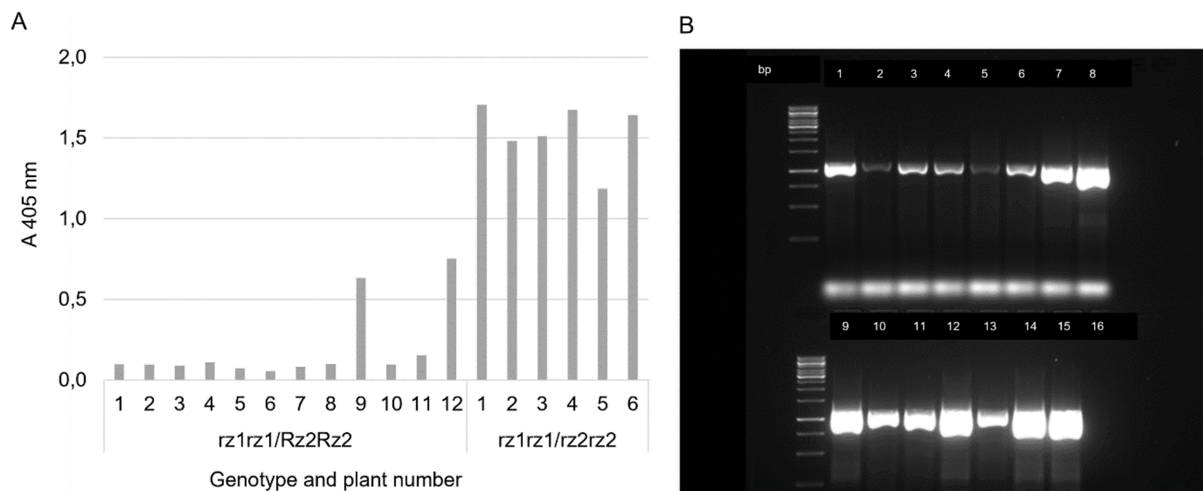

FIGURE 1 Determination of BNYVV infection of sugar beet plants either resistant (homozygous *Rz2*) or susceptible after grown in naturally infected soil. Plants were harvested at 42 dpi and virus titer was measured using DAS-ELISA (A). RT-PCR (B) was performed from total RNA extracts using BNYVV *TGB1* specific primers (fragment size 1152 bp). As template, cDNA generated with from resistant plants (lane 1-12) or susceptible plants (lane 13-15) was used. For control (lane 16), H_2O was used as template. As marker, gene ruler 1 kb DNA ladder was used.

Results showed elevated BNYVV values in all susceptible plants (mean absorption A_{405} = 1.5). In *Rz2* harboring plants grown in soil originated from Bondaroy two individual plants indicated an absorption A_{405} above 0.5 (plant 9 (0.63) and plant 12 (0.75), Figure 1). To confirm BNYVV infection, RNA was extracted from all plants grown on Bondaroy soil, cDNA synthesis was performed followed by PCR with BNYVV *TGB1* specific primers (Figure 1). As control, RNA was isolated from susceptible plants and treated the same. Gel electrophoresis indicated an amplification of BNYVV *TGB1* in all samples. Four of these samples (8,9,11 and 12) were sequenced and results were analyzed for double peaks in the electropherogram. In total 65 mutation were found in more than one sample, but only three of them resulted in amino acid mutations. These mutations, P23T, N90T and V118I were not found in the database (National Center for Biotechnology Information (NCBI)) or described before. From the isolated RNA of plant 12, *TGB1* was cloned, and plasmids were analyzed for SNP integration in 8 single plasmids by sequencing. Results indicated that the same three mutations leading to a change

in the amnio acid sequence did not occur in one of the plasmids. Interestingly, P23T appeared as single mutation, whereas N90T and V118T were found several times in the same plasmid.

HR elicitation of TGB1 mutants

After integration of the mutations in the TGB1 expression vector, all TGB1 variants were tested for their ability to elicit an HR reaction. Therefore, agroinfiltration of *N. benthamiana* leaf tissue was performed with TGB1 variants alone and in combination with isolated 35*S*-*Rz2* (Wetzel *et al.* 2021). As control, BNYVV A-type TGB1 was treated the same way. Three days after infiltration, cell death was detected in the infiltrated areas in all variants, including the control (Figure 2). In all cases, a recognition of TGB1 by Rz2 still led to a resistance reaction, meaning that none of these mutants seemed to be able to overcome *Rz2*-resistance.

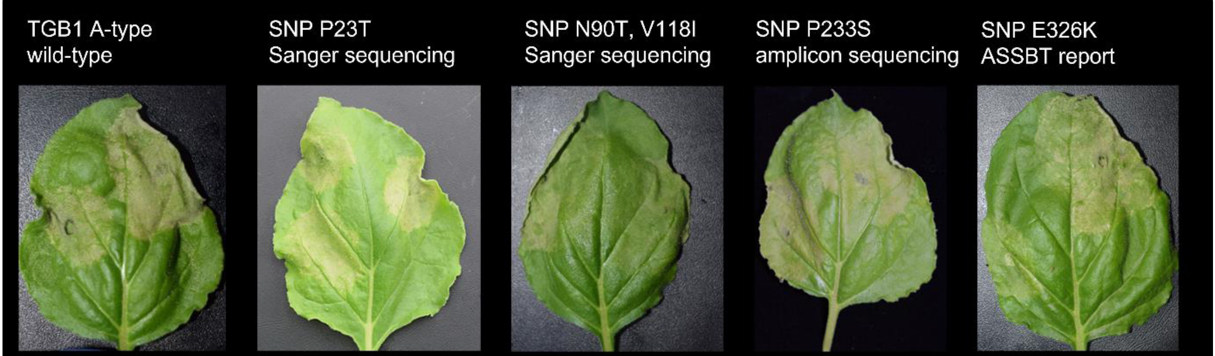

FIGURE 2 Coexpression of resistance protein Rz2 and BNYVV TGB1 A-type and SNP variants results in HR reaction 3 dpi. Different mutations (N90T, V118I, P23T, P233S and E326K) were detected in several approaches (indicated) and integrated in the BNYVV A-type TGB1 expression vector were tested for their ability to elicit HR in combination with *Rz2* expression by agroinfiltration of *N. benthamiana* leaf tissue.

Discussion

The aim of the present study was to investigate the variability of BNYVV *TGB1* to generate resistance-breaking strains to be able to evade *Rz2* mediated resistance. In the present study, naturally infected soils indicating BNYVV infection by Galein *et al.* (2018) and soils obtained from a region known for BNYVV strains able to overcome *Rz1* resistance were used to study the *TGB1* variability. By using a next generation sequencing approach and traditional sequencing, it was possible to identify several mutations leading to changes in the amino acid sequence. However, integrating these mutations into a BNYVV TGB1 expression vector and testing these mutations in coexpression experiments with *Rz2* still resulted in cell death. A similar approach was performed by Liebe *et al.* (2019) investigating the ability of BNYVV to evade *Rz1* mediated resistance by investigating the variability of P25. A deletion of amino acid 179 led to a higher accumulation of BNYVV in resistant varieties, but up to now this deletion has not been observed in nature. Although mutations in the same region was reported before, a complete deletion was only detected when a cDNA clone of BNYVV were used for infection (Chiba *et al.* 2013). While the deletion of amino acid 179 occurred after only one infection process, this might indicate a high fitness penalty of the virus carrying this deletion (Liebe *et al.* 2019). In the present study only one BNYVV *TGB1* mutation was identified by NGS, which occurred in plants possessing *Rz2* resistance which was leading to a change in the amino acid sequence of *TGB1*. Integration of this mutation, P233T, into the TGB1 expression vector and coexpression in leaf tissue of *N. benthamiana* with isolated *Rz2* resulted in an HR reaction comparable to the wild-type TGB1 variant. Even when HR reaction and resistance can be uncoupled, demonstrated for *Rx* against PVX and the *N* gene mediating resistance against *Tobacco mosaic virus* (TMV) (Ronde *et al.* 2014), several studies indicate that the elicitation of an HR reaction can be seen as a resistance mechanism. Although the study of Galein *et al.* (2018) indicated that BNYVV variants are able to overcome *Rz2*-mediated resistance, testing the same soils with elevated BNYVV content for their BNYVV *TGB1* variability gave no indication for resistance-breaking virus strains. In total three SNPs were identified resulting in change of the amino acid sequence. Plasmids generated with the naturally occurring TGB1 variants were produced and used for transient coexpression experiments, but still resulted in an HR reaction in combination with Rz2.

The durability of plant resistance, mediated by a single *R* gene, can hardly be predicted. It is assumed, that resistance based on a single is stable for less than a decade (Brown, 2015). In case of *Rz2*, which was introduced and used for breeding since beginning of the 20th century (Scholten *et al.* 1999), data on the use of varieties possessing *Rz2* are missing. But until now and demonstrated by the current study, no resistance-breaking strain of BNYVV was detected. In the current study a naturally infected BNYVV soil was used, with BNYVV variants able to overcome *Rz1* resistance. If other BNYVV variants exist able to break *Rz2* mediated resistance

can not be excluded. Former studies suggested that, according to the gene-for-gene hypothesis, resistance-breaking varieties often have fitness penalties compared to the wild-type virus in susceptible host (reviewed in Moreno-Pérez *et al.* (2016)). Viruses consist either of a small RNA or DNA genome and encode a small number of proteins responsible for the viral capsid protein, cell-to cell movement and long-distance movement, silencing suppressor, and pathogenicity factors. Often, viral proteins are multifunctional and ORFs overlap (Hull, 2014). To ensure protein expression, viruses are dependent on the host cell protein machinery as viruses are intracellular obligate pathogens. Therefore, changes in the viral genome sequence can have a major impact on the viral life cycle, and mutations that lead to resistance-breaking are restricted due to strong fitness penalties. On the other hand, RNA viruses like BNYVV have a high mutation rate as their RNA-dependent RNA polymerase lacks proof-reading function and they have a high potential to evolve against resistance (Drake and Holland, 1999). As consequence of the high mutation rate, it is assumed that a "swarm" of virus variants exits in a spatial area that infects the host plant (Drake and Holland, 1999). It was demonstrated several times that only one or a few mutations within the viral genome leads to in resistance-breaking (Harrison, 2002; Janzac *et al.* 2010; Lecoq *et al.* 2004). It was also validated by Janzac *et al.* (2009) that resistance that can be broken by a few substitutions is a less durable resistance gene (Ayme *et al.* 2007). But at the same time, the durable resistance gene *Pvr4* can be evaded by PVY by a single nucleotide substitution, which suggests that a prediction of durability is more complex (Janzac *et al.* 2010). In case of PVY, the mutation in the *avr* gene was generated in the laboratory but using this virus variant by transferring it into susceptible plants, the virus variant was not competitive (Janzac *et al.* 2010). In the present study, no resistance-breaking strains were identified. One possible explanation might be that the recognition site in the TGB1 protein is of high importance for biological functions that changes in the protein sequence cannot be tolerated by the virus. This is also the case in terms of *Pvr4* resistance against PVY. The change in the amino acid sequence, which is responsible for resistance-breaking, also resulted to a change in the protein charge (Janzac *et al.* 2010) and most probably affected the recognition or interaction with Pvr4. In case of TGB1, this is the most promising explanation. We demonstrated in former studies that the TGB1 proteins from distinct related plant viruses led to a resistance reaction in *N. benthamiana* (Wetzel *et al.* 2021). This might be indicative for the highly conserved structure leading to recognition of TGB1 protein by Rz2. Even when experimental evidence indicated TGB1 ability to break resistance are lacking and sequence alignments of the different TGB1 variants recognized by Rz2 were not helpful to predict the region for interaction, this might be the most reasonable explanation. Further experiments with random or specific mutations in conserved regions of the TGB1 might help to identify the region that is important for resistance, but the question whether a mutated version of TGB1 is still competitive in the virus population remains to be

addressed in future. Additionally, a stringent monitoring for BNYVV in field cultivation can be used to determine BNYVV variability. As nowadays regions with resistance-breaking strains for *Rz1* are known, for example in Pithiviers, France, monitoring for symptoms indicative for BNYVV infection can increase the knowledge regarding resistance-breaking variants for *Rz2*. The question remains whether BNYVV is able to adapt to *Rz2* resistance, and whether mutations leading to resistance -breaking are able to survive in nature due to fitness penalties. Therefore, a monitoring for rhizomania symptoms might help to get a better understanding and help to secure sugar beet cultivation in future.

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Table S1 List of primer used for indicated experiments. Attached adaptor for NGS sequencing are underlined.

Primer name ´	Primer sequence 5´>3
Primer for amplicon sequencing	
amp.seq.TGB1 1_1 s	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAATTGCGTGTTGGTGAAGG
amp.seq.TGB1 1_1 as	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCACCATTTTATGTTTTGCTCC
amp.seq.TGB1-2_1 s	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCTCCTGGTGTTGGGAAAT
amp.seq.TGB1-2_1 as	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAACTATCCTTTACCTCGTTA
amp.seq.TGB1-3_1 s	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTATGCTAGTAGACGGTTCG
amp.seq.TGB1-3_1 as	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCGGTTATTTCCCTAGACATTA
Primer for sample specific adaptors for NGS sequencing	
D502_s	ATAGAGGC
D701_as	ATTACTCG
D702_as	TCCGGAGA
D703_as	CGCTCATT
D704_as	GAGATTCC
D705_as	ATTCAGAA
D706_as	GAATTCGT
D707_as	CTGAAGCT
D708_as	TAATGCGC
D709_as	CGGCTATG
Primer for TGB1 amplification	
RNA2-TGB1_s	ATCGAATTCATGGTCCAAGTACAGCGTA
RNA2-TGB1_as	TGACTCGAGTCATCTATCTTCGCAAAAA
Primer used for sequencing by sanger	
Seq.TGB1_as	TCAACACATGAGCGAAACCCTAT
Seq.TGB1_s	TATCCTTCGCAAGACCCTTC
Primer for SNP PCR mutagenesis	
TGB1-SNP-697_s	CGTGAACTATAATTTTTCGATTATTGCTGAATGTTATGCTAGTAGAC
TGB1-SNP-697_as	CATTCAGCAATAATCGAAAAATTATAGTTCACGGCAGAACCGG
TGB1_SNP-978_s	CACTATCATTTTGGAAGACAAGTTTGATGATGCTGCCATTTGCG
TGB1_SNP-978_as	GCAGCATCATCAAACTTGTCTTCCAAAATGATAGTGACAACGTCG

CHAPTER 7: General Discussion

The focus of the presented work was to study the molecular characteristics of the resistance protein Rz2 to be able to improve the resistance. In the first instance, we were able to confirm *Rz2* as an independent monogenic resistance gene, which is mainly expressed in the root tissue of the natural host *Beta vulgaris*. Further, the TGB1 from BNYVV and other sugar beet infecting virus species were identified as *avr* determinant by using the experimental host *N. benthamiana* and transient *Agrobacterium*-mediated leaf infiltration as test system. We were able to functionally transfer *Rz2* into *N. benthamiana* by leaf-disk transformation to generate transgenic plants constitutively expressing *Rz2*. Moreover, interaction between the two proteins was investigated by different approaches but due to experimental problems, no validated results were obtained. Also, the ability of BNYVV to adapt to the resistance by evading recognition due to mutations was investigated but no virus variants were detected indicating their ability to break *Rz2* resistance.

In the following general discussion, the identified *avr* determinant and the role of TGB1 in plant immunity, the uniqueness of *Rz2* in terms of its broad recognition spectrum, the ability to confer resistance to BNYVV in transgenic *N. benthamiana* plants as well as the durability of *Rz2* although used for commercial cultivation of sugar beet for several years will be highlighted. Different aspects for future research question and improvement of the present work will be discussed. Several discussion points already presented in the individual manuscripts will not be repeated here except they have importance for the general discussion

It must be noted in advance, that by using the heterologous plant species, *N. benthamiana*, the results obtained in the present study remains to be verified in the host plant *B. vulgaris*. As the results in manuscript I show, *Rz2* is mainly expressed in sugar beet roots making experiments on sugar beet leaf tissue regarding resistance towards BNYVV not feasible. Furthermore, artificial root tissue infection by agroinfiltration is not well adapted in sugar beet. Therefore, *N. benthamiana* plants were used as model plant system to study resistance mechanisms in a non-destructive manner. However, *N. benthamiana* represents a plant species often used in plant virus research as the plant is susceptible to a huge variety of plant viruses as compared to the most popular model plant *A. thaliana.*

7.1 Plant virus movement proteins and their role in plant immunity

Nearly every plant virus encoded protein can act as *avr* determinant in *R* gene-mediated resistance (reviewed in Gouveia *et al.* (2016) and Ronde *et al.* (2014)). Although the CP of viruses is until know the most frequent *avr* identified, movement proteins are also often recognized by *R* genes (Ronde *et al.* 2014). In the present study, one of the BNYVV triple gene block proteins, TGB1, involved in viral cell-to-cell movement, was identified as the *avr* gene towards the *Rz2* resistance gene. Moreover, the TGB1 proteins derived from BSBMV and

BSBV were also recognized by Rz2 resulting in HR reaction in the experimental host *N. benthamiana*.

Movement proteins (MPs) are necessary for plant viruses to move along the site of infection through the plasmodesmata (Pd) into the neighboring cell. The first study on viral movement investigated the 30K MP of TMV, which forms a ribonucleinprotein (RNP) complex guiding the viral RNA through Pd (Melcher, 2000). Another strategy used by RNA plant viruses for movement is encoded by the TGB. Several models on plant virus movement were developed concentrating on TGB, including interaction with the CP or forming tubules to facilitate cell-to-cell distribution (summarized in Verchot-Lubicz *et al.* (2010)). Comprised of three proteins, TGB1, TGB2, and TGB3, these proteins accelerate the transport of viral RNA by building RNP complexes towards and through the Pd. The TGB strategy is used by individual plant virus families within nine plant virus genera, meaning that different strategies of movement occur within one plant virus genus. For example, within the genus *Virgavirida*e, the family *Pomovirus* utilizes TGB-mediated movement, whereas the family of *Tobamovirus*, with its most prevalent species TMV, uses the 30K-like movement strategy. The coexistence of different movement strategies within the same genus confirms the evolutionary model by genomes recombination and quick adaptation to ensure viral spread (Morozov *et al.* 1989). Due to phylogenetic properties and the necessity of the CP for proper movement, two classes of TGB models exist, the potex-like and the hordei-like models (Morozov and Solovyev, 2003). As the BNYVV encoded TGBs belong to the hordei-like family, the following discussion is based on this group. The TGB1 encodes for a protein with three structural and functional domains and can range in size from 42 kDa to 62 kDa. The C-terminal half contains an ATPase/helicase domain with six conserved domains (HELD) (indicated in manuscript 1, Figure 6) (Koonin and Dolja, 1993). Binding of the structural domains of TGB1 was investigated by Makarov *et al.* (2009) showing that self-interaction between all functional domains occurred. The same author postulated that binding ssRNA and dsRNA by the N-terminal domain, the internal domain, and the HELD domain was important for forming the RNP complex. The TGB1 from *Potato mop-top virus* (PMTV), *Pomovirus*, and *Barley stripe mosaic virus* (BSMV), *Hordeivirus*, possess a nuclear and nucleolar localization signal and mutation within these regions led to reduced cell-to-cell movement (Li *et al.* 2018; Lukhovitskaya *et al.* 2015). In addition, TGB1 from BSMV was found to interact with Fib2, a nucleolar protein involved in several plant-virus interactions promoting viral infection (Li *et al.* 2018). In case of BSMV, it is assumed that Fib2 is involved in proper cell-to-cell movement (Li *et al.* 2018), whereas RNA binding was verified for *Bamboo mosaic virus* derived TGB1 (Wung *et al.* 1999).

TGB2 and TGB3 are integral membrane proteins and range in size from 12 - 14 kDa and 7 kDa to 24 kDa, respectively. For PMTV TGB2, RNA binding activity was reported, and it is assumed that TGB2 is responsible for increasing the size exclusion limit of Pd to allow cell-to-cell

transport (Haupt *et al.* 2005). The TGB3 is the most divergent protein among the TGB proteins and contains two transmembrane domains and is also involved in increasing the size exclusion limit (Haupt *et al.* 2005). In addition, TGB3 is the main component responsible to guide viral RNA (vRNA) complexes to the cell wall (reviewed in Navarro *et al.* (2019)).

The TGB1 is localized in the cytoplasm and also in the nucleus when expressed alone, changing its location to the cell wall in presence of TGB3 (Lim *et al.* 2009). While BSMV TGB2 is located at the endoplasmic reticulum and TGB3 is localized at the cell wall in proximity to Pd, TGB2 is relocalized to the cell wall upon interaction (Lim *et al.* 2009). Lim *et al.* (2008) showed that TGB1 interacted with the vRNAs of BSMV and formed the RNP complex. Mediated by TGB3, the complex was transported along the endoplasmic reticulum towards the Pd (Lim *et al.* 2008; Schepetilnikov *et al.* 2008). Moreover, TGB2 increased the transport efficiency of the complex (Navarro *et al.* 2019). In a second movement model based on PMTV, the TGB3 formed motile granules which accumulated at the Pd (Haupt *et al.* 2005; Tilsner *et al.* 2010). Association between vRNA, TGB1, and TGB2 were recruited through interaction between TGB2 and TGB3 to these granules. In contact with Pd, TGB2 and TGB3 promoted the transport of vRNA in the ancient cell by forming a gate (Haupt *et al.* 2005; Shemyakina *et al.* 2011). Whereas TGB1 was transported to the neighboring cell, TGB2 and TGB3 remained in the cell and may recruited new viral particles (Haupt *et al.* 2005).

Besides cell-to-cell movement, several MPs are known to be involved in resistance, for example the MP from TSWV interacting with *Sw-5* (Hallwass *et al.* 2014; Peiró *et al.* 2014), the MP form *Tomato mosaic virus* with *Tm-2^2* (Lanfermeijer *et al.* 2003) and the P3 from *Soybean mosaic virus* with *Rsv4* (Luan *et al.* 2019; Wang and Hajimorad, 2016). But the TGB1 derived from BSMV which interacts with the resistance gene *BARLEY STRIPE RESISTANCE gene 1* (*Bsr1*) (Cui *et al.* 2012; Lee *et al.* 2012) and the PVX 25K protein against *Nb* resistance (Malcuit *et al.* 1999) are the only TGB1 identified as *avr* determinants while no TGB2 or TGB3 are known to act as effector. *Bsr*1 was identified in the wild grass *Brachypodium disachyon* as a single monogenic *R* gene encoding a CNL (Cui *et al.* 2012). A resistance-breaking viral strain derived from Norwich, Great Britain, indicated the importance of two amino acids (K^{390} and K^{392}) located in the helicase domain of TGB1 (Lee *et al.* 2012). Importance of the C-terminal end part of the TGB1 was former reported by Hu *et al.* (2015), indicating a phosphorylation site at amino acid position 395 to 398 important for viral movement, but it remains unknown whether this is also involved in *Bsr-1*-mediated resistance.

In case of PVX 25K protein, the TGB1 belongs to the class of potex-like TGBs and shows different properties compared to BNYVV TGB1. Potex-like TGB1s often represent the VSR next to the MP and to fulfill movement interaction with the CP is required (Verchot-Lubicz *et al.* 2010). Thus, in the 25K, a single amino acid mutation (Ile^6) has been associated with resistance braking abilities, and it has been proposed that the three-dimensional of the protein

structure is important for resistance elicitation (Malcuit *et al.* 1999). Additionally, it was speculated for *Nb* resistance and also for *Tm-2*[2] that resistance was independent of the cell-to-cell movement and resistance was not induced by suppression of cell-to-cell movement rather than recognizing protein modification by viral infection (Malcuit *et al.* 1999; Weber and Pfitzner, 1998).

Until now, no model for TGB1 recognition by R proteins exist. As speculated before, resistance is in most of the cases dependent on an indirect recognition mediated by host protein modifications rather than on direct interaction between resistance gene and pathogen. Since it was reported that TGB1 has nucleotide binding activities and until now no direct interaction was observed in the current study, indirect recognition seems more likely. Besides, it was demonstrated that TGB1 can be modified by host proteins by phosphorylation which can result in recognition (Hu *et al.* 2015). In the current study, direct interactions failed due to experimental problems (YTH and co-localization) but also by using BiFC no interaction was detected.

For further research regarding the durability of *Rz2*, it is important to identify either the interaction site of *Rz2* or the domain in the TGB1 responsible for recognition. As TGB1 is comprised of structural motifs, their involvement in proper function and elicitation of resistance might be interesting to investigate by site-directed mutagenesis or TGB1 deletion mutants. In several studies amino acid sequences which are important for resistance were detected by the occurrence of resistance-breaking strains, for example for BSMV and *Bsr-1* (Lee *et al.* 2012), PVX and *Rx* (Baurès *et al.* 2008), TMV and *Tm-2* (Meshi *et al.* 1989) and PVX and *Nb* (Malcuit *et al.* 1999). No resistance-breaking strains were found in BNYVV population yet and without already existing virus variants able to overcome *Rz2*, only artificial produced variants might give answers to this question. Besides recognition of BNYVV TGB1 Rz2 does also recognize other virus TGB1 variants, showing only 22% homology on amino acid level and ongoing studies indicate, that coexpression of TGB1 variants from PMTV and BVQ resulted in HR (Dr. Liebe, personal communication). This indicates that the recognition site of TGB1 is a highly conserved motif and can be found in different plant virus families and not only in sugar beet infecting viruses. A similar observation was done by Janzac *et al.* (2010) investigating the durability of *Pvr4* against *Potato virus Y* (PVY). The authors showed that a substitution of a nucleotide leading to an amino acid change in the *avr* determinant, the RdRp of PVY (NIb), led to resistance breakdown. But the mutation, produced *in vitro*, resulted in high fitness penalties, and most properly will not occur in nature. The NIb protein is highly conserved in the family of potyvirus*es*, and recognition of NIb protects several plant species against virus infection (Janzac *et al.* 2009). However, other potyviruses like *Tobacco etch virus* can infect pepper plants carrying the *Pvr4* gene. Analyses assumed that the region responsible for interacting is located within a loop structure in the NIb and the mutation leading to the resistance breakdown

resulted in a change in the charge of the residue at this position (Janzac *et al.* 2010). As *Pvr4* can be overcome by several potyviruses which also possess the loop structure and no obvious differences were detected in comparison of the different variants, it was suggested that the change of charge might resulted in conformational changes within the NIb leading to a loss of recognition by Pvr4 (Janzac *et al.* 2010). A similar situation might apply to TGB1 and Rz2 resistance, indicated by the missing resistance-breaking strains in nature and the recognition of several TGB1 variants. Even when it will be possible to generate TGB1 variants which are no longer leading to a resistance response without influencing the biological properties of TGB1, the survival of the mutated virus variant in the virus population remains unknown.
Additionally, it shows that although plant *R* genes are highly conserved in their structure, their corresponding *avr* gene can represent every gene derived from the virus. Until now, there is no evidence existing, explaining conserved structural dimensions preferably recognized by R proteins evaluating an *avr* determinant. Although R proteins can also recognize other pathogens besides viruses, most of them share the same domain structure. Moreover, they are able to interact with structurally and functionally diverse *avr* determinants. This raises the question of how R proteins select their corresponding *avr* determinant.

7.2 The role of the hypersensitive response in plant immunity

In the present study, the development of a hypersensitive response (HR), and with this local cell death, was used as a visible resistance phenotype against infection with different viruses. Although infection with BNYVV and BSBMV can result in local lesions, as demonstrated in manuscript I and II, this reaction occurred later and was not resulting in cell death rather in local yellowing. Nevertheless, the occurrence of cell death as phenotype of resistance was used in the complete study. As reported in manuscript III, fluorescent tagging and investigating the localization of both proteins, Rz2 and TGB1, alone and in coexpression, resulted in the development of HR in a very short timeframe. In the following part, the occurrence of local cell death in relation to plant resistance is discussed, as well as how HR is regulated and might be circumvented.
HR is a form of programmed cell death that occurs at the site of pathogen penetration and is believed to be the hallmark of plant resistance and was described the first time by Stakman (1916). Although first described about 100 years ago, the understanding of how cell death is activated during plant resistance is still unknown (Balint-Kurti, 2019). Following recognition of the pathogen, a signaling cascade is activated resulting in local cell death and restricting pathogen invasion. Hereby generation of biochemicals and cellular signals are important, including a burst ROS, increased SA content, induction of kinase signaling, and activation of *PR* genes. It has been suggested that cell death and plant resistance are physiologically and genetically linked, but several studies and reports indicated that cell death occurs as a consequence of overactivated defense response and can be uncoupled from resistance

(Balint-Kurti, 2019; Király *et al.* 1972; Kourelis and van der Hoorn, 2018; Künstler *et al.* 2016). Studies based on *Rx1* (Bendahmane *et al.* 1999), *N* gene (Wright *et al.* 2000), *RCY1* (Takahashi *et al.* 2012) and *HRT* (Kachroo *et al.* 2000) indicated that HR is not essential for resistance against invading viruses. For example, in *dnd1* mutant *A. thaliana* plants carrying the *RCY1* gene, no HR was detected after CMV inoculation, whereas CMV was not able to colonize the host plant (Takahashi *et al.* 2012). *A. thaliana* plants lacking the *dnd1* gene have been previously shown to lose the ability to react with HR to an infection with *Pseudomonas syringae* (*P. syringae*) strains but were still resistant to infection by activated *PR*-genes (Yu *et al.* 1998). The *dnd1* gene encodes a cation channel of the cyclic nucleotide gated ion channel involved in transport of Ca^{2+}, K^{+} and other cation into the cytoplasm (Clough *et al.* 2000). Ca^{2+} influx is known to be involved in resistance response and thought to be required for activation of MAP kinases and induction of oxidative burst (Jabs *et al.* 1997; Ligterink *et al.* 1997). This indicates that without the elicitation of HR, virus infection will still be stopped most properly by activation of *PR* genes. Investigation of the pathogen-plant interaction of TCV with the resistance gene *HRT* showed that the resistance gene alone was not sufficient for complete resistance and required the recessive gene *rrt* (Kachroo *et al.* 2000). While HR was elicited in the presence of HRT, resistance (stopping viral spread) was induced when both genes, *HRT* and *rrt,* were present (Kachroo *et al.* 2000). These two examples show that HR can be seen as part of resistance but not as essential for restricting pathogen invasion. It also demonstrated that plant-pathogen interactions and their outcome cannot be compared and nearly every pathosystem has its uniqueness (reviewed in Künstler *et al.* (2016) and Kachroo *et al.* (2006)). Nevertheless, several studies on plant virus *R* genes indicated that several proteins involved in resistance are conserved and play major roles in plant resistance. Proteins involved in salicylic acid synthesis like EDS1, EDS5, and PAD4 are required for *HRT*-mediated resistance whereas resistance mediated by *Rx* against PVX is dependent on SGT1 and HSP90 but does not require EDS1 (Chandra-Shekara *et al.* 2004; Peart *et al.* 2002). Even when different proteins are required for proper resistance, several studies, including the present one, demonstrated that conserved signaling is present in different plant species and functional transfer of *R* genes into different plant species is dependent on the proteins required for restricting the pathogen (Künstler *et al.* 2016).

In the current study the development of an HR response was assumed as phenotype for resistance mediated by the interaction between Rz2 and BNYVV. In case of the already discussed pathosystem *HRT* and TCV, the resistance gene *HRT* was able to elicit an HR response without the *rrt* gene, which is necessary for resistance against TCV (Kachroo *et al.* 2000). In the present study, it is not known how many and which genes are involved in a functional resistance against BNYVV, but it seems that *Rz2* is important for activation of mechanisms which result in an HR reaction. Moreover, the transfer of *Rz2* into the

heterologous plant species *N. benthamiana* demonstrated, that proteins required for functional resistance in the host plant sugar beet seemed to be present in *N. benthamiana*, too. In on-going work, it was shown that co-expression of *Rz2* and the TGB1 variant derived from the pomovirus *Potato mop top virus* results in the development of an HR reaction (Liebe personal communication). With this and the ability to confer resistance against several plant virus species, further research in context with functional transfer of *Rz2* into crop species belonging to the family of *Solanaceae* might result in additional resistance against viral diseases.

The development of a rapid HR response inhibited the determination of the localization of the potential Rz2 and TGB1 formed complex. Several plant-encoded proteins are known to play important roles in orchestrating HR development in a negative or positive way. For example, silencing the *N.bBECLIN1* gene, which is involved in programmed cell death and induced during *N* gene-mediated resistance against TMV, resulted in an uncontrolled programmed cell death even in absence of the pathogen (Liu, Y. *et al.* 2005). Contrary, silencing *SGT1, HSP90* or *RAR1* in *N. benthamiana* elicited an attenuated HR in plants challenged with *Pvr4* and the corresponding *avr* NIb, while silencing *EDS1*, *NDR1* and other genes involved in defense signaling had no effect on the occurrence of HR (Kim *et al.* 2018). SGT1*,* HSP90 and RAR1 are responsible for stabilization of *R* genes and their maturation and functionality (Zhang *et al.* 2010), while EDS1 participated in resistance response pathways. Contrary, the TNL resistance genes *RPS4* and *RAC*, derived from *A. thaliana,* mediate resistance against *P. syringae* and *Albugo candida,* respectively, are dependent on EDS1 to elicit HR (Borhan *et al.* 2004; Zhang *et al.* 2004). Silencing the vacuolar proteases (VPE) in *N. benthamiana* plants carrying the *N* gene caused inhibition of HR after TMV treatment, whereas control plants responded with HR in presence of the virus (Hatsugai *et al.* 2004). VPEs are caspases reported to be responsible for cell death associated with leaf senescence and maturation of proteins leading to disruption of the vacuole and consequent cell death (reviewed in Hatsugai *et al.* (2015) and Valandro *et al.* (2020)). Two additional caspases from *A. thaliana* were identified to be functional in plant HR, *At*MC1 and *At*MC2. Knockdown mutants of *AtMC1* abolished HR after pathogen treatment, while silencing of *AtMC2* resulted in an enhanced HR reaction (Coll *et al.* 2010). It is interesting to note here that silencing of At*MC1* still restricted pathogen invasion without causing HR (Coll *et al.* 2010).

These examples showed that HR can be suppressed by silencing different factors and might be helpful for further investigation in case of *Rz2* and *TGB1* interaction in the absence of cell death. The functionality of EDS1 demonstrates the uniqueness of every pathosystem once more. It also indicates that several resistance genes use the same downstream signaling pathways, allowing interspecific transfer of *R* genes. For further investigation of *Rz2* and *TGB1*, deficient mutants might be helpful to study the localization of both proteins essential for resistance. As cell death occurred in a short timeframe after transient expression by

agroinfiltration of both proteins, it was not possible to investigate the cellular distribution of both proteins and whether this changes when both proteins are present. Further, knockout mutants of specific plant immunity proteins will give information about interacting partners involved in the resistance mechanism and the conservation of them in distinct plant families. This will also help to figure out the ability to transfer *Rz2* and might help to increase resistance against other viral diseases.

7.2 Functionality and evolution of plant R proteins

One of the remaining questions is how Rz2 and TGB1 interact and who downstream signaling leads to cell death in *N. benthamiana* and *B. vulgaris*. As it was not possible to study direct interaction between *Rz2* and TGB1 due to experimental issues, indirect interaction might also be possible and cannot be excluded as discussed in manuscript III and seems more likely, as direct interaction occurs more seldom. Although indirect interaction needs additional protein partners for functionality, these are also needed for direct interaction models as no model exist until now, where resistance is mediated by a single resistant protein.

In the recent years, many studies were published about the functionality of *R* genes and their mechanism to mediate resistance. One of the most outstanding works was carried out by Wang *et al.* (2019a; 2019b). In their studies, the authors investigated the function of the CNL HOPZ-ACTIVATED RESISTANCE1 (ZAR1) from *A. thaliana*, which confers resistance against the bacterium *Xanthomonas campestris* pv. *camprestris* (*Xcc*) by recognizing the uridylyltransferase (AvrAC) of the bacterium. A complex formed of ZAR1 and the receptor-like cytoplasmic kinase RESISTANCE RELATED KINASE 1 (RKS1) is required to mediate cell death (Wang *et al.* 2019b). PBL2 (AVRPPHB SUSCEPTIBLE1-LIKE2), a protein kinase interacting with RKS1 is uridylated by AvrCR. Upon interaction with RKS1, a conformational change in ZAR will take place resulting in a dislocation of the NB domain and releasing of ADP (Wang *et al.* 2019a; Wang *et al.* 2019b). By binding ATP, this binding triggers a conformational change in the ZAR1 protein mainly in the CC domain leading to a pore forming tunnel, a so called resistosome, which is localized to the plasma membrane (Wang *et al.* 2019b). The authors proposed that this tunnel is initiating cell death by perturbing the plasma membrane which was further investigated by mutation analysis but remains under future experiments (Wang *et al.* 2019b). Moreover, it was shown that ZAR1 forms a pentamer in the plasma membrane and leads to a Ca^{2+} influx leading to cell death (Bi *et al.* 2021). Similar models were proposed for the resistance genes *RPP1* and *ROQ1* (Ma *et al.* 2020; Martin *et al.* 2020). In addition, analysis by Adachi *et al.* (2019a) showed the conservation of an N-terminal motif found in ZAR1 which is also present in nearly 20% of CNLs and proteins belonging to the helper NLRs required by many sensor NLRs to mediate resistance (Wu *et al.* 2017). This MADA motif, composed of the following amino acid sequence (MADAxVSFxVxKLxxLLxxEx), is located in a helix forming structure involved in the assembly of the resistosome (Adachi *et*

al. 2019a). In the same study the presence of the MADA motif in other CNLs was analyzed and it was demonstrated by exchanging the motif between ZAR1 and distinct NLRs, that the motif was conserved between NLRs derived from monocots and dicots (Adachi *et al.* 2019a). In their analysis, the authors included NLRs found in the sugar beet genome, but as the genome of a resistant line including *Rz2* is not published and comparison with the susceptible variety is not conclusive, there is no indication whether *Rz2* also possesses the MADA motif and might be a resistosome encoding protein.

The research by Adachi *et al.* (2019a) and similar studies also highlight the evolution of *R* genes and their conservation in plant genomes (Kourelis *et al.* 2020; Tamborski and Krasileva, 2020). The genomes of many angiosperms possess a high number of NLR genes (Baggs *et al.* 2017; Shao *et al.* 2016) and a common opinion is that NLRs evolved by a birth-and-death process (Michelmore and Meyers, 1998). In this model, new genes are emerging by gene duplication while others disappear or become non-functional (Nei and Rooney, 2005). This enables NLRs to adapt to fast evolving effectors and explains why NLRs represent one of the most diversified plant genes (Tamborski and Krasileva, 2020). The upcoming research assumed that NLRs appear as clusters as most of the known NLRs function as sensor and helper NLRs except for singleton NLRs that combine both function (Kourelis *et al.* 2020). In the work of Adachi *et al.* (2019b) the authors speculated that NLRs and especially sensor NLRs evolved from an ancestor NLR by massive expansion and building a network architecture. This is supported by a recent study of the helper NLR, NRC (NLR-REQUIRED FOR CELL DEATH), which interacted with several sensor NLRs to confer immunity (Wu *et al.* 2017). As consequence of coevolution with pathogens, sensor NLRs are assumed to evolve faster than helper NLRs (Wu *et al.* 2017). Investigations of CNLs against *P syringae* indicated that, in addition to *A. thaliana*, similar CNLs were found in soybean, guarding the same bacterial effectors, and conferring resistance (Ashfield *et al.* 2014).

Although in the present study no functional analyses of *Rz2* were conducted, the fact that *Rz2* can be functionally transferred to another plant family and several studies, indicating that parts of the plant immune system are highly conserved, suggest that *Rz2* operates in association with additional proteins. This would mean that proteins that Rz2 interacts with are present in two distinct plant families. In future, with upcoming new bioinformatic tools and increasing knowledge from other plant resistance genes, it will be possible to investigate the protein structure of Rz2 and it might be possible to determine the way cell death is mediated, by a pore-forming tunnel like ZAR1 or distinct pathways with additional helper proteins.

7.3 Generation of durable resistance

The development of resistance-breaking strains of pathogens, and thus the ability to overcome resistance, is one of the main obstacles in resistance breeding worldwide. Sugar beet cultivation is highly dependent on BNYVV control and resistance-breaking strains able to

overcome *Rz1* resistance have already been detected. Until now, no resistance-breaking strain has been reported for *Rz2,* although in field investigations elevated virus contents were measured in asymptomatic plants (Galein *et al.* 2018). In an NGS approach, the ability of BNYVV to adapt to the host resistance was examined and potential mutations for their ability to evade recognition were screened (manuscript IV). However, all SNPs that were identified resulted in an HR reaction after integration in wild-type TGB1 expression vector and used for transient coexpression experiments with Rz2. Reasons for this are discussed in manuscript IV. As viruses are missing a proofreading activity of their own RdRp, they have a high mutation rate (Steinhauer and Holland, 1987) and resistance is most often based on the recognition of short sequence of the pathogen (Wu *et al.* 2019). Since the durability of *Rz2* is of significant interest for the future, the following part discusses different approaches to strengthen *R* genes or to extend their recognition spectrum.

One way to generate durable resistance is to increase the recognition spectrum of the resistance gene by introducing mutations in the domain responsible for recognition. Generating random mutations in the LRR domain of *Rx* was the first report of this approach (Farnham and Baulcombe, 2006). Mutations in the LRR domains enabled *Rx* to confer resistance against a PVX strain able to overcome *Rx* resistance. Moreover, it broadened the recognition specificity of *Rx* and allowed control of the distinct related *Poplar mosaic virus* (Farnham and Baulcombe, 2006). Another example for this approach was the generation of *Sw-5b* mutants, able to recognize the resistance-breaking strains of TSWV (Huang *et al.* 2021). As stated in section 1.1.3, *Sw-5b* recognizes the viral *avr* in a two-step process through interaction with the NB-LRR and the SD domain (Li *et al.* 2019). In the study by Huang *et al.* (2021) it was shown that stepwise artificial mutagenesis in both domains (NB-LRR and SD) resulted in a resistance gene able to recognize resistance-breaking strains of TSVW, while mutation in only one of the domains involved in recognition did not result in a broader recognition spectrum. In order to use this strategy to increase the recognition spectrum of a particular *R* gene, viral strains that are able to evade the “wild-type” resistance are required. Since in the present study, no such virus variants are known and the critical part of TGB1 responsible for recognition has not yet been determined, it is essential to generate *in vitro* viral strains that are able to evade *Rz2*-mediated resistance. In addition, studying natural variation in other virus species recognized by *Rz2*, like BSBV, BVQ, BSBMV or even PMTV which are not controlled by *Rz1,* can help to identify TGB1 variants able to escape *Rz2*-mediated recognition.

Pyramiding resistance genes, demonstrated by the use of *Rz1* and *Rz2* for sugar beet cultivation, provides a promising way to prolong the lifetime of a single resistance gene. Gene pyramiding is defined as the combination of single plant *R* genes into one plant cultivar (Nicaise, 2014). By using different *R* genes that target the same virus, the use of *R* genes that recognize different effectors displays the most effective strategy to combat viruses. To evade

resistance, the virus needs to adapt to different R proteins. In this case, the random occurrence of viral strains that evade resistance with minor fitness penalties is lower compared to adaption to resistance mediated by a single *R* gene (Fuchs, 2017). Examples for resistance gene pyramiding are the combination of three monogenic genes conferring resistance against SMV, *Rsv1, Rsv3* and *Rsv4* (Shi *et al.* 2009) and pyramiding *Ty-2* and *Ty-3* against *Tomato yellow leaf curl virus* (Prasanna *et al.* 2015). In comparison to pyramiding resistance genes is the use of quantitative trait loci (QTL), conferring only partial resistance and are most of the time not race-specific. By combining *R* genes with QTLs, resistance can be increased, as it was demonstrated in the case of *pvr2*^{3} gene against PVX (Palloix *et al.* 2009). Comparison of the effectiveness of viral strains to evade resistance either provided by the single *R* gene or combined by QTLs indicated a breakdown of the single *R* gene whereas the combination remains stable (Palloix *et al.* 2009).

With the upcoming and rapidly increasing ability of gene editing technologies, the use of these technologies for plant breeding has increased. The key components of genome engineering are specific nucleases, which can be designed *in vitro* to recognize a specific nucleic acid sequence. Nucleases generate double strand breaks (DBS) which are repaired by the cell machinery. One of the commonly used nucleases is CRISPR/Cas. Originally it is part of the adaptive immune system of bacteria against invading viruses and phages (Bolotin *et al.* 2005). Upon pathogen attack, foreign RNA is integrated into the host genome, which is transcribed (CRISPR RNA) and will be recognized by Cas9. These CRISPR RNAs are used as template for generating DBS in the pathogen sequence by Cas nucleases. After the DBS, two naturally occurring cell machinery processes are required to repair the sequence, the non-homologous end joining (NHEJ) or homology-directed repair (HDR). Whereas NHEJ occurs more frequently and does not require a homologous sequence for repairing, NHEJ generated deletion or random mutations. A specific integration of a mutation or even foreign DNA can be fulfilled by HDR, which is dependent on a repair template (either the plants own one in form of the homologous template or by exogenous DNA templates). Genome editing is used worldwide for precise generation of mutations by engineering the CRISPR region specific to the target region in the genome not only in plants.

One way to fight against viruses is by integrating the CRISPR/Cas9 machinery in the host plant, which recognizes the virus sequence and render it harmless. This approach was used several times against DNA viruses, demonstrated by Ali *et al.* (2015a), Ali *et al.* (2015b), Baltes *et al.* (2015) and Ji *et al.* (2015) and also against RNA viruses, demonstrated by Aman *et al.* (2018). Common for these studies are the integration of the CRISPR/Cas system in the plant genome as a transgene and thereby generating resistance. A major drawback of this techniques is the ability of the virus to evade recognition, which was shown by Ali *et al.* (2016). Reasons are the recognition of a short sequence of the pathogen (Zaidi *et al.* 2016). A much

more promising way to use genome engineering in the field of plant immunity is by manipulating the plant genome and increase the resistance level of the plant itself, rather than introducing a foreign protein. This technique has already been used for targeting plant initiation factors (*eIF4E*) involved in recessive resistance (Truniger and Aranda, 2009). In this regard, disruption of *eIF4E* by CRIPR/Cas9 generated resistance towards *Turnip mosaic virus* in *A. thaliana* (Pyott *et al.* 2016) and against *Cucumber vein yellowing virus, Zucchini yellow mosaic virus* and *Papaya ringspot virus* in cucumber plants (Chandrasekaran *et al.* 2016).

Although genome editing can accelerate breeding for a broader recognition by targeting a specific site in the *R* gene and generating mutants is easier and faster, application for improved resistance has not yet been reported. Genome editing is expected to play an important role in breeding for crop protection and other important traits for sustainable agriculture in future, but there are several obstacles which cannot be ignored (further discussed in Langner *et al.* (2018), Chen *et al.* (2019) and Mushtaq *et al.* (2021)). For the present study, genome editing can be used to integrate mutations into *Rz2* to increase the durability. But in advance several research questions previously discussed needs to be answered before using genome editing techniques. However, when these questions, such as identification of the TGB1 recognition site, the Rz2 domain interacting with TGB1 or even helper NLRs involved in *Rz2*-mediated resistance, are answered, new technologies including CRISPR/Cas can be used to generate durable resistance against BNYVV. By identifying possible interaction domains or other aspect important for Rz2 functionality this information can be used to generate precise mutations to increase the durability or even the recognition spectrum. One future aspect may also be the identification of proteins similar to *Rz2* in plant species that are infected by TGB1 carrying viruses. As noted in section 7.2, the plant genome possesses several numbers of *R* genes which seem to be inactive. Using bioinformatic tools, it might be possible to identify these genes and genome editing might help to activate an *R* gene already present in the host plant. This approach would avoid the difficulties associated with introducing foreign genes into the host plant. Even though genome editing promises a significant role in future in terms of plant breeding, it has some limitations and research is still needed to secure this technique and its applications.

7.4 What makes Rz2 multi-talented?

The sugar beet derived resistance protein Rz2 exhibits some properties which were not reported before for one single *R* gene.

First, the broad recognition spectrum of Rz2. While the first experiments based on the resistance towards BNYVV, it could be shown that Rz2 is also able to control infection with BSBMV and BSBV, which was further verified by the elicitation of an HR reaction in coexpression experiments. Even when previous studies reported (reviewed in manuscript I)

that some *R* genes are able to interact with several virus species, no *R* gene was reported to confer resistance towards different members of plant virus families. It remains to be further clarified how this phenomenon can be explained, whether Rz2 can recognize several TGB1 variants by accident or whether *Rz2* has been selected to act against multiple TGB1 variants. Second, the fact that *Rz2* has been functionally transferred to the heterologous plant species *N. benthamiana*. Transgenic plants exhibit resistance towards BNYVV infection and agroinfiltration of TGB1 also resulted in an HR response. Although it was reported that NLRs can be transferred into distinct plant families (discussed in manuscript 2), there is a general rule that most of the times transfer is only possible between the same plant family.

And finally, the already existing durability of *Rz2*. The gene has been used for sugar beet cultivation since the beginning of the 21st century. Consequently, the durability of *Rz2* as a single *R* gene is relatively long, even if the varieties used for sugar beet cultivation carry *Rz1* in addition to *Rz2*. Because at least BSBV occurs in nearly every sugar beet cultivation area and is not controlled, the development of BSBV resistance-breaking strains might have occurred, but since no information on the BSBV diversity in naturally infested soils exists, this question requires further investigation.

In summary, the presented results of the thesis can help to improve *Rz2*-mediated resistance towards BNYVV and other virus species and will hopefully secure sugar beet cultivation in future.

CHAPTER 8: Summary

The viral disease, rhizomania, caused by the *Beet necrotic yellow vein virus* (family *Benyviridae*) is an increasing threat to efficient sugar beet cultivation. Transmitted by a soil-borne pathogen, *Polymyxae betae*, BNYVV infection results in a deformed taproot, due to an excessive lateral root growth. Control of the disease may only be achieved by using resistant varieties. At present, mainly two dominant resistance genes, *Rz1* and *Rz2,* are used either alone or in combination. While the genetic background of *Rz1* is unknown, *Rz2* has recently been identified as a classical *R* gene encoding a "coiled-coil nucleotide-binding and leucin-rich repeat" (CC-NB-LRR, NLR) protein (Capistrano-Gossmann et al. 2017). Due to the extensive widespread use of *Rz1* for decades in commercial cultivars, the occurrence of resistance-breaking strains had been observed. Mutations in the pathogenicity factor P25 encoded on RNA3 are responsible for this observation. Based on this discovery, it is assumed that P25 displays the avirulence gene (*avr*) towards *Rz1*, which is responsible for the recognition being responsible for recognition by *Rz1*. The *avr* corresponding for *Rz2* recognition was not identified and therefore is one of the main objectives of the present work. Further, a deeper knowledge of *Rz2*, as the identification of two individual resistance (*Rz1* and *Rz2*), and the expression pattern of *Rz2* were questioned. As experiments mediated by *Agrobacterium tumefaciens* infiltration (agroinfiltration) are hard to achieve in the natural host of BNYVV, the development of an experimental system was required to allow investigation of recognition and interaction between BNYVV and *Rz2*. Additionally, the identification of resistance-breaking strains of BNYVV, either naturally occurring or artificially produced, was part of the present study to generate a more durable resistance.

The identity of *Rz2* as an individual BNYVV resistance gene was verified by a resistance test. Next to BNYVV, the second sugar beet infecting benyvirus, *Beet soil-borne mosaic virus* (BSBMV) which is transmitted by the same vector, was used. An infection of both viruses was achieved by artificial infection of different breeding lines, possessing either homozygous *Rz1* or *Rz2* or being susceptible. Results of the resistance test indicated that *Rz2* is also mediating resistance against BSBMV infection in sugar beets, as no virus multiplication was measured in *Rz2* containing lines. In comparison to homozygous *Rz1* lines, where no BNYVV was detected but high BSBMV levels. This also indicated that *Rz1* and *Rz2* are independent BNYVV resistance genes. As former experiments indicated a root specific *Rz1* expression, the expression pattern of *Rz2* was investigated. Breeding lines were grown in naturally infected soil and expression was determined over time by quantitative real time Reverse Transcription PCR) (qRT-PCR). Results indicated a root specific and constitutive expression of *Rz2*, which was slightly increased due to an BNYVV infection. Because of the root specificity and the hardly achieved transient expression mediated by agroinfiltration in sugar beet leaf tissue, the experimental host, *Nicotiana benthamiana* was used in the following experiments.

After isolation and cloning of *Rz2* into a binary plant expression vector, coexpression of the infectious BNYVV full length cDNA clone and *Rz2* in *N. benthamiana* leaf tissue resulted in a resistance reaction in form of local cell death (hypersensitive response, HR). To identify the *avr* determinant, the individual ORFs of BNYVV were cloned into a binary plant expression vector and used for coexpression studies with transiently expressed *Rz2*. Only in one combination, when the RNA2 encoded “triple gene block protein” (TGB1) was expressed together with *Rz2*, a resistance reaction was visible and verified by DAB staining. TGB1 is involved in the cell-to cell movement of the virus and can be found in several plant virus families. Since the previous resistance test showed that *Rz2* is also active against BSBMV, coexpression of cloned BSBMV *TGB1* and *Rz2* also resulted in an HR reaction. Besides BNYVV and BSBMV, *P. betae* is transmitting additional sugar beet infection viruses. For example, the *Beet soil borne virus* and *Beet virus Q*, which also possess a TGB1 but belong to a different plant virus family. In the present work and beyond, it was shown that *Rz2* recognized the TGB1 variants from BSBV and BVQ both belong to the genus *Pomovirus*. Furthermore, it was shown that coexpression of *Rz2* and the *TGB1* from *Potato mop top virus*, another pomovirus which is not infecting sugar beets, resulted in an HR reaction.
These results indicated that TGB1 represents the *avr* determinant towards *Rz2* and seems to be functionally effective in the heterologous plant species *N. benthamiana*. To study the resistance mechanism in more detail, transgenic *N. benthamiana* constitutively expressing *Rz2* were produced by leaf disk mediated transformation. *Rz2* was cloned into a plant transformation vector, which is known for stable transformation and selection of homozygous plants, which can be performed by kanamycin selection. After transformation and selection, two independent lines were identified that constitutively expressed *Rz2* and homozygous integration of the transgene was confirmed. No systemic infection was detected after agroinoculation of BNYVV cDNA clone, while agroinfiltration of BNYVV *TGB1* resulted in HR. After mechanical inoculation, in one line infection foci were detected, but no systemic infection was developed. In the second line no infection lesions or systemic infection were detected. Two different types of resistance are known in ETI mediated by R genes in terms of viral diseases. Hypersensitive response, where the pathogen is able to multiplicate in the host and a small number of cells before a programmed cell death is activated. And extreme resistance (ER), where no pathogen movement occurs, and first infected cells are the target of programmed cell death. The present study indicated, that after transformation, both resistance phenotypes developed. A possible explanation can be different expression levels, indicated by a two-fold higher expression of *Rz2* in the transgenic line showing ER after infection. But still, conclusion to the original host reaction cannot be drawn. The present study showed that it is possible to functionally transfer an *R* gene into a heterologous plant family and some downstream signaling pathways are conserved in the plant kingdom involved in resistance. It

also indicated that the resistance mechanisms is rather dependent on the integration site and its genetic background, instead of the resistance gene itself.
To study the interaction between *Rz2* and TGB1, different approaches studying protein-protein interaction were applied. Because of a strong autoactivation of TGB1, the yeast two-hybrid system was not suitable to investigate the interaction. *In silico* analysis indicated a strong transcription activation domain, which can be the reason for autoactivation. By using BiFC, a reconstituted mRFP was detected when *Rz2* and TGB1 were labelled with split parts of mRFP. But in the same approach, the biological function to elicit HR, was not observed. Even when mRFP might influence the biological function of the complex, this was not observed in the colocalization experiments. When *Rz2* was tagged at the C-terminal part with *smRSGFP* and *TGB1* was tagged also at the C-terminal part with *mRFP*, a strong HR was detected indicating that the fluorochromes do not influence biological function. But in the same combination, no signal was detected in BiFC assay. On the other side, the development of HR in colocalization experiments was quite rapid, making it impossible to detect the proteins in coexpression experiments and allowing no speculation about the distribution upon interaction of the proteins.
In nature, the development of a resistance-breaking strain is responsible for the loss of the resistance. Described by the zigzag model by Jones and Dangl (2006), the identification of resistance-breaking strains before the breakdown of the resistance in nature, might help to strengthen the resistance gene. In case of *Rz2*, no natural resistance-breaking strains of BNYVV were reported yet. To investigate the TGB1 variability a Next Generation Sequencing approach was carried out. Naturally infected plants were analyzed for their TGB1 variability, indicating one mutation (P233T) leading to an amino acid exchange. The mutation was integrated in the TGB1 expression vector and tested for HR elicitation in coexpression with *Rz2* in *N. benthamiana.* In this test, HR developed as former observed when applying wild-type TGB1. In a recently published study, elevated BNYVV levels harboring *Rz1* and *Rz2* were observed, and soil samples were used to analyze the TGB1 variability. In total, three mutations were identified. Integration into the TGB1 expression vector and coexpression with *Rz2* still resulted in an HR reaction, indicating no resistance-breaking ability of these mutants.
This work showed that *Rz2* is able to recognize virus species derived from different plant virus families by direct or indirect interaction with *TGB1*. Furthermore, it was demonstrated that *Rz2* can be functionally transferred into a heterologous plant species and until now no resistance-breaking strain was identified. Taken together, *Rz2* seems to be a multitalented *R* gene, with a broad range of recognition, a strong durability and being able to function in a distinct plant family. With this, *Rz2* is a very powerful tool for breeding programs and might also be of interest for breeding viral resistance traits not only for sugar beet cultivation.

CHAPTER 9: References

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Wetzel, V., Brault, V., & Varrelmann, M. (2018).: „Production of a Beet chlorosis virus full-length cDNA clone by means of Gibson assembly and analysis of biological properties." *Journal of General Virology*, *99*(11), 1522-1527.,DOI 10.1099/jgv.0.001146

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Wetzel, V., Willlems, G., Darracq A., Galein, Y.,Liebe, S., Varrelmann, M.(2021): „ *The Beta vulgaris* derived resistance gene *Rz2* confers a broad-spectrum resistance against soil-borne sugar beet infecting viruses from different families by recognizing the triple gene block protein 1.", *Molecular Plant Pathology* , DOI 10.1111/mpp.13066

Oral presentations

German Society for Plant Protection and Plant Health R.S., study group Plant Virology, Bonn, 27.03.17 – 28.03.17, oral presentation: „Construction of an infectious full-length cDNA clone of Beet chlorosis virus for agroinfection using Gibson Assembly."

German Society for Plant Protection and Plant Health R.S., study group Plant Virology, Bad Herrenalb, 19.03.18-20.03.18, oral presentation: „Das *Rz2* kodierte R-Protein aus *Beta vulgaris* erkennt das *Beet necrotic yellow vein virus* (BNYVV) Transportprotein (TGB1) als Elicitor in *Nicotiana benthamiana.*"

Association of Applied Biologists, Plant Virology conference, Birmingham, UK, 11.04.18-13.04.18, oral presentation: "Identification and characterization of *Beet necrotic yellow vein virus* RNA2 encoded movement-protein TGB1 as avirulence gene targeting resistance gene *Rz2* in *Beta vulgaris.*"

International Institute of Sugar Beet Research, study group meeting Pest & diseases, Dinteloord, Netherlands, 04.09.18-05.09.18, oral presentation: „*Beta vulgaris* resistance protein *Rz2* recognizes *the Beet necrotic yellow vein virus* RNA2 encoded movement protein TGB1 and triggers cell death."

Deutsche Pflanzenschutztagung, Hohenheim, 11. 09.18 – 14.09.18, oral presentation: „Das *Rz2* kodierte R-Protein aus *Beta vulgaris* erkennt das *Beet necrotic yellow vein virus* (BNYVV) Transportprotein (TGB1) als Elicitor in *Nicotiana benthamiana* und löst Zelltod aus."

Plant science student conference, Halle (Saale), 18.06.19-21.06.19, oral presentation: „*Rz2* - a plant virus resistance protein derived from *Beta vulgaris* targets the viral movement-protein TGB1 and is still functional in a heterologous model plant."

Poster presentations

International Plant Immunity Symposium 2018, Göttingen, 14.06.-15.06., poster presentation: „*Beta vulgaris* resistance protein *Rz2* recognizes *the Beet necrotic yellow vein virus* RNA2 encoded movement protein TGB1 and triggers cell death.”

German Society for Plant Protection and Plant Health R.S., study group Plant Virology, Goettingen, 25.03.19-26.03.19, poster presentation: „The anti *Beet necrotic yellow vein virus* resistance protein *Rz2* from *Beta vulgaris* mediates resistance in transgenic *Nicotiana benthamiana.*”

International Advances in Plant Virology 2019, Rome, 29.10.19-31.10.19, poster presentation: “Development of a transgenic heterologous plant system for characterization of an anti-viral plant resistance gene.”

International Institute of Sugar Beet Research, 77th IIRB Congress, 11.02.20-12.02.20, Brussels, Belgium, poster presentation: „*Rz2* - a plant anti *Beet necrotic yellow vein virus* resistance protein derived from *Beta vulgaris* targets the viral movement-protein TGB1 as avirulence gene.”

Eidesstattliche Erklärung

1. 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.

Heidelberg, den 09.08.2021

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

Heidelberg, den 09.08.2021

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