INTRODUCTION

Sugar beet growth and beet sucrose production are highly dependent on an effective control of the viral soilborne 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 sugar beet-growing areas and the long persistence of the soilborne protist vector, Polymyxa betae. 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 in 1952, the causative agent of the disease was detected from infected sugar beet plants in Japan a few years later (Tamada & Baba, 1973). Infection with BNYVV results in a reduced taproot weight, massive proliferation of lateral rootlets, and a brownish discolouration 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).

**Beet necrotic yellow vein virus** belongs to the genus *Benyvirus*. The species has a multipartite plus-sense single-stranded RNA genome. RNA1 contains one open reading frame (ORF) encoding a replicase protein that harbours 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 (RT) domain, responsible for encapsidation and vector transmission (Tamada & 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, which is required for virus pathogenicity, long-distance movement, and symptom development in *Beta vulgaris* (Koenig et al., 1991; Lauber et al., 1998; Tamada et al., 1989). P31, translated from RNA4, is responsible for efficient vector transmission and suppression of gene silencing (Rahim et al., 2007; Tamada & Abe, 1989). There are three different subgroups of BNYVV that can only be differentiated according to sequence polymorphism of the different RNA components and phylogenetic relationships (Koenig et al., 1995, 1997; Koenig & 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 has been extremely high because, 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 1980s (Scholten & Lange, 2000). A second dominant resistance gene, Rz2, was identified in a *B. vulgaris* subsp. *maritima* population in Kalundborg, Denmark, conferring an even 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 & Varrelmann, 2011; Pferdenges & 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 Rz2 gene was identified as encoding a coiled-coil nucleotide-binding and leucine-rich repeat (CC-NB-LRR) protein (Capistrano-Gossmann et al., 2017), representing a major class within resistance (R) genes. R genes confer a dominant resistance by an active recognition of pathogens due to pathogen-derived effectors, namely avirulence (Avr) determinants. In response to pathogen recognition, a hypersensitive response (HR) occurs in the form of localized cell death at the infection site (Jones & Dangl, 2006). Dominant R genes can be classified into two groups, the major class encoding NB and LRR domain (NLR) proteins and all others having a 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 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, which displays the highest variability and is therefore assumed to be mainly responsible for the recognition of target proteins (Moffett, 2009). The central domain of R proteins is characterized by an NB site and an Apaf-1, R proteins, CED-4 (ARC) domain, which is responsible for hydrolysing ATP. These domains are known to be involved in cell death signalling in animals also (Leipe et al., 2004). As indicated by the review of de Ronde et al. (2014), nearly 20 dominant antiviral R genes with this architecture have already been 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 Rz2 gene enabled us to investigate the resistance mechanism in the present study. First, we confirmed that Rz1 and Rz2 represent different BNYVV resistance genes using a resistance test with homozygous breeding lines and an artificial infection method. Using the heterologous host and model plant *Nicotiana benthamiana*, it was possible to trigger a resistance response when Rz2 and BNYVV RNA1–4 were coexpressed in leaf tissue. Moreover, we demonstrate with this plant system that BNYVV TGB1 represents the Avr determinant of R2z. Besides BNYVV TGB1, two distinct TGB1 protein variants from related and unrelated viruses also triggered cell death in the presence of R2z. Thus, this study provides evidence on a sugar beet dominant resistance gene conferring resistance against plant viruses derived from two different families.

## RESULTS

### 2.1 Sugar beet resistance test

Two different BNYVV-resistant sugar beet lines, homozygous for Rz1 or Rz2, and two susceptible lines were tested in a resistance test investigating the ability of BNYVV to infect genotypes with different genetic backgrounds. For inoculation, systemically infected leaf material from artificially infected *Beta macracarpa* 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 soilborne mosaic virus (BSBMV) was tested. Seven-day-old seedlings of each genotype (12 replicates) were inoculated, planted in sterile soil, and grown for 6 weeks before virus detection was conducted by means of double antibody sandwich (DAS)-ELISA (Table 1). The results revealed no detectable virus multiplication in the case of BNYVV infection in lateral roots of both resistant lines but a clear propagation in both susceptible lines. In the case of BSBMV, considerable amounts of virions could be detected
in the Rz1 line and in both susceptible lines but not in the Rz2 line. This indicates that Rz2 confers resistance against BSBMV in addition to BNYVV, whereas Rz1-mediated resistance is restricted to BNYVV.

2.2 | Tissue-specific expression of Rz2

In the study of Liebe et al. (2020), Rz1 tissue-specific resistance was revealed using a monomeric red fluorescent protein (mRFP)-labelled BNYVV clone (BNYVV-mRFP) (Laufner et al., 2018). Following vortex inoculation, no fluorescent signal was detectable in roots of the resistant genotype, indicating root-specific Rz1 expression. In contrast, following mechanical leaf inoculation local lesions 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 susceptible, harbouring homozgyous Rz2, or a combination of Rz1 and Rz2 resistance genes. Mechanical inoculation was performed with plant sap from BNYVV-mRFP systemically infected \textit{B. macrocarpa} leaf material as described below. After 8 days, virus symptoms in the form of yellow spots were visible on the inoculated leaves of all genotypes in similar appearance and local virus replication was detected by means of epifluorescence microscopy (data not shown). At 13 days post-inoculation (dpi), enlargement of the lesions in all genotypes was observed, but a systemic infection was not detectable in any genotype (Figure 1a).

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, either a variety harbouring heterozygous Rz1 and Rz2 or a susceptible variety was used. Confocal microscopy of newly emerging leaf tissue showed fluorescence signals in both genotypes (Figure 1b). This is in accordance with the mechanical inoculation of leaf material, showing local lesions on all genotypes, even in the presence of both resistance genes. In lateral roots of the susceptible genotype, fluorescent signals were detected at 28 dpi, indicative of 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 later time points (up to 60 dpi). These two different inoculation experiments indicated that in leaves a tissue-specific resistance mechanism 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 systemic virus 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 this resistance type is time-independent.

To provide further evidence for the root-specific resistance mechanism by Rz2, tissue-specific expression of Rz2 was measured by quantitative reverse transcription PCR (RT-qPCR). For this purpose, sugar beet seedlings of the homozgyous Rz2 breeding line and 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 compared to leaf tissue was calculated in healthy and infected plants (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. At 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 increase in Rz2 expression over time could be detected in both tissues and treatments, but this induction was lower in infected samples. At each time point after infection, higher Rz2 expression was detected in infected tissue with a ratio between 1.5 and 10.

2.3 | Transient coexpression of BNYVV and Rz2 in \textit{N. benthamiana} induces cell death

As expression analysis in sugar beet indicated root-specific expression of Rz2, further analysis by transient \textit{Agrobacterium}-mediated expression in the host plant was not feasible. As shown by several studies using \textit{Agrobacterium}-mediated inoculation (agroinoculation), R genes can be expressed to recognize the corresponding Avr determinant, resulting in a similar response comparable to that of the original host in the presence of essential cofactors (Bendahmane et al., 2000; Hallwass et al., 2014; Peiró et al., 2014; Tran et al., 2015). Additionally, agroinoculation of BNYVV cDNA and transient expression in sugar beet roots is extremely inefficient (Laufner, Mohammad, Christ, et al., 2018).

\textit{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 \textit{N. benthamiana}, the Rz2

| Genotype               | BNYVV | BSBMV |
|-----------------------|-------|-------|
|                       | Mean A405 | SD   | Mean A405 | SD   |
| Rz1Rz1 + rz2rz2       | -0.09  | 0.08  | 0.41      | 0.22 |
| rz1rz1 + Rz2Rz2       | -0.11  | 0.04  | -0.16     | 0.04 |
| rz1rz1 + rz2rz2 line 1| 0.21   | 0.08  | 0.37      | 0.23 |
| rz1rz1 + rz2rz2 line 2| 0.52   | 0.13  | 0.62      | 0.31 |

Table 1: Results of BNYVV and BSBMV resistance tests with different sugar beet breeding lines after mechanical inoculation.

*Significant difference between treatments, p < .002.
ORF was cloned under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter in a binary vector. Transient expression was achieved by *N. benthamiana* leaf tissue infiltration with *Agrobacterium tumefaciens* cells harbouring the gene of interest. In parallel BNYVV-mRFP (including RNA1–4) was agroinoculated to analyse autoactivation of the HR or a local plant resistance response (Laufer, Mohammad, Christ, et al., 2018). Agroinoculation of BNYVV RNA1–4 resulted in local lesions in the infiltrated patches and systemic infection. In contrast, local cell death was visible at 5 dpi when BNYVV was coexpressed with Rz2 (Figure 3) and no systemic infection was detected. By infiltration of Rz2 alone (Figure 4), no visible symptoms in the form of local cell death were detected. HR-like cell death was also confirmed by fluorescence microscopy, indicated by a strong autofluorescence of dying cells using the green fluorescent
protein (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 precipitate, which is produced during cell death. To confirm that the Rz2 protein is responsible for the resistance reaction, a nontranslatable version of Rz2 was also
coexpressed 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.

2.4 | 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 host plant N. benthamiana. A resistance phenotype in the 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 genes encoded by BNYVV, all individual viral ORFs were cloned under control of the 35S promoter and applied for transient coexpression 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 the form of an HR was only detected by coexpression of Rz2 and the gene encoding the movement protein TGB1 (Figure 4). For control, cell death was monitored by the absence of fluorescence signals in tissues coexpressing 35S-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 using the GFP filter (Koga et al., 1988). HR was verified by DAB staining as a brownish precipitate (Figure 4). For protein expression analysis, Rz2 and TGB1 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 coexpression 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 P25 from potato virus X (PVX) and TGB1 from Alternanthera mosaic virus as well as potato virus M (Lim et al., 2010; Senshu et al., 2011; Voinnet et al., 2000). Furthermore, the PVX-derived P25 also elicits an HR in the presence of the resistance gene Nb (Malcuit et al., 1999). To exclude the ability of HR elicitation by coexpressed VSRs, we coinfiltrated the BNYVV VSR P14, encoded by RNA2, and a second VSR derived from tomato bushy stunt virus, P19. In both treatments, no HR was detected at 4 dpi, excluding the ability of VSR to lead to nonspecific HR (Figure S2).

2.5 | TGB1 from both BSBMV (Benyviridae) and BSBV (Virgaviridae) is 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 is 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 coexpression 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 the form of an HR when coexpressed with Rz2. Cell death was verified by
DAB staining and fluorescence microscopy (Figure 5). Furthermore, protein expression was confirmed by western blot after the addition of a C-terminal HA tag to BSBMV TGB1 (Figure S1). As reported above, the HA tag had no influence on the resistance mechanism.

Because 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 soilborne 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 & Tamada, 2016). To test the ability of Rz2 to mediate resistance towards a broad spectrum of TGB1 variants, sugar beet plants were analysed 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 content could be detected in Rz2 resistant plants, validating the ability of Rz2 to control another virus belonging to the genus Pomovirus. Finally, TGB1 from BSBV was used for coexpression experiments with Rz2. Coexpression of BSBV TGB1 and Rz2 resulted in an HR, which was verified by DAB staining (Figure 5). Expression of HA-tagged BSBV TGB1 was analysed by western blot (Figure S1).

Sequence alignment of the different TGB1 proteins shows that the BNYVV and BSBMV proteins share 74.6% homology, whereas BNYVV TGB1 and BSBV TGB1 only share 22.4%. The TGB1 of plant viruses contains an NTPase/helicase sequence domain, belonging to the helicase superfamily I with seven conserved motifs (Koonin
Sequence alignment of the TGB1 variants from BNYVV, BSBMV, and BSBV indicated 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.

3 | DISCUSSION

3.1 | 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 with previous genetic mapping work (Barzen et al., 1997; Scholten et al., 1999; Scholten & 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.

Previous 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 harbouring Rz2. No virus was detectable in roots after vortex inoculation, but local lesions occurred following mechanical leaf tissue inoculation, comparable to local lesions after infection of susceptible plants (Liebe et al., 2020). Local lesions represent a symptom that is associated with resistance in Chenopodiaceae (Canto & Palukaitis, 1999; Loebenstein, 2009), which is independent of 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 were verified by measuring transcript levels of Rz2 in leaf and root tissue in naturally infected and healthy plants. In this test, the relative expression level of Rz2 in root tissue was remarkably higher compared to that in leaf tissue, indicating tissue-specific expression in roots. Considering that BNYVV is vectored by the soilborne 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 of a resistance host response in aboveground tissue that unsuccessfully or incompletely attempts to limit the spread. The reason for the infrequent movement to

| Genotype               | BNYVV  | BSBV  |
|------------------------|--------|-------|
|                        | Mean A405 | SD     | Mean A405 | 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   |

Note: Absorbance values (A405) determined by double antibody sandwich ELISA on lateral roots of BNYVV- and BSBV-infected and healthy plants of homozygous breeding lines harbouring different resistance genes.

*Significant difference between infected and healthy plants, p < .002.

Figure 6: Multiple sequence alignment of amino acid sequences 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 acids conserved in two (three) sequences are highlighted in grey (black). Motifs I–IV of RNA helicase superfamily I, including a P-loop, according to Koonin et al. (1993), are indicated.
abovementioned tissue, where obviously a deviating mechanism limits the spread, is unknown. Tissue-specific expression of NLRs has been reported in other studies and seems to be related to the tissue where infection occurs (Lai & 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 the tobacco mosaic virus resistance (N) gene before and after viral inoculation. Constitutive N gene expression was detected in noninoculated 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 & Nayak, 2011; Schornack et al., 2005; Van Der Biezen & Jones, 1998). It was shown that, after inoculation, N gene expression was increased 165-fold and the elevated mRNA level remained constant for at least 10 days (Levy et al., 2004). In the case of R2, an increase of expression was also observed, with the highest expression level at 60 days after infection. In contrast to the study of the N gene, where only a single inoculation was used, in the present study continuous infection occurs when plants are grown in infected soil. This is due to the multiple P. betae zoospore-mediated infection cycles. This could be an explanation for the steady increase of the R2 mRNA level over time, whereas the mRNA level of the N gene decreases after 10 days.

3.2 | Agrobacterium-mediated expression of Rz2 in combination with BNYVV leads to an 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 the case of sugar beet, agroinoculation of leaf tissue with BNYVV cDNA cannot be applied as local lesions are formed, and considering the tissue-specific expression of R2, investigations on the natural host are 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; Bendahmane, 1999; Tomita et al., 2011; Tran et al., 2015). 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 promoter like 35S from CaMV can result in an autoimmune reaction (Bendahmane et al., 2002; De Oliveira et al., 2016; Joshi & Nayak, 2011; Takahashi et al., 2012), this was not observed in terms of Rz2 expression under the control of CaMV 35S in N. benthamiana. Even no autoinduction was observed when VSRs were coexpressed.

3.3 | 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 its expression is achieved by subgenomic RNA at an early stage of infection (Morozov & Solovyev, 2003). There are two additional TGB1s known to represent an Avr determinant towards a plant resistance gene: TGB1 from barley stripe mosaic virus (BSMV) (Lee et al., 2012) and P25 from PVX. BSMV TGB1 is recognized by a CC-NBS-LRR 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 acids 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 analysis. The second TGB1 representing an Avr determinant is P25 from PVX, which is the elicitor of the resistance gene Nb (Malcut et al., 1999). The P25 protein 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 from 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 observed in this study by coexpression 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. The TGB1s from BSBMV and BSBV were both recognized, resulting 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 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 genus. For example, both the Phaseolus vulgaris resistance gene I and Pvr4 from pepper mediate resistance against several species in the genus Potyvirus (Cadle-Davidson & Jahn, 2006; Janzak et al., 2009). Similarly, the L gene from pepper, the Tm-2 and Tm-22 genes from tomato, and the resistance gene Krish from sorghum act against several species in the genus Tobamovirus (Moury & Verdin, 2012; Seifers et al., 2012). Moreover, the Rx1 gene recognizes and defends against several viruses in the genus Potexvirus (Baurès et al., 2008). In the study of Baurès et al. (2008) it was shown that Rx1 is able to recognize not only the CP derived from PVX but also the more distinct CPs encoded by white clover mosaic virus, narcissus mosaic virus, and Cymbidium mosaic virus, 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.
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 almost 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 the phylogenetic analysis performed by Adams et al. (2009). According to the gene-for-gene hypothesis it was formerly expected that one R protein specifically recognizes a single Avr determinant. With the data provided, we suggest that recognition of more than one virus species might be the rule rather 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 the 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 colonization, preventing or at least delaying the selection of variants in the population with resistance-breaking abilities. Furthermore, this might be also a hypothesis for the development of resistance, recognizing 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, but also to a variety of other plant viruses, when the recognition is based on a highly conserved TGB1 motif. Although studies exist reporting about elevated virus content in sugar beet genotypes carrying both resistance genes (Galein et al., 2018), TGB1 variants leading to resistance breaking were not yet identified. Population analysis by deep-sequencing of TGB1 from suspicious soils might identify resistance-breaking virus strains and will help to characterize the mode of action between the resistance gene Rz2 and the corresponding Avr determinant TGB1 in more detail.

4 | EXPERIMENTAL PROCEDURES

4.1 | Virus, plant, and soil materials

B. vulgaris subsp. vulgaris homozygous resistant (Rz1Rz1 + rz2rz2 and rz1rz1 + Rz2rz2) and susceptible (rZ1rZ1 + rz2rz2) breeding lines were used 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 + Rz2rZ2) KWS Angelina were used. These genotypes were formerly used for mechanical rub inoculation additionally to the homozygous Rz2 breeding line. For production of virus inoculum, B. vulgaris subsp. macrocarpa was used. N. benthamiana wild-type plants were used for coexpression experiments. All plants were grown and maintained under greenhouse conditions (24 °C/14 hr light, 18 °C/10 hr dark), unless otherwise stated. For agroinoculation the BNYVV A-type and BSBMV full-length infectious clones were used (Laufer, Mohammad, Maiss, et al., 2018).

The origin of soils used in the study is as follows. For expression analysis, plants were planted in soil possessing BNYVV A-type soil, origin Thornhof, Straubing, Germany (Pferdmenges et al., 2009). For the BSBV resistance test, soil derived from Pithiviers, France, was used carrying BNYVV P-type.

4.2 | Cloning of the viral genes in binary plant expression vector

For the generation of constructs for agroinoculation of individual viral genes and Rz2, the full-length clone plasmids of BNYVV RNA1, RNA2, RNA3, and RNA4 and total root cDNA from Rz2 sugar beet were used as template and the binary vector pDIVA (acc. no. KX665539) was used. To ensure high protein expression an optimal ribosomal-binding site was added and the ribozyme sequence was removed. The following sequence accessions served as a basis for primer design: BNYVV KX665336, KX665337, KX665338, and MF476800 and patent EP3011037 for the resistance gene Rz2 (Laufer, Mohammad, Maiss et al., 2018; Törjék et al., 2014); BSBMV cDNA clone and BSBMV TGB1 sequence based on KX352033, KX352170, KX352171, and KX352034, and TGB1 of BSBV based on NC_003519.1) (Laufer, Mohammad, Maiss, et al., 2018). PCR was performed using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) following the manufacturer’s instructions and primers listed in Table S1 (Eurofins Genomics). Cloning was performed using the isothermal one-step Gibson assembly, followed by transforming recombinant plasmids into chemically competent Escherichia coli DH5α cells (Gibson et al., 2009) and verification by Sanger sequencing. For the generation of nontranslatable genes, two TGA stop codons were inserted directly after the start codon of each ORF by PCR mutagenesis. The coding sequence of the HA tag (consisting of YPYDVPDYA) was inserted as a C-terminal translational fusion to the target protein for western blot analysis. TSBV P19 was kindly provided by Edgar Maiss (University of Hannover) (Zilian & Maiss, 2011).

4.3 | Sequence alignment and protein structure prediction

Sequence homology of the different TGB1 variants was determined using the Clustal Omega online service, MultAlin online software
was used for multiple sequence alignment, and visualization was done by GeneDoc. Accession numbers were as follows: 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).

### 4.4 | Plant infection

Mechanical virus inoculation of sugar beet seedlings (vortex inoculation) and mechanical rub inoculation of different sugar beet genotypes were performed using 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 coexpression experiments, recombinant 35S expression vectors with viral genes or *Rz2* were electroporated into *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) strain C58C1, and experiments were conducted as described by Voinnet et al. (1998). *R. radiobacter* suspensions were adjusted to an OD$_{600}$ value of 0.5. In addition to viral genes and *Rz2*, a fluorescent marker gene under 35S control (35S-*dsRed*) was used to confirm cell vitality by transient protein expression and to facilitate detection of the HR. To ensure equal cell densities 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.

### 4.5 | Detection of cell death

Hypersensitive cell death was detected by staining the infiltrated parenchymatic leaf tissue with 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 phosphate-buffered saline (PBS) and incubated for 2–3 hr at room temperature. Then leaves were boiled at 96 °C in 96% ethanol until complete chlorophyll bleaching. Documentation was done using a Nikon camera (Nikon DX, AFS mikro Nikkor 40 mm) on a white light transilluminator table.

Viable and dying cells were further analysed by marker gene fluorescence (DsRed). As a consequence of cell death, the cells showed autofluorescence by the release of cell compartments. This can be visualized in the GFP filter (Koga et al., 1988). Virus replication was detected using the mRFP-labelled BNYVV clone (Lauder, Mohammad, Christ, et al., 2018). Fluorescence was detected with an epifluorescence microscope (Leica DMR) using specific DsRed filters (Emitter HQ 620/60, Beamsplitter Q 585 LP, Exciter HQ 565/30) and a GFP filter (Emitter D 510/540, Beamsplitter 425 DCLP, Exciter D 395/40). Photographs were taken using a Leica DFC camera (DFC300 FX). Virus-mediated fluorescence was analysed using a Leica TSC SP2 confocal laser scanning microscope. mRFP fluorescence was excited at 561 nm and detected at 580–590 nm. Images were processed with LAS-AF software v. 2.6.3.8173.

### 4.6 | Immunodetection of viral CP and HA-tagged proteins in planta

Western blot analysis was conducted using monoclonal high-affinity anti-HA (rat, 1:500, Sigma Aldrich) and mouse anti-rat (IgG-AP, 1:10,000, Sigma Aldrich). Protein extraction was performed as described by Thiel and Varrelmann (2009) and total protein extracts were separated by 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting on a nitrocellulose membrane Protran BA85 (Whatman Schleicher & Schuell). Signal detection was performed by the colourimetric 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, AS-0737) was used. Dilution of samples was increased to 1:200; the remainder of the procedure was performed following the manufacturer’s instructions. BSBV was detected by means of triple antibody sandwich ELISA (DSMZ, AS-0576) following the manufacturer’s instructions. The incubation time for antibodies was 4 hr, and the 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-hr incubation) at 1:1,000 dilution and an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit-AP, Sigma A3687) (1:1,000, 4-hr incubation). Final staining was performed for 60 min. A$_{405}$ values of the healthy controls were subtracted from those of the samples. Samples were considered positive if they exceeded the mean plus three standard deviations of the healthy controls. Statistical independence of the DAS-ELISA values was calculated for each genotype between treatment (healthy and infected) using the unpaired t test by SigmaPlot 14.

### 4.7 | RT-qPCR analysis

*Rz2* mRNA was quantified in total RNA extracts by means of RT-qPCR as described by Fernando Gil et al. (2020). Primers were designed to discriminate between the susceptible line and the Rz2 resistant line. Sugar beet elongation factor 1β (*EEF1B2*, XM_010679634.2) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, XM_010679634.2) were used as reference genes. Amplification was performed using a CFX96 Touch Real-Time PCR Detection System (BioRad) using the iTaq Universal SYBR Supermix (BioRad) and primers listed in Table S2 (Eurofins Genomics). For each time point five biological replicates were tested. Data normalization and relative expression values were calculated using the 2$^{-\Delta\Delta Ct}$ method after Livak and Schmittgen (2001). Statistical independence of the data was analysed using R.
software using the Tukey honestly significant difference (HSD) test.
The statistical independence between root tissue and leaf tissue was calculated for the individual ΔCₜ values at each time point.

ACKNOWLEDGEMENTS
The authors thank S. Vogler, H. Korf, M. Müllender, and J. Lange for technical assistance; G. Steinruence, A. K. Mahlein, H. Tschep, O. Amand, and P. DeDiesbach for discussions; and O. Eini for critical review.

CONFLICT OF INTEREST
All authors declare no conflict of interest.

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.