Effect of sugar beet genotype on the *Beet necrotic yellow vein virus* P25 pathogenicity factor and evidence for a fitness penalty in resistance-breaking strains

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SUMMARY

Beet necrotic yellow vein virus (BNYVV), vectored by *Polymyxa betae*, causes rhizomania in sugar beet. For disease control, the cultivation of hybrids carrying *Rz1* resistance is crucial, but is compromised by resistance-breaking (RB) strains with specific mutations in the P25 protein at amino acids 67–70 (tetrad). To obtain evidence for P25 variability from soil-borne populations, where the virus persists for decades, populations with wild-type (WT) and RB properties were analysed by P25 deep sequencing. The level of P25 variation in the populations analysed did not correlate with RB properties. Remarkably, one WT population contained P25 with RB mutations at a frequency of 11%. To demonstrate selection by *Rz1* and the influence of RB mutations on relative fitness, competition experiments between strains were performed. Following a mixture of strains with four RNAs, a shift in tetrad variants was observed, suggesting that strains did not mix or transreplicate. The plant genotype exerted a clear influence on the frequency of RB tetrads. In *Rz1* plants, the RB variants outcompeted the WT variants, and mostly vice versa in susceptible plants, demonstrating a relative fitness penalty of RB mutations. The strong genotype effect supports the hypothesized *Rz1* RB strain selection with four RNAs, suggesting that a certain tetrad needs to become dominant in a population to influence its properties. Tetrad selection was not observed when an RB strain, with an additional P26 protein encoded by a fifth RNA, competed with a WT strain, supporting its role as a second BNYVV pathogenicity factor and suggesting the reassortment of both types.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV), the causal agent of the virus disease rhizomania, leads to devastating effects on sugar beet production. *In vivo* transmission by the soil-borne plasmodiophoromycete *Polymyxa betae* enables the virus population to survive for decades in contaminated soils (Abe and Tamada, 1986). Following the first description in northern Italy in the late 1950s (Canova, 1959), a world-wide spread of the disease was

noted within a few decades, now involving almost all sugar beet-growing areas (Asher, 1993; McGrann *et al.*, 2009). As a result of the high yield losses that threatened economic sugar production, resistance screening started as early as the mid-1960s (Biancardi *et al.*, 2002). In California, the single dominant resistance gene *Rz1* was selected (Lewellen *et al.*, 1987). *Rz1* plants display a phenotype of quantitative resistance (Heijbroek *et al.*, 1999; Scholten *et al.*, 1994). Resistant plants are characterized by a reduction in virus accumulation in small rootlets, the inhibition of virus spread to the taproot, and the lack of rootlet proliferation and other rhizomania-associated symptoms. As *Rz1* has been used in most of the commercial varieties since its identification (Biancardi *et al.*, 2002), it was anticipated that, sooner or later, resistance-breaking (RB) BNYVV variants would be selected. Other resistance genes, such as *Rz2* and *Rz3*, as well as additional minor genes, are known (Biancardi *et al.*, 2002; Gidner *et al.*, 2005); however, because of the observed yield penalty, currently only *Rz1* and *Rz2* are used in a few genotypes.

The virus consists of four to five plus-sense single-stranded RNA components, depending on strain origin (Tamada *et al.*, 1989). RNA1 and RNA2, encoding for viral housekeeping genes responsible for replication, assembly and cell-to-cell movement (reviewed in Peltier *et al.*, 2008), show high sequence homology between different strains (Saito *et al.*, 1996). The RNA4-encoded P31 protein is responsible for vector transmission and suppression of RNA silencing (Peltier *et al.*, 2008). The protein P25, encoded by RNA3 and well known for its role as a pathogenicity factor and in the control of disease symptoms, is supposed to be an avirulence factor in *Rz1* genotypes (Chiba *et al.*, 2008). Four consecutive amino acids (tetrad) at positions 67–70 of P25, which is subjected to strong positive selection, have been identified (Schirmer *et al.*, 2005). Comparison of the selective constraints on this P25 tetrad with those of other viral avirulence factors revealed the highest ratio between synonymous and nonsynonymous substitution rates (ω) encountered so far in plant viruses (Janzac *et al.*, 2009; Schirmer *et al.*, 2005).

The prediction of BNYVV resistance durability is of great importance for several reasons: (i) the huge impact on crop profitability; (ii) the limited number of natural resistance genes; and (iii) the long persistence of the virus population in the soil. It has been suggested that resistance durability to viruses could be, at least in part, a consequence of the number of mutations in the virus

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genome required for shifts in virulence (Harrison, 2002). However, mutations associated with RB may have negative fitness effects in susceptible genotypes (Aramburu *et al.*, 2010; Carrasco *et al.*, 2007; Desbiez *et al.*, 2003; Janzac *et al.*, 2010; Jenner *et al.*, 2002). Fitness is defined as the ability of a viral variant to reproduce and propagate infectious progeny under certain environmental conditions within a viral quasispecies (Holland *et al.*, 1991). It was hypothesized that resistance genes that impose a high penalty to pathogen fitness would probably be more durable than those that select variants with a lower loss of fitness (Leach *et al.*, 2001). A knowledge of fitness costs not only provides an insight into host–pathogen interactions, but can also be useful to help predict resistance gene durability for resistance management. For example, counter-selection for RB strains was shown by Vera Cruz *et al.* (2000) using susceptible crops grown in rotation. Experimental approaches were suggested that might allow a comparison of the fitness penalty of RB mutations with wild-type (WT) strains for the prediction of resistance durability (Leach *et al.*, 2001). Following the analysis of 20 virus host resistance gene combinations, Janzac *et al.* (2009) identified that the selective constraints applied on the corresponding avirulence factor correlate with the observed resistance durability, and proposed a model for its prediction.

BNYVV can be distinguished by RNA1 and RNA2 marker mutations into four strain groups (Koenig *et al.*, 1995; Schirmer *et al.*, 2005). BNYVV types A and B contain four RNAs and are most widespread. Their origin is unknown, but a mixture of both types in nature is rarely observed (Koenig *et al.*, 1995). Types P and J possess a fifth RNA-encoding protein, P26, which is thought to represent an additional pathogenicity factor (Tamada *et al.*, 1996). Type P only occurs in a few small areas in France, England and Kazakhstan (Harju *et al.*, 2002; Koenig and Lennefors, 2000; Koenig *et al.*, 1997), and type J mainly in Japan and at a single field site in Germany (Koenig *et al.*, 2008). Strains with five RNA components show increased aggressiveness, even in *Rz1* plants (Heijbroek *et al.*, 1999; Tamada *et al.*, 1989), which is suggested to be mainly caused by a possible synergism between P25 and P26 (Pferdmenges *et al.*, 2008). As RNAs-containing strains were identified long before the large-scale cultivation of *Rz1* genotypes, an evolutionary interrelation or selection by *Rz1* has never been speculated. Moreover, these strains do not spread rapidly, but mix with types A and B at the border of infected areas, suggesting the reassortment of both strain types (Koenig *et al.*, 2009a). The occurrence of the more widely distributed strains carrying four RNA components with RB properties in different geographical regions has been associated with specific P25 tetrad compositions: VCHG, VLHG and AYPR (Liu and Lewellen, 2007; Liu *et al.*, 2005; Pferdmenges *et al.*, 2008; Bornemann *et al.*, unpublished results). A positive selection on amino acids 67 and 70 in RB strains by *Rz1* was reported by Acosta-Leal *et al.* (2008). With the use of pseudorecombinants and reverse genetics, it was demonstrated that an

alanine (A)-67 substitution to valine (V) induces RB abilities in a WT BNYVV type A strain possessing P25 with a ACHG tetrad (Koenig *et al.*, 2009b). However, attempts to select RB variants from natural soil-borne BNYVV populations failed, although an increased P25 tetrad variation was found after serial planting experiments in the *Rz1* genotype (Bornemann *et al.*, unpublished results; Acosta-Leal *et al.*, 2010).

In the past, P25 tetrad analysis was mainly based on consensus Sanger sequencing of viral polymerase chain reaction (PCR) amplicons or clones from single plants grown in field soil samples (Schirmer *et al.*, 2005). P25 tetrad diversity in a certain soil (sample) was never analysed, although it is widely accepted that virus populations are continuously subjected to genetic variation, competition and selection, thereby forming a genetic structure, known as a quasispecies (Domingo and Holland, 1997; Domingo *et al.*, 1998; Eigen *et al.*, 1988). The introduction of deep sequencing (DS) technology (Mardis, 2008) now offers the possibility to overcome these limitations. The increased number of reads also allows for the detection of low-frequency mutations and supplies information about the population structure, e.g. avirulence factor variability.

In this study, the P25 tetrad variability of several BNYVV WT and RB strains of different geographical origin was analysed by means of DS to find evidence of whether RB P25 tetrads are present at lower concentration in WT populations. Competition experiments for different BNYVV populations with WT and RB abilities in *Rz1*-resistant and *Rz1*-susceptible genotypes were performed to determine the following: (i) the spatial separation of types A and B; (ii) the possible reassortment of four and five RNA-carrying strains; and (iii) the estimation of the relative fitness of RB variants and further evidence for their selection by *Rz1*. This work represents the first high-density analysis of pathogenicity factor variability in the field of plant virology for the study of several soil-borne BNYVV populations.

RESULTS

Inoculum production of BNYVV populations for competition experiments

Soils with BNYVV populations of varying aggressiveness and different geographical origins were selected and used for the initial determination of the RNA3-encoded P25 (consensus) sequence and encoded tetrad amino acid composition (Table 1). In order to obtain homogeneous virus inoculum in vector zoospores from natural soil, bait plants of a susceptible sugar beet genotype were grown in each soil sample for 6 weeks. BNYVV infection was confirmed by means of enzyme-linked immunosorbent assay (ELISA). The consensus sequence of the P25 tetrad in the BNYVV populations of the soils used, as determined by Sanger sequencing (Table 1), confirmed the consensus tetrad composition of previous

Soil origin	BNYVV type	Consensus P25 tetrad	Rz1 phenotype*
Rovigo (Italy)	A	ACHG	WT
Harting (Bavaria, Germany)	B	AYHR	WT
Pithiviers (France)	P	SYHG	RB
Zeewolde (the Netherlands)	A	AYPR	RB
Dilworth (MN, USA)	A	VCHG	RB

*WT, wild-type (a nonresistance-breaking soil used as a reference soil); RB, resistance-breaking (Bornemann & Varrelmann, 2011; Liu *et al.*, 2005; Liu and Lewellen, 2007; Pferdmenges *et al.*, 2008; Bornemann *et al.*, unpublished results).

findings (Bornemann *et al.*, unpublished data; Bornemann and Varrelmann, 2011). However, DS showed that the different populations contained considerable P25 tetrad variability (see below). The presence of RNA5 in the type P soil, as well as its absence in all other soil samples, was verified by means of reverse transcription-polymerase chain reaction (RT-PCR).

DS control experiment

To determine possible bias during emulsion-PCR (Tsibris *et al.*, 2009) and to allow the calculation of the sequencing error (Gilles *et al.*, 2011), an additional PCR subpool was produced and sequenced in parallel. BNYVV RNA3 cDNAs with VCHG, SYHG and ACHG were each PCR amplified and added to the DS at an input ratio of 85 : 14 : 1 (VCHG/SYHG/ACHG). DS of this control PCR subpool resulted in a total of 14 194 reads containing 82.47% reads of VCHG, 15.11% of SYHG and 0.91% of ACHG, respectively. This demonstrates that the initial ratio was well preserved throughout amplification and that no relevant PCR bias had influenced the depth of DS assay. The total sequencing error for all three plasmids was calculated to be 0.229% at the nucleotide level.

P25 tetrad DS analysis of inoculum BNYVV populations

The amplicon pools were Multiplex Identifier (MID) sorted and clustered on the basis of the different P25 tetrad coding sequences, translated and counted. Tetrad variants at the nucleotide level with a frequency of occurrence below the sequencing error rate of 0.229% were not considered for tetrad frequency evaluation. The total read number of the P25 tetrad coding sequence DS of the source strains varied between 13 104 and 15 473 reads (Table S1, see Supporting Information). Initially, the results of the tetrad proportion evaluation confirmed the dominant tetrad of each BNYVV population as determined by initial consensus sequencing. However, additional variants were detected at significant frequencies (Table 2). The WT type A population from Italy showed the highest tetrad variability in the inoculum of all populations studied. In addition to the dominant ACHG tetrad with 45.1% of all reads, 32.05% of the tetrads were composed of ALHG. Remarkably, although Rz1 RB was not observed (Bornemann and Varrelmann, 2011), 11.7% of the Italian type A population contained the VCHG tetrad, well known for its RB

Table 1 *Beet necrotic yellow vein virus* (BNYVV)-infested field soils applied for bait plant test in the glasshouse to produce virus-infected roots of a BNYVV-susceptible sugar beet genotype for use as inoculum.

Table 2 Frequencies of P25 tetrad compositions in the *Beet necrotic yellow vein virus* (BNYVV) inoculum used in competition experiments, obtained from lateral roots of a susceptible (rz1rz1) sugar beet genotype following deep sequencing analysis. Tetrad variants with frequencies below the calculated sequencing error of 0.229% are not displayed.

BNYVV strains (type, consensus sequence, Rz1 aggressiveness)	Tetrads	%
A ACHG (WT)	ACHG	45.1
	ALHG	32.05
	VCHG	11.7
	TCHG	1.02
	AHHG	0.72
B AYHR (WT)	AYHR	96.51
	AYHK	0.86
P SYHG (RB)	SYHG	95.10
	SYHR	1.83
	SFHG	0.78
	AYPR	97.26
A AYPR (RB)	VCHG	97.34
A VCHG (RB)	VCHG	82.47
Control*	SYHG	15.11
	ACHG	0.91

RB, resistance-breaking; WT, wild-type.

*Read frequencies of P25 plasmid amplicons applied to estimate a possible polymerase chain reaction (PCR) bias of the deep sequencing process.

abilities (Koenig *et al.*, 2009b; Liu *et al.*, 2005). Two other tetrad variants (TCHG and AHHG) were detected at frequencies of about 1%. In the analysed type A sequences, only amino acids 67 and 68 showed polymorphisms. In contrast, only two tetrad variants, AYHR (96.51%) and AYHK (0.86%), were detected in the German type B soil population, displaying variation only in amino acid position 70. The RB types A originating from the Netherlands (consensus tetrad AYPR) and USA (VCHG) showed an unexpected low variability, as only one single RB tetrad composition was detected above the threshold. Three different tetrad compositions were found in the type P population. However, the consensus SYHG composition dominated (95.1% of all reads). Only tetrad amino acids 68 and 70 displayed variation.

Competition experiments with different BNYVV populations

The influence of the plant genotype on the P25 tetrad frequencies was analysed in susceptible and Rz1 genetic backgrounds to

Table 3 Beet necrotic yellow vein virus (BNYVV) inoculum combinations used for infection and in competition experiments in the glasshouse in susceptible and *Rz1* resistant sugar beet plants: mean BNYVV enzyme-linked immunosorbent analysis (ELISA) absorption values measured in small rootlets of sugar beet genotypes after 6 weeks of cultivation and infection rate.

BNYVV competitor strains (type, consensus sequence, <i>Rz1</i> aggressiveness)		Genotype	Mean A_{405}^*	MSD	No. of replications	% infected plants
A ACHG (WT)	B AYHR (WT)	<i>rz1rz1</i>	1.16	0.87	15	80
A ACHG (WT)	B AYHR (WT)	<i>Rz1rz1</i>	0.3	0.41	15	40
A ACHG (WT)	A AYPR (RB)	<i>rz1rz1</i>	0.91	0.28	15	100
A ACHG (WT)	A AYPR (RB)	<i>Rz1rz1</i>	0.34	0.42	15	46.7
B AYHR (WT)	A AYPR (RB)	<i>rz1rz1</i>	0.71	0.29	15	93.3
B AYHR (WT)	A AYPR (RB)	<i>Rz1rz1</i>	0.66	0.53	15	80
A ACHG (WT)	A VCHG (RB)	<i>rz1rz1</i>	0.42	0.49	15	66.7
A ACHG (WT)	A VCHG (RB)	<i>Rz1rz1</i>	0.45	0.62	15	60
A ACHG (WT)	P SYHG (RB)	<i>rz1rz1</i>	1.13	0.41	15	100
A ACHG (WT)	P SYHG (RB)	<i>Rz1rz1</i>	0.67	0.33	15	93.3
B AYHR (WT)	P SYHG (RB)	<i>rz1rz1</i>	1.38	0.37	15	100
B AYHR (WT)	P SYHG (RB)	<i>Rz1rz1</i>	0.59	0.28	15	93.3
A ACHG (WT)†		<i>rz1rz1</i>	0.65	n.a.‡	5	n.a.‡
B AYHR (WT)†		<i>rz1rz1</i>	1.02	n.a.‡	5	n.a.‡
P SYHG (RB)†		<i>rz1rz1</i>	1.77	n.a.‡	5	n.a.‡
A AYPR (RB)†		<i>rz1rz1</i>	1.14	n.a.‡	5	n.a.‡
A VCHG (RB)†		<i>rz1rz1</i>	1.24	n.a.‡	5	n.a.‡

MSD, mean standard deviation of absorption; RB, resistance-breaking; WT, wild-type.

*Calculated from the number of infected plants.

†Infected roots served as inoculum for competition experiments.

‡n.a., not applicable as sample was taken as a mixture of five pots.

determine the relative fitness of the different populations and tetrads present. The combinations applied are displayed in Table 3. A high percentage of susceptible plants displayed infection in all treatments (Table 3). The combination of the WT type A ACHG + RB strain VCHG showed unexpectedly low infection rates in all plants treated. Therefore, only infested plants were used for further processing. The infection rate for susceptible plants infected in combination with type P was 100%, and 93.3% for the resistant variants. Competition experiments were followed by DS analyses of the P25 gene. A total read number of 148 626 over all variants of the competition experiments was obtained. The sequencing depth showed some variation, as between 9133 and 16 252 reads were obtained (Table S1).

Competition between WT strains

WT types A and B are widespread and are known to occur in most cases as separate populations, as well as in neighbouring sugar beet-growing areas, i.e. in the Netherlands and Belgium, with high probability to mix and compete according to their relative fitness (Schirmer *et al.*, 2005). The combination of types A and B WT populations was assayed to understand the missing observation of mixed infection under natural conditions. In susceptible plants, the most frequent AYHR tetrad of the type B inoculum dominated, with 78.65% of all reads (Table 4). In *Rz1*-resistant plants, type A tetrads were found most frequently. The P25 VCHG variant with known RB properties increased from 11.7% in the inoculum to 68.49%, and the consensus tetrad ACHG decreased from 45.1% to 20.97%.

Rz1-resistant sugar beet plants select P25 tetrad compositions associated with *Rz1* RB, and RB variants suffer from a fitness penalty in the susceptible genotype

Mixtures of WT and RB populations with four RNAs should supply evidence for *Rz1* selection of RB tetrads and the fitness penalty of RB isolates in susceptible plants. Both WT populations were combined in competition experiments with all three RB populations possessing either four or five RNA components. As expected, in *Rz1* plants, the RB tetrad variants (AYPR and VCHG) outcompeted all other P25 variants. In mixed infections of the RB strains from the Netherlands with WT types B and A, the AYPR tetrad reached 82.18% and 95.93% of all reads, respectively. In comparison with the frequencies detected in the Italian type A inoculum, the VCHG tetrad, however, did not accumulate to increased density when mixed with the AYPR soil from the Netherlands. Similar frequencies of the RB tetrad were obtained when the RB type A from Minnesota was used for competition with the Italian type A. In susceptible plants, a contrasting situation was observed. The most frequent tetrads of the WT populations, type A ACHG and type B AYHR, clearly outcompeted the RB tetrad AYPR, reaching 59.26% and 96.21%, respectively. The RB P25 variant AYPR showed much lower densities, 22.94% or 1.54%, depending on the competitor. A slightly different situation was observed when WT type A was mixed with the RB population, with a dominant VCHG tetrad in susceptible plants. Here, the ACHG tetrad attained 35% of all reads, which was nearly twice as much as in *Rz1* plants, but was,

Table 4 Beet necrotic yellow vein virus (BNYVV) P25 tetrad compositions following competition of different BNYVV populations in susceptible (*rz1rz1*) and resistant (*Rz1rz1*) sugar beet genotypes obtained by means of deep sequencing*.

BNYVV competitor strains (type, consensus sequence, aggressiveness)	Tetrads	<i>rz1rz1</i> (%)	<i>Rz1rz1</i> (%)
A ACHG (WT) + B AYHR (WT)	AYHR	78.65	0.43
	VCHG	7.88	68.49
	ACHG	6.51	20.97
	ALHG	4.52	7.6
A ACHG (WT) + A AYPR (RB)	ACHG	59.26	0.54
	AYPR	22.94	82.18
	VCHG	14.00	13.32
	ALHG	1.02	1.24
B AYHR (WT) + A AYPR (RB)	AYHR	96.21	1.23
	AYPR	1.54	95.93
A ACHG (WT) + A VCHG (RB)	VCHG	56.45	69.23
	ACHG	35.73	17.72
	AHHG	4.06	—
A ACHG (WT) + P SYHG (RB)	SYHG	40.59	78.44
	ACHG	36.20	6.11
	ALHG	10.88	0.29
	VCHG	3.69	12.16
	AHHG	2.16	—
	SFHG	1.67	—
	SYHR	1.09	—
B AYHR (WT) + P SYHG (RB)	AYHR	58.16	49.35
	SYHG	36.84	47.21
	SFHG	1.38	—
	SYHR	0.86	0.34
	AYHG	0.53	0.39

RB, resistance-breaking; WT, wild-type.

*An error rate of 0.229% was calculated to determine the error occurring during amplification. For detailed explanations, see text.

nevertheless, unable to outcompete the RB tetrad VCHG. In most combinations in which the WT type A was applied, it more or less lost the ALHG tetrad, which was present at a density of 32.05% reads in the initial inoculum. Only in combination with type P did it retain nearly 11% of the ALHG tetrad.

Mixed infection of WT isolates with RB BNYVV type P does not result in clear competition phenotypes between P25 variants

The competition of types A and B with the RNA5-containing type P should supply information about the hypothesized and above-described reassortment phenomenon. Remarkably, a different scenario of the P25 tetrad proportions was always observed when the fifth RNA-containing type P was co-infected with type B (Table 4). No clear dominance of a single tetrad in either resistant or susceptible plants was detectable. In WT type A plus type P mixed infection, a balance of P25 reads with WT and RB tetrads was observed in susceptible plants, suggesting similar fitness and/or transreplication. In *Rz1* plants, however, SYHG nearly outcompeted type A tetrads except VCHG, which shifted from 3.69% to 12.16%.

DISCUSSION

This is the first study to apply DS for high-resolution BNYVV population analysis. DS has been proven to be a valuable tool for the detection of P25 variability. The results show, in most cases, that the sugar beet genotype has a strong selective effect on the accumulation of different P25 tetrads, and that RB tetrad mutations are selected with a loss of relative fitness, with the exception of type P. To our knowledge, this is also the first application of DS in the field of virus–plant interactions to compare the relative viral fitness of several populations with different properties. In mammalian virological studies, DS is already a well-suited method for the detection of the effect of certain types of selection pressure on virus populations, i.e. low-frequency mutations of drug resistance in human immunodeficiency virus-1 (HIV-1) (Wang *et al.*, 2007), bottlenecks of hepatitis C virus (HCV) transmission (Wang *et al.*, 2010) and hepatitis B virus (HBV) diversity (Solmone *et al.*, 2009).

In the current study, using 454 DS technology and *c.* 9000–16 000 reads per base pair, as well as with the consideration of the PCR amplification and sequencing error, mutations at the nucleotide level above a frequency of 0.229% were detectable. Compared with conventional Sanger sequencing, which can only detect mutants that reach approximately 10% of the template, and DS of small RNAs, which results in *c.* 500× coverage (Kreuze *et al.*, 2009), DS read depth is several orders of magnitude higher. DS therefore enabled us to study P25 tetrad diversity, the key for BNYVV population aggressiveness towards *Rz1* resistance, with high sensitivity.

Remarkably, the different populations applied as inoculum showed considerable P25 tetrad variability in DS analysis. No link between RB properties and diversity was detected, as all RB populations displayed relatively few P25 variants and the two WT soil samples showed high (type A) and low (type B) P25 variability, respectively. When these findings are regarded in relation to the strong genotype effect on P25 tetrad accumulation detected in competition experiments, it can be proposed that the high tetrad variability found in the Italian type A soil can be explained by the cultivation of diverse sugar beet genotypes with different BNYVV resistance genetics in the past. All other populations analysed displayed less tetrad variability. This may be regarded as an indication of fitness differences between the populations. A homogeneous population may be an indication for a recent infection of the soil or a uniform selection pressure exerted during previous sugar beet rotations. The finding that different tetrad amino acid positions displayed variation, depending on the population origin and virus type, remains unexplained. Whether this reflects type-specific interactions between virus and host plant genotype, or whether it is caused by epistatic effects between different viral gene mutations (Sanjuán *et al.*, 2004), needs to be analysed in further experiments.

In competition experiments, a shift in P25 variants was observed, indicating that competition between inoculum sources occurred with the use of mixed infections, and was dependent on the combinations of population and plant genotype. First, the huge influence of genotype on P25 variant frequency supports the finding that this protein acts as the main pathogenicity and avirulence factor (Chiba *et al.*, 2008) and the hypothesis that RB variants (VCHG and AYPR) have been selected by *Rz1* (Acosta-Leal *et al.*, 2008; Bornemann and Varrelmann, 2011). The P25 variant shift also suggests that strains possessing four RNAs with different P25 variants do not transreplicate, and that the *Rz1* resistance does not seem to act via a systemic signal, but more likely at the cellular level. This indicates either spatial separation or incompatibility of virus variants. Spatial separation of two populations from the same species seems to be more probable, as it has been demonstrated previously for two other plant virus species (Dietrich and Maiss, 2003; Takahashi *et al.*, 2007). By means of labelling the virus with two different markers, it was shown that two populations of the same virus species (*Plum pox virus* and *Apple latent spherical virus*) distribute separately during colonization of the host plant. However, experimental evidence is missing as to whether two slightly different BNYVV populations perform a similar host colonization strategy. It can be assumed that transreplication with RNA4 and RNA5 strains takes place, and the presence of P26, as a second pathogenicity factor, acting independently of P25, leads to a more balanced proportion of tetrads.

The changes in tetrad frequency as a function of the genotype enable an estimation of the RB properties and relative fitness of the pathogen. The variant shift observed in different treatments suggests a ranking of relative fitness of P25 tetrad compositions depending on the plant genotype. The fitness of the main tetrads of each population in susceptible plants, as deduced from the competition experiments, is AYHR > SYHG > VCHG > ACHG > AYPR > ALHG. The relative fitness of RB mutations, as determined in competition experiments, might represent a good estimation of resistance durability. Obviously, in *Rz1* plants, all RB P25 tetrads from strains with four genome components had a huge selective advantage over WT strains. Because no clear competition effects were observed between WT strains and the RNA5-containing type P, it is assumed that RB populations with four RNAs might have gained higher relative fitness relative to type P strains in resistant plants as a consequence of direct *Rz1* selection. However, in order to obtain a good estimation of the relative RB properties in *Rz1* plants of different populations, competition experiments between RB strains with different P25 compositions are required.

The transferability of the population analysis to natural field conditions also needs to be evaluated. The inoculation in glass-house experiments was performed with minute amounts of lateral roots and cannot be compared directly with the situation in field soils. Although the number of primary infection sites from inde-

pendent penetration of *P. betae* zoospores loaded with BNYVV is unknown, the tetrad shift occurred quite rapidly during only 6 weeks of cultivation. Therefore, it can be deduced that each sugar beet growth period in the field with a certain genotype may influence considerably the genetics of the BNYVV soil population. Selectively propagated P25 variants become a part of the vector population as *P. betae* cystosori and will infect one of the next sugar beet plants grown in the rotation until the spores die. In a specific field soil, therefore, the proportion of tetrads with different properties may reflect the genotype history. We conclude that the P25 tetrad composition of a certain population is a genetic footprint of the sugar beet genotypes grown in the past in a certain soil. Possibly, even small changes in the ratio of different sequence variants may be used to predict the resistance durability of other natural resistance sources, such as *Rz2* and *Rz3*, in the future, a pro-active approach already suggested by Leach *et al.* (2001) for fungal and bacterial pathogens.

By the combination of this population analysis with previously performed resistance tests (Bornemann and Varrelmann, 2011), it can be concluded that 11.7% of the VCHG tetrad in the Italian type A soil is not sufficient to enable the population to overcome *Rz1*. The type A and B competition experiment in *Rz1* plants clearly shows an increase in P25 with VCHG composition, although neither population is able to overcome *Rz1* in single infections (Pferdmenges *et al.*, 2008). Therefore, we suggest that a specific tetrad needs to become dominant in a population to determine its RB properties and fitness. However, future mixed infection experiments with WT isolates are required to determine whether synergistic effects can be found. In addition, the comparison of diverse virus isolates most suitable in serial planting experiments, followed by population DS analysis, is important to confirm this hypothesis. The variable fitness of WT types A and B during host plant colonization observed in this study may explain why the two types do not mix rapidly under natural conditions in field soils. A spatial separation of strains, speculated here, supports this conclusion. In extensive monitoring studies, as performed by Schirmer *et al.* (2005), no type A and B mixed infections were detected in natural soils. The observation of no clear WT tetrad dominance in susceptible plants when WT isolates compete with RNA5-containing type P supports the co-replication of RNA5 by the two isolates in mixed infections, although this needs to be confirmed in more detailed analysis.

One important question was raised by the observation that, to date, in addition to RNA5 strains, only type A populations carrying four RNAs have been observed to overcome *Rz1*. The results obtained in this study clearly show that the type B BNYVV population, compared with the type A (WT) analysed here, not only possesses another P25 tetrad including different variable amino acids, but, in addition, displays a relatively higher fitness in susceptible plants. It needs to be determined experimentally whether the rarely detected type B P25 variants in the population analysed

possess RB properties, following their isolation on local lesion hosts.

Especially in the case of extremely persistent soil-borne viruses, the DS approach is an excellent technique for the identification and enrichment of newly selected variants under experimental conditions or in the field, their functional characterization, and for the detection and introgression of alternative resistance sources, before RB strains spread widely. The use of different resistance sources is necessary to control the disease and to inhibit the selection and accumulation of RB strains. Moreover, DS technology allows the monitoring of selection pressure. Further studies need to be addressed in order to show the influence of the *Rz2* resistance gene on the virus variability. As it is still unclear whether the *Rz2* gene possesses the same target as *Rz1*, the BNYVV *Rz2* avirulence gene must be identified first.

Host plant resistance genes from natural resources are available, but their number is finite. Large-scale cultivation of crop plants with uniform resistance genetics may currently be the method of choice for profitable production, but exerts selection pressure on the pathogen population. DS, in association with experiments that determine the relative fitness of the virus, is a valuable tool for the analysis of rapid changes in the avirulence gene of a plant pathogen adapting to resistance genes. If resistance factors and corresponding avirulence genes are known, this method can be easily adapted to other host–pathogen combinations in order to increase the lifetime of resistance sources when implemented into resistant management strategies.

EXPERIMENTAL PROCEDURES

Preparation of inoculum

To obtain sugar beet lateral roots infected with divergent BNYVV strains, a bait plant test with five different field soils containing different BNYVV types (Table 1) was conducted in the glasshouse. A susceptible sugar beet genotype (see below) was cultivated in these soils for a 6-week period. Roots were harvested separately, washed gently under running tap water and air dried.

The soil sample from Rovigo (Italy) was collected in an area with a history of 40 years of sugar beet breeding activities, including the selection of rhizomania resistance (Biancardi *et al.*, 2002). This soil, as well as the German type B soil, has so far been unable to overcome *Rz1* (Bornemann and Varrelmann, 2011; Pferdmenges *et al.*, 2008). The type P soil was collected in the Pithiviers area from a patch with strong rhizomania symptoms in *Rz1* plants and has the ability to overcome *Rz1* (Bornemann and Varrelmann, 2011; Pferdmenges *et al.*, 2008). The two RB soils containing BNYVV populations with four RNA components were collected in Zeewolde (the Netherlands) (Bornemann *et al.*, unpublished data) and Dilworth (MN, USA). The Minnesota-derived BNYVV with the P25 VCHG tetrad composition has been shown to overcome *Rz1* in previous experiments (B.-L. Lennefors, Syngenta Seeds, Landskrona, Sweden, personal communication). The Dutch soil with AYPR as the consensus P25 tetrad

sequence possesses similar aggressiveness (Bornemann *et al.*, unpublished data).

Competition experiments in the glasshouse with different sugar beet genotypes

The different BNYVV-infected lateral roots were used as inoculum of sterilized soil in different combinations (Table 3). Equal minute amounts (50 mg/pot) of the different treatments were crumbled onto the sterile soil in half-full 700-mL plastic pots. After the pots had been filled to capacity with sterile soil, 10-day-old sugar beet plants were transplanted. A susceptible sugar beet genotype (*rz1rz1*) and an *Rz1*-resistant genotype (*Rz1rz1*) were used, each with seven plants per pot. Both genotypes have been described previously (Bornemann and Varrelmann, 2011; Liu *et al.*, 2005). Fifteen replications for each combination resulted in a total of 240 pots. Plants were kept in the glasshouse at 24 °C (day)/18 °C (night) with a 10-h photoperiod of additional light. To control damping-off diseases, plants were sprayed with the fungicide Tachigaren 70 WP (Sumitomo, Tokyo, Japan) at 90 g per 100 000 plants. After 6 weeks, the plants were harvested as described above. One part of the harvested roots was stored at –80 °C until nucleic acid preparation. The other part served to determine the concentration of BNYVV by ELISA, performed as described previously (Saito *et al.*, 1996).

Detection of BNYVV

Five root samples of each variant, which were considered positive by ELISA, were selected for RNA preparation. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The detection of the presence of BNYVV was performed by means of RT-PCR partial amplification of the RNA3- and RNA5-encoded P25 and P26 genes, respectively, as described in Bornemann and Varrelmann (2011). RT-PCR chemicals and conditions were the same as described in Pferdmenges *et al.*, 2008. The presence of BNYVV RNA5 was detected with primers that amplified the entire P26 open reading frame (ORF) (nucleotides 447–1103 of GenBank Accession No. U78293). To determine the consensus P25 tetrad composition before starting DS (see below), one PCR sample per variant was purified using SureClean reagent (Bioline, Luckenwalde, Germany) and sequenced by commercial capillary Sanger sequencing (Eurofins MWG Operon, Ebersberg, Germany) with specific primers.

Sample preparation for DS

Five RNA samples of each treatment were chosen for DS analysis. For RT-PCR amplification and DS of partial P25, containing the tetrad coding sequence (nucleotides, 465–824; product length, 360 bp; Accession No. M36894), specific primers were designed (Upper-465, 5'-GCAGTTTATGATTTAGGGCA-3'; Lower-824, 5'-ACGATCATAGTGATGACGA-3'). Oligonucleotides were supplied with 25 3'-adaptor nucleotides for on-bead amplification and DS (Roche, see below) and a unique MID of 10 nucleotides in length (provided by the sequencing company). The whole cDNA reaction mixture of 10 µL was divided to obtain three PCR samples per RNA (15 RT-PCRs per variant), and the following PCR programme was performed: 96 °C for 2 min, 36 cycles of 96 °C for 45 s, 52 °C for 1 min,

72 °C for 1 min, and a final synthesis for 10 min at 72 °C. PCR products were visualized on agarose gels and subsequently pooled. An amount of 150 ng of DNA was applied for DS. The pooled PCR products were cleaned with QiaExII (Qiagen), following the manufacturer's instructions, to remove primer dimers. Fluorometric quantification and adjustment to equal concentrations were performed before all different RT-PCR subpools were finally pooled for sequencing. To determine the DS error, plasmids (pBluescript, Stratagene, Waldbronn, Germany) containing full-length BNYVV RNA3 cDNAs from isolates with known tetrad compositions (VCHG, SYHG and ACHG) were used as templates for PCR amplification with specific primers with one MID for all three variants. For DS PCR, products of all three variants were quantified and adjusted to an input ratio of 85:14:1 (VCHG/SYHG/ACHG) by the sequencing company. DS was conducted using 454 GS FLX sequencing platforms (Roche, Mannheim, Germany) at Eurofins MWG Operon. The 454 libraries were amplified on beads with emulsion-PCR according to Roche standard protocols, and deep sequenced in both sense and antisense orientations, as described in Margulies *et al.* (2005).

The emulsion-PCR and sequencing-induced error was calculated as follows. Nucleotide sequences of the control reaction were aligned initially with the program MAFFT (<http://mafft.cbrc.jp/alignment/server/>) and visualized with the program Jalview (<http://www.jalview.org/>). Furthermore, scripts were developed to perform clustering of the three tetrad variants and counting of the mutations, insertions and deletions. The percentage of insertions and deletions (total DS error) was determined by calculating the ratio of the total number of read nucleotides (including incomplete reads) and the total number of errors (both per variant) (data not shown). Bioinformatic evaluation of the sequencing results of the amplicon pools, including MID sorting and clustering according to P25 tetrad coding sequences, translation and counting, was performed by the sequencing company.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Deep sequencing analysis read numbers obtained in competition experiments with different *Beet necrotic yellow vein virus* (BNYVV) strains and sugar beet genotypes.