Diversity of viruses and viroids in the rhizosphere of common bean cultivars differing in resistance to the fungal root pathogen *Fusarium oxysporum*

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Abstract

The rhizosphere microbiome plays a key role in plant protection against soil-borne pathogens. Plant breeding for resistance against soil-borne pathogens can alter the rhizosphere microbiome. However, most studies have focused on bacterial and fungal communities, leaving the role of the virus and viroids unassessed. Here, we tested the influence of resistance breeding on the composition of rhizosphere viruses and viroids. By analyzing metatranscriptomes from the rhizosphere of common bean (*Phaseolus vulgaris*) cultivars with varying resistance to the soil-borne pathogen *Fusarium oxysporum*, we recovered sequences representing 78 and 23 novel populations of viruses and viroids, respectively. We compared the abundances of these infectious agents across the different cultivars and found that the *Fusarium*-resistant cultivar harbored >1.2 times more viroids and a more different composition of viroids and viruses than less resistant plants. Given their role in interfering with host metabolism and their potential influence on plant-fungi associations, our study suggests that changes in the rhizosphere infectome are an important consideration in breeding for resistance against soil-borne pathogens.

Key words

rhizosphere microbiome; plant-microbe interactions; infectome; food security

The rhizosphere is the first line of defense against soil-borne pathogens (Mendes et al., 2013), from which plants are able to recruit protective microbes when they are attacked by pathogens (Berendsen et al., 2012). Recent studies have shown that plant breeding can change the composition of the rhizosphere microbiome (Mendes et al., 2018; Wei and Jousset, 2017). To date, however, rhizosphere microbiome studies have almost exclusively focused on bacterial and fungi, with little attention given to other microbiome members including protists, viruses, and viroids. The latter two microbiome members have the potential to affect plant-mycorrhizal interactions in the context of disease (Andika et al., 2017; Wei et al., 2019). For example, viral infections can convert a phytopathogenic fungi into a beneficial endophyte (Zhang et al., 2020), whilst viroids have been experimentally demonstrated to reduce the growth and virulence of different phytopathogenic fungi, including *Fusarium* (Wei et al., 2020, 2019). However, how the composition of rhizosphere viruses and viroids differ between disease susceptible and resistant plants remains unassessed.

Here, we tested the hypothesis that the composition of viruses and viroids in the rhizosphere microbiome of the common bean (*Phaseolus vulgaris*) differed between cultivars that were resistant versus susceptible to a soil-borne pathogen. To this end, we analyzed the RNA sequences obtained from the rhizosphere metatranscriptomes of three common bean cultivars that span a gradient of disease susceptibility to the widespread, root pathogenic fungus *Fusarium oxysporum*. These cultivars were either resistant (cultivar IAC Milenio (Carbonell et al., 2014)), moderately resistant (cultivar BRS Estilo (Melo et al., 2010)), or susceptible (cultivar IAC Alvorada (Carbonell et al., 2008)). To simulate realistic field conditions for rhizosphere selection of plant-microbe interactions, the plants were not infected with *Fusarium* in the experiment, as artificial infections interfere with the natural process of root colonization by soil-borne microbes. Metatranscriptomes were obtained from the rhizosphere of three replicates from each of the three cultivars grown in pots under controlled greenhouse conditions. Plants were harvested at the early flowering stage, which is most representative for our studies because rhizosphere microbial communities converge once they reach this stage (Chaparro et al., 2014).

To recover viral sequences, metatranscriptomes were quality filtered and assembled with IDBA-UD (Peng et al., 2012). Contigs (>3,000bp) were then screened with VirSorter2 (Guo et al., 2021), Seeker (Auslander et al., 2020) and DeepVirFinder (Ren et al., 2017). These analyses resulted in 251 sequences predicted as viruses that clustered into 155 viral operational taxonomic units (vOTUs), *i.e.* sharing >95% average nucleotide identity across 80% of the length of the shorter contig (Roux and Emerson, 2022). Only sequences classified by at least two of these tools were considered in further analyses. The vOTUs were further quality controlled following the

standard operational procedure using CheckV (Nayfach et al., 2021), including annotations of encoded functional domains (Supplementary Material and Methods). A total of 78 sequences were subsequently considered as bona fide viral sequences. Half of these sequences were predicted to be medium- or high-quality genomes, with 32% expected to be >90% complete genomes (Supplementary Table 1). Forty-two sequences were further confirmed as RNA viruses based on the presence of viral RNA-dependent RNA polymerase (RdRp) identified with Palmscan (Edgar et al., 2022). Based on their RdRp sequences (*i.e.*, palmprints), vOTUs 54 and 57 represented variants from the same clade. The same was true for vOTUs 63 and 64. All the other RNA virus sequences were inferred to represent distinct species (Supplementary table 1). Taxonomic classifications and host predictions were inferred based on sequence similarity against the NCBI Virus database (Hatcher et al., 2017).

The viral RNA genomes were found to represent novel populations closely related with viruses infecting plants, phytopathogenic fungi, insects, invertebrates or rhizobacteria. They were classified as Leviviricetes (26.92%), Picornavirales (7.69%), Sobemovirus (2.56%), Tombusviridae (2.56%), Totiviridae (2.56%), Endornaviridae (2.56%), including two Phaseolus vulgaris Alphaendornaviruses (PvEVs), Amalgaviridae (1.28%), Partiviridae (1.28%), Reovirales (1.28%), and other reference Riboviria viruses (5%) still unclassified at higher taxonomic ranks. The two genomes classified as PvEV were predicted to be high-quality (>90% completeness) and produced whole genome alignments with 100% coverage and ≥97% identity against reference PvEV genomes.

To recover viroid sequences, the assembled contigs (>300bp) were screened with Vdsearch, a tool tailored to identify viroid covalently closed circular RNAs (Lee et al., 2022). We found 23 viroid-like sequences, hereafter referred to as viroids for simplicity (Supplementary Table 2). Twelve were symmetric, containing one ribozyme per polarity, and 11 were asymmetric, containing a ribozyme only in one polarity. All but one of the symmetric sequences contained well described autocatalytic hammerhead ribozymes (HHRs), with HHR3 being the predominant form among them. The symmetric viroid sequence that was the exception contained a twister ribozyme (twister-P1), which has been recently described (Roth et al., 2014), and is less commonly distributed in nature as the HHR pattern (Lee et al., 2022; Roth et al., 2014). Among the asymmetric sequences, HHRs were also predominant, and one of them matched the twister-P1 motif. Generally, viroids had >70% of nucleotides self-paired and several even had perfect rod-shaped conformations. The size and structure of the identified sequences all matched expected viroid-like patterns (Lee et al., 2022; Roth et al., 2014).

We then compared the abundances of viruses and viroids between the cultivars and found differences in their composition in the rhizosphere (Figs. 1 and 2). We found that resistance breeding was positively correlated with the total abundance of viroids in the rhizosphere. Viroids were >1.2-times more abundant, on average, in the *Fusarium*-resistant cultivar compared to the susceptible ones ($p<0.05$; Fig. 1b). This effect was not detected for the total fraction of viral sequences (Fig. 1a). Nevertheless, the abundance of individual viral populations was influenced by plant resistance to *Fusarium*, with most of the changes being observed when comparing resistant against susceptible cultivars ($p<0.05$; Fig. 2). Most vOTUs were related with uncultivated viruses that were first detected in Chinese soils (Supplementary Table 1), including the rhizosphere (Chen et al., 2022). The differences in the abundance of vOTUs were observed in all pairwise comparisons, but greater impacts were detected comparing the resistant against the susceptible cultivar ($p<0.05$; Fig. 2). Similarly, individual populations of viroids also diverged significantly when comparing the *Fusarium*-resistant cultivar against the moderately susceptible and susceptible cultivars ($p<0.05$; Fig. 2). Although the total abundance of viroids was greater in the resistant cultivar (Fig. 1b), three viroid populations were enriched in the more susceptible cultivars (Fig. 2). These results highlight that viral and viroid diversity in the rhizosphere may be driven by different mechanisms, likely arising from the many possible trophic interactions between plants and their microbiomes.

To explore the consequences of resistance breeding for the community composition of viruses and viroids in the rhizosphere, we performed a co-occurrence analysis for each cultivar. This analysis allowed us to assess the stability of rhizosphere interactions under resistance breeding and identify keystone taxa (Freeman et al., 1991; Salavaty et al., 2020), which are typically considered to be disproportionately important for the network (Röttjers and Faust, 2018). We estimated the co-occurrence networks only using pairwise correlations between viruses and viroids that were statistically significant when correcting for multiple comparisons for each virus and viroid (see Methods). We found that the number of correlations between viruses and viroids in the rhizosphere of resistant cultivars was higher than for other cultivars, with nearly twice as many correlations as the susceptible cultivars (Fig. 3; $p < 0.001$). From the network, we extracted topological features that described the stability of co-occurrence patterns, such as betweenness centrality and degree scores (Supplementary Table 3). Based on these metrics, the rhizosphere of resistant cultivars was more stable than the other cultivars, as it was composed of nodes with significantly higher values of both betweenness centrality and degrees (Fig. 4; $p<0.05$). Moreover, the identity of keystone viruses and viroids was completely altered in response to resistance breeding (Supplementary Table S3). Most keystone taxa represent novel viral populations, and some included members from the

Leviviricetes and Picornavirales, which are groups that infect prokaryotes and eukaryotes (including plants), respectively. Compared to the other genotypes, the much lower betweenness centrality scores in the susceptible cultivar suggests absence of keystone nodes in this network (Supplementary Table 3).

Overall, 94% and 95% of the associations found in the resistance network were absent from the moderately resistant and the susceptible cultivars, respectively. These results consistently support the finding that resistance breeding altered the composition of viruses and viroids in the rhizosphere. Resistance against soil-borne pathogens led to more unique, complex, and stable interactions between rhizosphere viruses and viroids (Figs. 3 and 4). This pattern mirrors that observed for rhizosphere bacterial communities analyzed in these systems (Mendes et al., 2018) and elsewhere, where more complex networks have been proposed to represent communities that are better able to resist pathogen invasions (Mallon et al., 2015; Wei et al., 2015).

Here we found two lines of evidence that crop breeding for resistance against a specific fungal pathogen was also associated with large changes in the rhizosphere-associated "infectome", that is, the collection of infectious agents composed of viruses and viroids. First, more resistant plants generally harbored more viroids and had altered infectome composition (Figs 1 and 2). Second, co-occurrence patterns dramatically changed across the gradient of disease resistance, with resistant cultivars showing evidence of having more stable infectomes (Figs. 3 and 4). Plant traits can influence the rhizosphere infectome by selecting microbial hosts from the soil stock and also by releasing infectious agents replicated in their cells to the rhizosphere. For example, we found that the core infectome in the resistant cultivars had more active bacteriophages than the other cultivars, and the recovery of complete PvEVs genomes from the rhizosphere in our experiment further confirms that viruses produced in plant cells can be released by the roots. The same mechanism can apply to viroids, where their release into the rhizosphere is facilitated by their accumulation in root cells (Góra-Sochacka et al., 2019; Wang et al., 2011). Furthermore, plant viruses and viroids can be transmitted vertically (Matsushita et al., 2018; Mutuku et al., 2018), so, in addition to the soil stock (horizontal transmission), the rhizosphere infectome can be influenced by seed-borne infectious agents (vertical transmission) (Johnston-Monje et al., 2021; Simonin et al., 2022). Therefore, plant genotype should affect rhizosphere infectome composition and that may alter the interactions between plants and their soil-borne pathogens.

Our study outlines the importance of considering changes in the rhizosphere infectome when breeding for resistance against soil-borne pathogens. Plant pathogens represent a significant threat to crop production, contributing, on average, to 11-30% of global yield losses (van Esse et al., 2020). Resistance breeding is one of the most important and sustainable strategies to protect against

pathogens. However, resistance breeding is challenging given the complex arms-race dynamics involving plant-pathogen interactions, and often has short-term durability due to pathogen evolution (McDonald and Linde, 2002; Nelson et al., 2018). It is important to explore further the direction of causality between the rhizosphere infectome and plant protection against soil-borne pathogens. For example, some viruses and viroids may be able to infect phytopathogens or influence the composition of the rhizomicrobiome, both of which may confer protection to plants against colonizing pathogens. At the same time, the composition of these viruses and viroids can also be influenced by the plant genotype, that determines the recruitment of specific bacteria and fungi. Hence, bidirectional causality may also be considered in this case. In our system, the mechanisms determining the differences in infectome composition still needs to be determined. Nevertheless, we provide evidence suggesting that a better understanding of the role of viruses and viroids in the rhizosphere, as well as their response to plant breeding against soil-borne pathogen, are an important consideration for breeding programs aimed at improving food security.

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Author contribution

LPPB, SMT, JOS, RM and LWM designed the research; LPPB and LWM performed the research. LPPB, BL and AJT analyzed the data; LPPB and AJT wrote the manuscript. All authors read and approved the final manuscript.

Data availability

The metatranscriptomes used for this analysis are available at the MG-RAST server (http://v4-web.metagenomics.anl.gov) under the project ‘Common Bean Rhizosphere Metatranscriptome’ (mgp20659). Assembled virus and viroid sequences are publicly available on PATRIC server in the workspace named RhizoB2023.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Figure 1 – Abundance of viral and viroid sequences in common bean cultivars differing in resistance to the fungal root pathogen *Fusarium oxysporum*. Boxplots show the relative abundance of (a) viral and (b) viroid sequences in the rhizosphere metatranscriptomes of *P. vulgaris* cultivars that are either resistant, moderately resistant or susceptible to *F. oxysporum*. Different letters indicate statistically significant differences between cultivars according to a linear model ($p<0.05$). Horizontal lines denote medians with boxes spanning inter-quartile range and whiskers extending to minimum and maximum values.

Figure 2 - Composition of viral and viroid communities in common bean cultivars differing in resistance to the fungal root pathogen *Fusarium oxysporum*. Taxonomic classification of viral populations was inferred based on similarity searches against the NCBI Virus database (Hatcher et

al., 2017), and the best hits were used to infer putative hosts for the recovered viral sequences. Viroids were classified according to the ribozyme domains identified in both plus and minus strands using Vdsearch (Lee et al., 2022). Italics denote sequences confirmed as RNA viruses and viroids from those representing transcripts of DNA viruses or incomplete RNA virus genomic sequence not encoding sufficient information for confident classification (see Supplementary Material and Methods and Supplementary Table 1). Taxonomy of viruses and ribozyme domains of viroids are indicated between parentheses (see Supplementary Tables 1 and 2). For symmetric ribozymes, semicolons separate identifications of different strands. HHR: hammerhead ribozyme. Populations of viruses and viroids that respond to resistant breeding were detected by pairwise comparison of differential abundances among *P. vulgaris* cultivars with different levels of susceptibility to *F. oxysporum*. We used the effect size estimated from linear discriminant analysis (LEfSE) to detect populations of viruses and viroids that were statistically different among cultivars (see Supplementary Material). Relative abundances were obtained by normalizing read counts by sequence length and total abundances using the LEfSE software according to default parameters (Segata et al., 2011). * denotes populations that statistically differ in abundance between cultivars ($p<0.05$).

Figure 3 - Potential rhizosphere-associated virus-viroid infectome. Co-occurrence analysis detected strong positive associations between viral (ellipse) and viroid (triangle) populations. Associations (*i.e*. edges connecting nodes) were only visualized if the strength of a Spearman rank correlation between virus and viroid populations was > 0.99 or < -0.99, which was statistically significant when adjusted for the multiple comparisons calculated for each population ($p=0.0004$). Nodes (indicate populations of viruses and viroids) with the top-10 highest betweenness centrality across all three networks, *i.e*. keystone populations, were scaled in size to represent their importance from greater (largest node) to smallest (Supplementary Table 3). Italicized labels denote sequences confirmed as RNA viruses and viroids from either DNA or unclassified viruses (see Supplementary Material and Methods and Supplementary Table 1). Taxonomy of viruses and ribozyme domains of viroids were indicated where possible between parentheses (see Supplementary Tables 1 and 2). HHR: hammerhead ribozyme; na: polarity without ribozyme.

Figure 4 – Infectome stability increased with disease resistance. Boxplots show the (a) betweenness centrality and (b) degree of nodes from the networks in Fig. 3. This analysis was performed using the nodes with the top-10 highest betweenness centrality and degree (see Supplementary Table 3). Different letters indicate statistically significant differences between

cultivars according to a linear model ($p<0.05$). Horizontal lines denote medians with boxes spanning inter-quartile range and whiskers extending to minimum and maximum values.