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Formation of a pool of different-sized virus-specific short RNAs in *Solanum tuberosum*

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Motivation and Aim: Potato plants are an important agricultural crop. However, potatoes can be affected by various diseases, including viral ones. RNA-based technologies, including external application of double-stranded RNA (dsRNA), can be used to protect crops from pests. While it is generally accepted that the mechanism of dsRNA-mediated antiviral RNA silencing reflects natural RNA interference (RNAi) defense against RNA viruses, there is little direct evidence to support this notion.

Methods and Algorithms: Potato virus Y strain NTN (PVY) was propagated on potato plants of the Manhattan variety. For the experiments, we used two-week-old Indigo potato plants grown in soil from seedlings propagated in vitro. For each experimental condition, potato plants were treated with dsRNA solution or buffer, and the next day they were inoculated with buffer or PVY and allowed to grow in a controlled environment chamber. The photoperiod of which was 16/8 hours day/night, relative humidity 40 % and light flux density 250 μmol m-2 s-1. Leaf samples were taken 7 days after the plants were treated with the potato virus.

Small RNA was prepared for sequencing using the NEXTFlex Small RNA-Seq v3 kit (Bio Scientific, Gymea, Australia) which were then sequenced using the Illumina NovaSeq 6000 with a single-read length of 50 nucleotides. After preparing the reads, reads were mapped to the PVY-NTN genome, with zero mismatches. Mapping results were visualized. To analyze viral siRNAs, the reference sequences of PVY-NTN genomes were used to map 18–30 nt reads from each library. The sorted sRNAs were then counted by size, polarity, and 5′-terminal nucleotide identity (5′A, 5′C, 5′G and 5′U).

Results: High-throughput sRNA sequencing was conducted to analyze the sRNA populations resulting from the application of dsRNApvy and the virus. Three treatments were examined: (i) PVY, (ii) dsRNApvy, and (iii) PVY + dsRNApvy in both treated and untreated leaves. The analysis revealed that PVY-infected plants contained virus-specific siRNA species of 21 nt and 22 nt in size, with 21 nt species being predominant, consistent with sRNA profiles induced by other RNA-containing plant viruses. In contrast, dsRNA-treated leaves of PVY-uninfected plants showed a range of sRNAs in length from 18–30 nt, suggesting a non-canonical origin. However, all samples exclusively matched the PVY genome segment used for dsRNA design, indicating a sequence-specific origin for the non-canonical sRNAs. Investigation into systemic movement of these non-canonical sRNAs showed that dsRNA applied to bottom leaves could move and accumulate in upper untreated leaves. In plants with deficient systemic movement, sRNA accumulation in untreated leaves was minimal. These findings suggest

that systemically moving dsRNA can be processed into non-canonical sRNAs in upper untreated leaves similar to treated leaves.

Upon aligning the mRNA with the PVY-NTN genome sequence, it was observed that in dsRNApvy-treated leaves of uninfected plants, the number of reads with sequences complementary to the viral genome (referred to as antisense mRNAs) exceeded the number of opposite strand-specific reads (sense mRNAs) across all mRNA size classes (18–30 nt). A similar pattern was noted in untreated leaves of dsRNApvy-treated uninfected plants. In PVY-infected plants, both strands of the entire PVY genome contributed to virus-induced mRNAs detected in inoculated and non-inoculated (systemically infected) leaves, albeit with a bias towards the sense strand.

In addition to mRNA size, the 5'-terminal nucleotide is crucial in determining the selective loading of mRNA into specific AGOs. To assess the potential number of RISC-producing mRNAs that could actively suppress PVY, the relative abundance of four different 5′ nucleotide identities in antisense mRNAs was examined. In PVY infection, two major classes of antisense mRNAs (21 nt and 22 nt) were enriched in 5'U (36–40 %) and 5'A (28–29 %), followed by 5'C (20 %) and 5'G (21 %) in both inoculated and systemically infected leaves, indicating their association with AGO1-, AGO2-, and AGO5-like proteins, respectively.

Conclusion: Thus, the presented results from high-throughput sequencing (HTS) analysis of virus-specific small non-coding RNAs (mRNAs) may help elucidate the precise mechanisms induced by external dsRNAs.

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