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RNA Polymerase Slippage as a Mechanism for the Production of Frameshift Gene Products in Plant Viruses of the *Potyviridae* Family

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Modifications of RNA sequences by nucleotide insertions, deletions, or substitutions can result in the expression of multiple proteins in overlapping open reading frames (ORFs). In the case of viruses, polymerase slippage results in the alteration of newly synthesized RNA. The mechanism has been well characterized in animal RNA viruses such as *Ebolavirus* (1) (EBOV) or *Hepatitis C virus* (HCV) (2). For plant viruses of the *Potyviridae* family, polymerase slippage has been proposed as a general process of evolution (3), although a lack of experimental systems has precluded confirmatory data, and most pieces of evidence are indirect (4).

Translation of a large ORF that results in a polyprotein, later processed into mature factors, is the canonical strategy of potyviral protein production. Along with this, in all members of this family, an overlapping ORF named PIPO was identified in the middle of the P3 coding region. The translation of PIPO begins at a specific GA_6 motif (5). Interestingly, GA_6 and other A_n motifs ($n = \geq 6$) are misrepresented among members of the *Potyviridae* family (1.2 A_6 motifs in the coding region per viral genome versus the expected 8.1 motifs). This additional ORF of potyviruses produces a P3N-PIPO fusion protein, which was originally identified in *Turnip mosaic virus* (5) and was shown to be essential for cell-to-cell movement during viral infection (6). Recently, another extra ORF located downstream of a GA_6 motif was informatically identified inside the large P1 coding region of sweet-potato-infecting potyviruses (7, 8). This new ORF, named PISPO, harbors the possibility of producing a frameshifted P1N-PISPO gene product, whose existence is still to be determined.

To explore the mechanism by which these additional potyviral proteins can be synthesized, we analyzed available RNA sequencing (RNA-seq) data of two *Plum pox virus* (PPV) isolates (9). After data filtering (10, 11), sequences were mapped versus the references (12) allowing a maximum of three mismatches per read. The expected indel error was modeled as a Poisson distribution calculating λ from the Illumina indel calling error rate, PCR error rate, and sample indel frequency. This analysis revealed the presence of A residue additions in the PIPO GA_6 motif in 1.6% of the reads (Fig. 1A). Interestingly, the presence of an additional A residue in this motif was also detected in libraries of PPV-derived small RNAs (not shown). Besides, published data on another potyvirus, *Zucchini yellow mosaic virus*, showed a minor variant with an extra A in all samples of a *Cucurbita pepo* vine studied by deep sequencing of long RNAs (13), and our analysis located this modification in the PIPO GA_6 motif as well.

To assess the scope among potyviruses of the extra A at the PIPO junction, we subjected a sample of sweet potato (*Ipomea batatas*) infected with the potyvirus *Sweet potato feathery mottle virus* (SPFMV) to RNA-seq analysis. SPFMV reconstruction (SRR1693230 and SRR1693363) showed that there were 1.8% sequence variants in this PIPO ORF, with the insertion of an A residue as the most prominent modification (Fig. 1B).

Altogether, these data strongly suggest that P3N-PIPO is produced, at least partially, through polymerase slippage. This possibility, previously considered by Chung et al. (5), could not be demonstrated at that time, likely because of the low rate of nucleotide insertion into this site.

Reconstruction of the SPFMV genome confirmed the previously described PISPO ORF imbedded in the P1 coding sequence. But more importantly, the RNA-seq data also revealed the presence of a high proportion of molecules (11.8%) with a single A nucleotide addition in the upstream GA_6 motif, which is indicative of polymerase slippage (Fig. 1B). This change would result in the production of the hypothetical P1N-PISPO, and these results not only support the existence of this alternative product but also suggest that this protein might play an important role during sweet potato potyvirus infection.

Considering the evolutionary relatedness of polymerases of the members of the families *Picornaviridae* and *Potyviridae* (14), it is reasonable to envision similar behaviors in both viral families and, consistent with that idea, the six-adenine repetition motif is underrepresented in picornaviruses (0.67 motif in the coding region per viral genome versus the expected 1.9 motifs), as was the case in the family *Potyviridae*. There is no previous report of polymerase slippage in the *Picornaviridae* family; nonetheless, when an A_6 motif was present, as in the case of the enterovirus *Human rhinovirus C* (SRR363436), there were 2.4% A residue insertions at this location. These data suggest that polymerase slippage can occur in both *Picorna*-like families but that *Potyviridae* take more frequent advantage of this mechanism.

A common denominator in RNA slippage is the low fidelity of viral RNA polymerases and their tendency to stutter when encountering repetitive motifs. It is known that polymerases of EBOV, HCV, *Vaccinia virus*, or T7 bacteriophage, given the appropriate contexts, are prone to slippage (1, 2, 15). Repetitive

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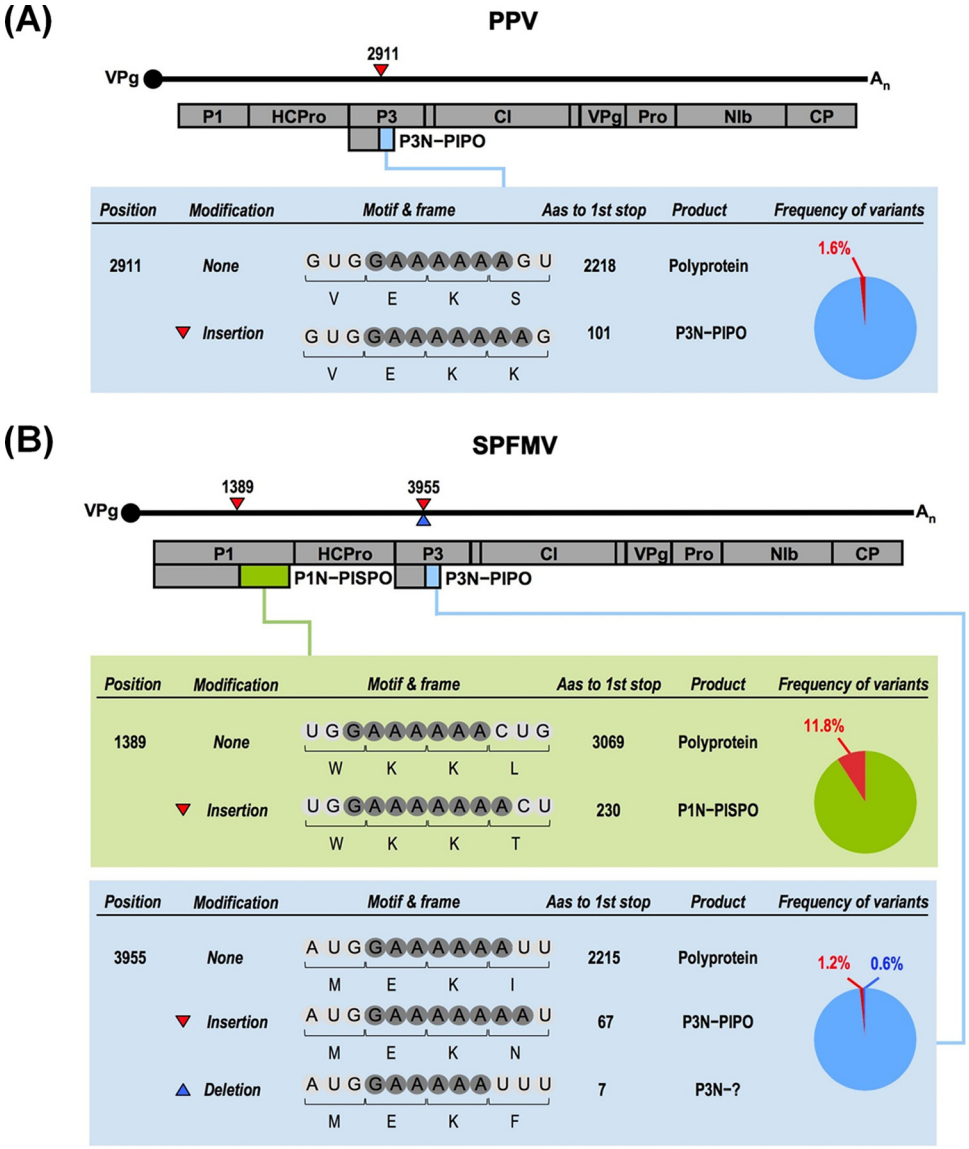

FIG 1 RNA slippage in viruses of the *Potyvirus* genus. The genomes of the potyviruses PPV (A) and SPFMV (B) are depicted schematically. Mature gene products are shown as boxes. Additional ORFs corresponding to out-of-frame PIPO and PISPO regions are also depicted. Sites where indels were detected after RNA-seq analysis are shown with red (insertion) or blue (deletion) triangles. Details of the motif, the resulting frame for the modifications, and the length of the expected products, as well as the RNA slippage percentages, are indicated for each modification. Color codes in the pie charts refer to insertions and deletions compared to the genomic sequence.

motifs, however, are not the rule but the exception, probably because of selective negative pressure supported by nonsense-mediated decay (16) or other mechanisms. Nonetheless, in certain situations, slippage of the polymerase would give rise to the production of new protein variants that are used by the virus, opening the door to new ways of adaptation and evolution.

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