Pervasiveness of exoribonuclease-resistant RNAs in plant viruses suggests new roles for these conserved RNA structures

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1 **ABSTRACT**

2 Exoribonuclease-resistant RNAs (xrRNAs) are discrete folded RNA elements that block the processive
3 degradation of RNA by exoribonucleases. xrRNAs found in the 3' untranslated regions (UTRs) of animal-
4 infecting flaviviruses and in all three members of the plant-infecting *Dianthovirus* adopt a complex ring-
5 like fold that blocks the exoribonuclease; this ability gives rise to viral non-coding subgenomic RNAs.
6 The degree to which these folded RNA elements exist in other viruses and in diverse contexts has been
7 unclear. Using computational tools and biochemical assays, we discovered that xrRNA elements are
8 widely found in viruses belonging to the *Tombusviridae* and *Luteoviridae* families of plant-infecting RNA
9 viruses, demonstrating their importance and widespread utility. Unexpectedly, many xrRNAs are located
10 in intergenic regions rather than in the 3'UTR and some are associated with the 5' ends of subgenomic
11 RNAs with protein-coding potential, suggesting that xrRNAs with similar scaffolds are involved in the
12 maturation or maintenance of diverse subgenomic RNAs, not just the ones generated from the 3'UTR.

13 INTRODUCTION

14 During infection, positive-sense RNA viruses produce full-length genomic RNA and many
 15 produce subgenomic RNAs (sgRNA) that can encode viral proteins or act as “riboregulators” that interact
 16 with and influence the cellular and viral machinery to benefit viral infection ¹⁻⁶. Most viral sgRNAs are
 17 thought to be produced directly through transcription; however, recent discoveries show that some
 18 noncoding viral sgRNAs result from discrete RNA elements that block the progression of 5′ to
 19 3′ exoribonucleases (Figure 1) ⁷⁻¹⁵. Discrete, compactly-folded exoribonuclease-resistant RNA (xrRNA)
 20 elements were first identified in mosquito-borne flaviviruses (e.g. Dengue virus, Zika virus, West Nile
 21 virus), where they protect the genome’s 3′ untranslated region (UTR) from degradation ⁸. The resultant
 22 decay intermediates accumulate and comprise biologically active viral non-coding sgRNAs (Figure 1)
 23 ^{8,9,12,16-21}.

25 Extensive functional and high-resolution structural studies show that mosquito-borne flaviviral
 26 xrRNA (xrRNA_F) function is conferred by a specific three-dimensional fold containing an interwoven
 27 pseudoknot stabilized by extensive conserved secondary and tertiary interactions; this creates an unusual
 28 ring-like conformation that protectively wraps around the 5′ end of the RNA structure ^{22,23}. xrRNAs are
 29 found broadly within flaviviruses including those that are tick-borne, are specific to insects, or have no
 30 known vector ^{15,24,25}. Comparison of diverse xrRNA_F sequences revealed two classes; class I (xrRNA_{F1}) is
 31 exemplified by mosquito-borne flaviviruses while class II (xrRNA_{F2}) is found in diverse flaviviruses ²⁵.
 32 Although aligned xrRNA_{F2} sequences show patterns, their three-dimensional structures are unknown, as
 33 are the structures of recently reported xrRNAs from most other viral clades ^{7,13}.

35 Recently, we structurally and functionally characterized xrRNAs from the 3′UTRs of
 36 dianthoviruses, plant-infecting positive-sense RNA viruses in the *Tombusviridae* family; similar to the
 37 xrRNA_F, they function to produce a non-coding RNA derived from the viral 3′UTR ^{10,26}. Dianthoviral

38 xrRNAs (xrRNA_D) also rely on a pseudoknot that forms a protective ring-like structure²⁶, but they have
 39 very different sequences and secondary structures compared to xrRNA_{F1} and the ring is formed by a
 40 different set of interactions (Figure 1). Although xrRNA_F elements pervade the flaviviruses with
 41 associated sequence and structural diversity, xrRNA_D have only been identified in the three closely related
 42 members of the *Dianthovirus* genus. This raises the question of whether xrRNAs similar to xrRNA_D are
 43 more widespread and diverse than currently known, and thus if they may be an underappreciated way to
 44 produce or protect viral RNAs. Moreover, the only available xrRNA_D crystal structure is in an “open”
 45 conformation that likely represents a necessary folding intermediate before the pseudoknot forms²⁶
 46 (Figure 1). Thus, we still do not know the full repertoire of secondary and tertiary interactions required to
 47 form and stabilize the exoribonuclease-resistant pseudoknot state of xrRNA_D. The lack of diverse
 48 xrRNA_D sequences prevents conclusions about the role, prevalence, and structural diversity of this fold.

49

50 To begin to address these questions, we used a bioinformatic approach to identify more xrRNA_D
 51 sequences among plant viruses, identifying over 40 putative new xrRNA_D-like elements in viruses
 52 belonging to the *Tombusviridae* and *Luteoviridae* families. *In vitro* assays show that these elements are
 53 indeed resistant to Xrn1 and analysis of these new xrRNAs reveals both conservation and variability.
 54 Furthermore, the genomic location of these new xrRNAs suggests new roles in the generation of sgRNA
 55 species that have protein-coding potential, providing evidence that xrRNA-based RNA maturation
 56 pathways may be more widespread than previously anticipated.

57 RESULTS AND DISCUSSION

58 To search for xrRNA_D-like elements, we used the *Infernal* software (Eddy lab), which enables
 59 screening of massive datasets of DNA sequences for conserved structure patterns with poor sequence
 60 conservation²⁷. Because the *Dianthovirus* genus only comprises three members (Red clover necrotic
 61 mosaic virus (RCNMV), Sweet clover necrotic mosaic virus (SCNMV), and Carnation ringspot virus

(CRSV))²⁶, we expanded our search to other plant-infecting positive-sense RNA viruses. The initial search within a library of viral reference genomes (see Methods) identified two potential sequences among *Luteoviridae*; *Poleroviruses*: wheat leaf yellowing-associated virus isolate JN-U3 (GenBank ID # NC_035451; Infernal E-value = 0.00025, score = 44.3) and sugarcane yellow leaf virus (GenBank #NC_000874; Infernal E-value = 6.5, score = 24.2). With these sequences added to the alignment, subsequent searches identified > 40 candidates within the public repository of all available sequences for *Tombusviridae* and *Luteoviridae*, demonstrating how powerful this tool is for computationally identifying functional elements in viral RNAs²⁸.

Alignment of the putative xrRNA_D-like elements revealed that their predicted secondary structures contain conserved helices P1, P2, and the pseudoknot, which are supported by covariation but have little sequence conservation (R-scape²⁹ E-values for the 12 covarying base pairs in the stems and the pseudoknot are within 3.10^{-4} – 8.10^{-13} (95th percentile = 1.10^{-12}); Figure 2A). L1 and L2B are > 97% conserved in sequence. In the case of L1 and L2B, this is consistent with their role in creating a specific folded motif that promotes pseudoknot formation²⁶. Also, two of the three nucleotides immediately upstream of the 3' side of the pseudoknot are >97% conserved, but their role is not obvious from the crystal structure of the open state. Likewise, the non-Watson-Crick A8-G33 base pair identified in the crystal structure (Figure 1) cannot be reconciled with the predominant presence of G at position 8 and G/A at position 33 in all the other sequences. These observations support the previous assertion that the crystallized open state represents a folding intermediate and that structural adjustments and additional interactions are present in the “closed” pseudoknot state.

Viruses with putative novel xrRNAs include members of the *Machlomovirus* and *Umbravirus* genera of the *Tombusviridae* family, as well as members of the *Polerovirus* and *Enamovirus* genera of the *Luteoviridae* family. To experimentally determine if these are authentic xrRNAs, we tested representative

87 sequences from viruses of both families using our established *in vitro* Xrn1 resistance assay ¹¹.
 88 Specifically, *in vitro*-transcribed and purified RNA sequences from opium poppy mosaic virus (OPMV),
 89 Maize chlorotic mottle virus (MCMV), Potato leafroll virus (PLRV) Maize yellow dwarf virus-RMV
 90 (MYDV-RMV) and Hubei polero-like virus 1 (HuPLV1) were challenged with recombinant Xrn1. All
 91 RNAs stopped Xrn1 degradation similarly to RCNMV xrRNA_D (Figure 3A, B), demonstrating that they
 92 are authentic xrRNAs and do not require additional *trans*-acting proteins for function. Moreover,
 93 mutations to disrupt the putative pseudoknot in the MCMV, PLRV and HuPLV1 xrRNAs abolished Xrn1
 94 resistance, while compensatory mutations that restore pseudoknot base pairing rescued the activity
 95 (Figure 3C-E). In addition, the mapped Xrn1 stop site is at the base of P1 in all newly identified xrRNAs,
 96 matching the xrRNA_D stop site (Figure 3F-H, Supplementary Figure S2). Overall, the conserved
 97 secondary structure (Figure 2B), the location of the exoribonuclease halt site, and the strict dependence on
 98 the pseudoknot for Xrn1 resistance suggest that these newly-identified xrRNAs use a similar molecular
 99 fold and mechanism as the xrRNA_D, thus we classify them as such, and hereafter refer to the class as
 100 xrRNA_{TL} (for *Tombusviridae* and *Luteoviridae*).

101

102 A notable structural difference between diverse xrRNA_{TL} elements is that a subset of xrRNAs
 103 found in the *Tombusviridae* family (RCNMV, SCNMV, CRSV, OPMV, MCMV) possess a P3 stem-loop
 104 immediately downstream of the pseudoknot (Figure 2A; Tables 1 and S1). We previously showed that
 105 this part of the sequence is not required for Xrn1 resistance by xrRNA_{TL} *in vitro* ²⁶. Consistent with this,
 106 an analogous stem-loop (P4) found in xrRNA_{F1} is also dispensable *in vitro*; the crystal structure indicates
 107 it may stabilize the pseudoknot through stacking interactions (Figure 1) ²². Thus, in xrRNA_{TL} coaxial
 108 stacking of P3 on P1/P2 could help to stabilize the RNA structure in the cellular context during infection.

109

110 The location of the new xrRNAs reveals unexpected variation (Figure 4). Only two of the newly
 111 identified xrRNAs are in the 3'UTR of the viral genome (Table 1), as are the previously characterized

112 dianthoviral xrRNA_{TL} and xrRNA_{F1-F2}. In MCMV, the first nucleotide of the P1 helix matches the 5' end
 113 of sgRNA2³⁰, thus this new xrRNA element probably blocks Xrn1 to generate non-coding sgRNAs
 114 derived from the 3'UTR, as with the dianthoviruses, flaviviruses, and other xrRNAs. However, for some
 115 members of the *Tombusviridae* family as well as for *Poleroviruses*, xrRNA_{TL} is located in an intergenic
 116 region, within 5–20 nt from the translation start site of ORF3a, and ~ 135 nt from the start site of a
 117 readthrough protein encoded by ORF3-5 (our data suggest that ORF3a has not been annotated for all
 118 *Poleroviruses*; Table S1). ORF3a codes for protein P3a, which is essential for long-distance movement of
 119 the virus in plants³¹. Translation of ORF3a occurs from sgRNA1, generally at a non-AUG codon (Tables
 120 1 and S1)³¹⁻³³. This implies that these xrRNAs, rather than functioning in non-coding RNA production,
 121 act to produce or maintain protein coding RNAs (Figure 4); sgRNAs could be produced from full-length
 122 genomic RNAs without requiring a subgenomic promoter, or sgRNAs produced by other means could be
 123 protected from 5' to 3' degradation. Since the *Tombusviridae* and *Luteoviridae* families use 3' proximal
 124 cap-independent translation enhancers (3'-CITEs) to initiate translation, uncapped sgRNAs with xrRNAs
 125 on their 5' ends could still be translationally active^{34,35}. Thus, these xrRNAs could be part of complex
 126 translation regulation mechanisms involving these 3'-CITEs and different sgRNAs³⁶.

127

128

129 Various roles for xrRNAs are possible, depending on their genetic context. The presence of
 130 xrRNAs in diverse locations within viral genomes suggests that new xrRNA scaffolds may emerge from
 131 analyzing sgRNA 5' termini from other viruses, as certainly not all xrRNA elements were identified by
 132 the algorithm used here^{5,7,37}. Intriguing candidates for novel xrRNA identification are viruses in which no
 133 obvious promoter elements for sgRNA production were identified, or viruses in which putative promoter
 134 sequences are downstream of the sgRNA 5' end^{1,5,30,37}. Many questions remain that pertain to
 135 understanding the structural/sequence requirements for Xrn1 resistance, the degree to which structural
 136 variation is tolerated, and how sequence diversity is integrated into similar folds³⁸. The now-expanded set

137 of xrRNA_{TL} candidates provides a broader phylogeny for future bioinformatic and structural studies that
138 will address these points.
139

correspond to positive hits but with a larger sequence or structure variation. By the time the alignment reached a size of 10–12 sequences, we were able to retrieve most of the sequences that made it into the final alignment through further iterations of *Infernal* searches and manual addition to the alignment. Hits for unclassified viruses were also retrieved from large-scale transcriptomics data of invertebrate and vertebrate-associated RNA viruses, using the deposited sequences^{40,41}.

A statistical validation of the final proposed alignment of 47 sequences was performed using the latest version of R-scape available at <http://eddylib.org/R-scape/>²⁹ (last accessed on August 17, 2018). The corresponding conserved structure and sequence patterns were rendered using R2R v. 1.0.5⁴².

188

Design of RNAs for *in vitro* assays. DNA templates for *in vitro* transcription were gBlocks ordered from IDT, cloned into pUC19 and verified by sequencing. RNA constructs for Xrn1 degradation assays contained the xrRNA sequence plus ~30 nucleotides of the endogenous upstream sequence (‘leader sequence’) to allow loading of the exoribonucleases. Below are the sequences used in *in vitro* Xrn1 degradation assays with the T7 promoter underlined, the leader sequence in italic and the first protected nucleotides (experimentally validated as described below) in bold. Lower case letters indicate extra nucleotides inserted to allow better transcription.

OPMV xrRNA:

TAATACGACTCACTATA*GGAATTGCCTCCACCAGTAACTAAACCA***ACCACAGCCAAGCATTA**
GTTGCAAGCGTTGGAGTGGCAGGCTTAACGTCCGACAGTACGACAACTGCGG

MCMV xrRNA:

TAATACGACTCACTATA*GGTTCCAGGCCAGGGCTGGCAAATCATTGAGCACAAGGTGAGCCG*
GCATGAGGTTGCAAGACCGGAACAACCAGTCCTTCTGGCAGAGTCCTGCCAA

PLRV xrRNA:

203 TAATACGACTCACTATAgGCCACCACAAAAGAACACTGAAGGAGCTCACTAA^{**A**}ACTAGCCAAGC
 204 ATACACGAGTTGCAAGCATTGGAAGTTCAAGCCTCGT

205 **MYDV-RMV xrRNA:**

206 TAATACGACTCACTATAgGTCCAGAAACAAAAAGTTTAA^{**A**}ACAGAA^{**A**}GCTCTCAAGTCAGCCAGGC
 207 AAATTCGAGTTGCAAGCACTGGATGACCTAGTCTCGATA

208 **HuPLV1 xrRNA:**

209 TAATACGACTCACTATAgGCCACAAAACGAATAAAGGAAGAACGCACGAGAGTCAGCCAAACA
 210 AACACAAGTTGCAAGTGTTGGAGACTCATTCTAGTCTTGT

211

212 **RNA preparation.** DNA templates for *in vitro* transcription were amplified by PCR using custom DNA
 213 primers (IDT) and Phusion Hot Start polymerase (New England BioLabs). 2.5 mL transcription reactions
 214 were assembled using 1000 µL PCR reactions as template (~0.2 µM template DNA), 6 mM each NTP, 60
 215 mM MgCl₂, 30 mM Tris pH 8.0, 10 mM DTT, 0.1% spermidine, 0.1% Triton X-100, T7 RNA
 216 polymerase and 2 µL RNasin RNase inhibitor (Promega) and incubated overnight at 37°C. After
 217 inorganic pyrophosphates were precipitated by centrifugation, the reactions were ethanol precipitated and
 218 purified on a 7 M urea 8% denaturing polyacrylamide gel. RNAs of the correct size were gel-excised,
 219 eluted overnight at 4°C into ~40 mL of diethylpyrocarbonate (DEPC)-treated milli-Q filtered water
 220 (Millipore) and concentrated using Amicon Ultra spin concentrators (Millipore). Mutations were
 221 introduced using mutagenized custom DNA reverse primers.

222 **Primers used in this study (mutated residues in bold):**

223 OPMV_WT_rev
 224 5'-CCGCAGTTGTCGTACTGTCCG-3'

225 OPMV_PKmut1_rev
 226 5'-CCGCAGTTGTCGTACTGTCCGACGA^{**A**}TTGCCTGCCACTCCAACGC-3'

227 OPMV_PKmut2_rev
 228 5'-CCGCAGTTGTCGTA~~CT~~GTCGGACGTTAAGCCTGCCACTCCAACGCTTGCAACA**A**TTTGCTT
 229 GGCTGTGGTTGG-3'

230 OPMV_PKcomp_rev
 231 5'-CCGCAGTTGTCGTA~~CT~~GTCGGACGA**A**TTGCCTGCCACTCCAACGCTTGCAACA**A**TTTGCTT
 232 GGCT GTGGTTGG-3'

233 MCMV_WT_rev
 234 5'-TGGCAGGACTCTGCCAGAAGG-3'

235 MCMV_PKmut1_rev
 236 5'-TGGCAGGACTCTGCCAG**CT**CCACTGGTTGTTCCGGTCTTGC-3'

237 MCMV_PKmut2_rev
 238 5'-TGGCAGGACTCTGCCAGAAGGACTGGTTGTTCCGGTCTTGCAAG**GAG**ATGCCGGCTCACC
 239 TTGTGCTC-3'

240 MCMV_PKcomp_rev
 241 5'-TGGCAGGACTCTGCCAG**CT**CCACTGGTTGTTCCGGTCTTGCAAG**GAG**ATGCCGGCTCACC
 242 TTGTGCTC-3'

243 PLRV_WT_rev
 244 5'-ACGAGGCTTGA**A**CTTCCAATGC-3'

245 PLRV_PKmut1_rev
 246 5'-**TG**CTGGCTTGA**A**CTTCCAATGCTTGC-3'

247 PLRV_PKmut2_rev
 248 5'-ACGAGGCTTGA**A**CTTCCAATGCTTGCAAC**AGC**AGTATGCTTGGCTAGTTTTAGTG-3'

249 PLRV_PKcomp_rev
 250 5'-**TG**CTGGCTTGA**A**CTTCCAATGCTTGCAAC**AGC**AGTATGCTTGGCTAGTTTTAGTG-3'

251 MYDV-RMV_WT_rev
 252 5'-TATCGAGACTAGGTCATCCAGTGC-3'

253 huPLV_WT_rev
 254 5'-ACAAGACTAGAATGAGTCTCC-3'

255 huPLV_PKmut1_rev
 256 5'- **TG**TTGACTAGAATGAGTCTCCAACACTTGC-3'

257 huPLV_PKmut2_rev
 258 5'- ACAAGACTAGAATGAGTCTCCAACACTTGCAACA**A**CAGTTTGT**T**TGGCTGACTCTCG-3'

259 huPLV_PKcomp_rev
 260 5'- **TG**TTGACTAGAATGAGTCTCCAACACTTGCAACA**A**CAGTTTGT**T**TGGCTGACTCTCG-3'

261 ***In vitro* Xrn1 resistance assays.** 4 µg RNA was resuspended in 40 µL 100 mM NaCl, 10 mM MgCl₂, 50
 262 mM Tris pH 7.5, 1 mM DTT and re-folded at 90°C for 3 minutes then 20°C for 5 minutes. 3 µL
 263 recombinant RppH (0.5 µg/µL stock) was added and the samples were split into two 20 µL reactions (-/+

264 exoribonuclease). 1 μ L of the recombinant Xrn1 (0.8 μ g/ μ L stock) was added where indicated. All
 265 reactions were incubated for 2 hrs at 30°C using a thermocycler. The degradation reactions were resolved
 266 on a 7 M urea 8% denaturing polyacrylamide gel and stained with ethidium bromide.

267

268 **Mapping of the exoribonuclease halt site.** To determine the Xrn1 stop site at single-nucleotide
 269 resolution, 30 μ g *in vitro*-transcribed RNA was degraded using recombinant RppH and Xrn1 as described
 270 above (the reaction volume was scaled up to 300 μ L, and 20 μ L of each enzyme was used). The
 271 degradation reaction was resolved on a 7 M urea 8% polyacrylamide gel, then the Xrn1-resistant
 272 degradation product was cut from the gel and eluted overnight at 4°C into ~20 mL of
 273 diethylpyrocarbonate (DEPC)-treated milli-Q filtered water (Millipore) and concentrated using Amicon
 274 Ultra spin concentrators (Millipore). Once recovered, the RNA was reverse-transcribed using Superscript
 275 III reverse transcriptase (Thermo) and a 6-FAM (6-fluorescein amidite)-labeled sequence-specific reverse
 276 primer (IDT) with an (A)₂₀ -stretch at the 5' end to allow cDNA purification with oligo(dT) beads. 10 μ L
 277 RT reactions contained 1.2 pM RNA, 0.25 μ L 0.25 μ M FAM-labeled reverse primer, 1 μ L 5x First-Strand
 278 buffer, 0.25 μ L 0.1 M DTT, 0.4 μ L 10 mM dNTP mix, 0.1 μ L Superscript III reverse transcriptase (200
 279 U/ μ L) and were incubated for 1 hour at 50°C. To hydrolyze the RNA template after reverse transcription,
 280 5 μ L of 0.4 M NaOH was added and the reaction mix incubated at 90°C for 3 min, followed by cooling
 281 on ice for 3 min. The reaction was neutralized by adding 5 μ L of acid quench mix (1.4 M NaCl, 0.57 M
 282 HCl, 1.3 M sodium acetate pH 5.2), then 1.5 μ L oligo(dT) beads (Poly(A)Purist MAG Kit (Thermo))
 283 were added and the cDNA purified on a magnetic stand according to the manufacturer's instructions. The
 284 cDNA was eluted in 11 μ L ROX-HiDi and analyzed on a 3500 Genetic Analyzer (Applied Biosystems)
 285 for capillary electrophoresis. A Sanger sequencing (ddNTP) ladder of the undigested RNA was analyzed
 286 alongside each degradation product as reference for band annotation.

287 **CONTRIBUTIONS**

288 Q.V., A.-L.S., J.S.K. designed and analyzed research; Q.V. performed the computational search; A.-L.S.
289 performed the biochemical experiments; Q.V., A.-L.S. & J.S.K wrote the paper.

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TABLE

Table 1. Selected set of plant viruses possessing an xrRNA_{TL}. Viruses are grouped by their genomic context (last column). The complete list of sequences used for comparative sequence alignment is shown in Table S1.

Name	Abbreviation	Classification	GenBank ID	Total ssRNA length (nt)	Genomic location*	Genomic context
Red clover necrotic mosaic virus	RCNMV	Tombusviridae; Dianthovirus	NC_003756	3890	3461-3504	3' UTR
Sweet clover necrotic mosaic virus	SCNMV	Tombusviridae; Dianthovirus	NC_003806	3876	3446-3489	3' UTR
Maize chlorotic mottle virus (isolate KS1)	MCMV	Tombusviridae; Machlomovirus	NC_003627	4437	4101-4143	3' UTR
Opium poppy mosaic virus (isolate PHEL5235)	OPMV	Tombusviridae; Umbravirus	NC_027710	4230	3585-3629	3' UTR
Carrot mottle mimic umbravirus	CMoMV	Tombusviridae; Umbravirus	NC_001726	4201	2664-2706	74 nt to AUG from ORF3
Chickpea chlorotic stunt virus	CpCSV	Luteoviridae; Polerovirus	NC_008249	5900	3489-3534	11 nt to AUA from ORF3a; 129 nt to AUG from ORF3-5
Cowpea polerovirus 1 (isolate BE167)	CpPV1	Luteoviridae; Polerovirus	NC_034246	5845	3380-3425	11 nt to CUG from ORF3a; 129 nt to AUG from ORF3-5
Cotton leafroll dwarf virus	CoLRDV	Luteoviridae; Polerovirus	NC_014545	5866	3451-3499	13 nt to CUG from ORF3a; 131 nt to AUG from ORF3-5
Cereal yellow dwarf virus-RPV	CYDV-RPV	Luteoviridae; Polerovirus	NC_004751	5723	3566-3622	14 nt to AUU from ORF3a; 132 nt to AUG from ORF3-5
Maize yellow dwarf virus-RMV (Formerly BYDV)	MYDV-RMV	Luteoviridae; Polerovirus	NC_021484	5612	3335-3384	14 nt to ACG from ORF3a; 132 nt to AUG from ORF3-5
Potato leafroll virus	PLRV	Luteoviridae; Polerovirus	NC_001747	5987	3509-3557	18 nt to AUA from ORF3a; 136 nt to AUG from ORF3-5
Hubei polero-like virus 2 (strain QTM26674)	HuPLV2	Unclassified	NC_033229	6083	3706-3753	133 nt to AUG from ORF3-5
Beet western yellows luteovirus (strain bwv-1, isolate 28a)	BWVY	Luteoviridae; Polerovirus	L39983	973	341-389	135 nt to AUG from ORF3-5
Hubei polero-like virus 1 (strain WHCC118254)	HuPLV1	Unclassified	NC_032224	4213	3357-3410	135 nt to AUG from ORF3-5
Sugarcane yellow leaf virus	ScYLV	Luteoviridae; Polerovirus	NC_000874	5899	3467-3512	136 nt to AUG from ORF3-5
Beet western yellows virus	BWVY	Luteoviridae; Polerovirus	NC_004756	5666	3346-3393	138 nt to AUG from ORF3-5

*xrRNA boundaries defined as the 1st nucleotide of the P1 stem and the last nucleotide of the pseudoknot.

FIGURES

Figure 1 | An expanding repertoire of structured RNAs for blocking exoribonuclease degradation.

Top: xrRNAs adopt a three-dimensional structure that blocks the progression of 5' to 3' exoribonucleases such as Xrn1 (grey). In the case of flaviviruses and dianthoviruses, xrRNAs are in the 3'UTR and this results in accumulating sgRNAs that comprise the 3'UTR. Middle: Secondary structure diagrams are shown for the two classes of xrRNAs from flaviviruses ($xrRNA_{F1}$ and $xrRNA_{F2}$)^{15,24,25}, and from dianthoviruses ($xrRNA_D$)²⁶. Secondary structure features are labeled, and nucleotides involved in tertiary interactions are shown in colors connected by dashed lines (pseudoknot shown in blue). Experimentally determined Xrn1 stop sites are indicated. Bottom: The grey shaded boxes below each secondary structure contain diagrams reflecting the currently available three-dimensional structures^{22,23,26}. The A8-G33 pair is highlighted in the open state of the Sweet clover necrotic mosaic virus (SCNMV) xrRNA.

Figure 2 | Widespread occurrence of Xrn1-resistant RNAs among plant viruses. (a) Consensus sequence and secondary structure of xrRNA_{TL} based on a comparative sequence alignment of 47 sequences of viruses belonging to the *Tombusviridae* and *Luteoviridae* families (shown in Figure S1). Y = pyrimidine; R = purine. Non-Watson-Crick base pairs are shown using the Leontis-Westhof nomenclature⁴³. The numbering is that of the crystal structure of the SCN MV xrRNA_D (now referred to as xrRNA_{TL})²⁶. **(b)** Phylogenetic relationship between various plant viruses, based on the RNA-dependent RNA polymerase amino acid sequence³². The viruses and corresponding genera in which we identified xrRNA_{TL} structures are marked by a star. Numbers at the nodes refer to bootstrap values as percentages obtained from 2000 replications, shown only for branches supported by more than 40%. Branch length is proportional to the number of changes. Additional analysis will likely reveal xrRNA_{TL} elements in more of these viruses with additional sequence and structural variation.

Figure 3 | Biochemical characterization of representative plant virus xrRNA_{TL} elements. (A) *In vitro* Xrn1 resistance assay of putative xrRNA_{TL} from various plant RNA viruses (Table 1). The xrRNA from RCNMV was included as a positive control. Arrows indicate the size of full-length RNA and Xrn1-resistant degradation product. **(B)** Classification of viruses used in A (Table 1). **(C-E)** *In vitro* Xrn1 resistance assay of WT and PK mutant versions of MCMV (C), PLRV (D) and HuPLV1 (E) xrRNAs. **(F-H)** Reverse transcription (RT) mapping of the Xrn1 halt site. Distribution of RT products of Xrn1-resistant fragments of MCMV (F), PLRV (G) and HuPLV1 (H) degradation fragments. Experimentally validated halt sites are indicated on the secondary structure diagram for all tested xrRNA_{TL} in Figure S2.

Figure 4 | xrRNA_{TL} can produce or protect both coding and noncoding sgRNAs. The presence of xrRNA_{TL} in different contexts suggests an expanded role for these elements. Shown here, full-length viral genomic RNA (top) could be processed by exonucleases that stop at xrRNAs (depicted as dashed structures) to yield both sgRNAs with protein coding potential (middle) and noncoding sgRNAs (bottom). Also, sgRNAs produced by subgenomic promoters could be “trimmed” or protected by xrRNAs (not shown). Note that only some *Umbraviruses* (e.g. OPMV) possess two xrRNA_{TL} elements. Colored boxes symbolize ORF organization in the plant viruses examined in this study.