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Stem-loop structure of *Cocksfoot mottle virus* RNA is indispensable for programmed –1 ribosomal frameshifting

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The –1 programmed ribosomal frameshifting (–1 PRF) mechanism utilized by many viruses is dependent on a heptanucleotide slippery sequence and a downstream secondary structure element. In the current study, the RNA structure downstream from the slippery site of cocksfoot mottle sobemovirus (CfMV) was proven to be a 12 bp stem-loop with a single bulge and a tetranucleotide loop. Several deletion and insertion mutants with altered stem-loop structures were tested in wheat germ extract (WGE) for frameshifting efficiency. The impact of the same mutations on virus infectivity was tested in oat plants. Mutations shortening or destabilizing the stem region reduced significantly but did not abolish –1 PRF in WGE. The same mutations proved to be deleterious for virus infection. However, extending the loop region to seven nucleotides had no significant effect on frameshifting efficiency in WGE and did not hamper virus replication in infected leaves. This is the first report about the experimentally proven RNA secondary structure directing –1 PRF of sobemoviruses.

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**1. Introduction**

Many positive strand RNA viruses use –1 programmed ribosomal frameshifting (–1 PRF) for regulating the expression of viral polymerases. Two cis-acting elements are required for an efficient –1 PRF. First, a seven nucleotide slippery sequence is the place where the ribosome will slip back by one nucleotide. The sequence motif for the slip-site is X XXX YYY (preframeshift codons are indicated), where X can be any nucleotide, Y either A or U, and Z can be any nucleotide except G (Brierley et al., 1992). Secondly, the shifty heptanucleotide is followed by the sequence that is forming a downstream secondary structure. The investigated downstream stimulatory elements can be divided into three groups. The best studied group includes hairpin-type RNA pseudoknot structures formed when the nucleotides of hairpin loop base pair with single-stranded downstream complementary nucleotides (reviewed by Giedroc and Cornish, 2008). The second group of structures consists of pseudoknots having an unusual structure. For example, a threestemmed pseudoknot structure has been reported for *Severe acute respiratory syndrome coronavirus* (Baranov et al., 2005; Brierley and Dos Ramos, 2006; Plant et al., 2005; Su et al., 2005). This structure is composed of two double-stranded stems connected by a single-stranded loop and a second loop which itself folds into a stem-loop. The stimulatory RNA of the *Visna-Maedi virus* –1 ribosomal frameshifting signal is another unusual pseudoknot with a seven nucleotide interstem element between two stems (Pennell et al., 2008). The third group includes stable stem-loop structures sufficient to promote efficient –1 PRF. Examples include the frameshift-promoting elements at the gag-pol junction in *Giardia lamblia virus* (GLV), *Human immunodeficiency virus* type 1 (HIV-1), *Simian immunodeficiency virus* (SIV) and related lentiviruses (Brierley and Dos Ramos, 2006; Li et al., 2001; Marcheschi et al., 2007; Staple and Butcher, 2005). The requirement for a simple hairpin loop in frameshifting has been also demonstrated in *Human astrovirus* serotype-1 (HAst-1), *Human T-cell leukemia virus* type II (HTLV-II) and *Red clover necrotic mosaic virus* RNA-1 (RCNMV) (Falk et al., 1993; Kim and Lommel, 1994, 1998;Marczinke et al., 1994).

*Cocksfoot mottle virus* (CfMV, unassigned genus *Sobemovirus*) is a plant virus with a monopartite, single-stranded, positive-sense RNA genome (Mäkinen et al., 1995b). The polyprotein of CfMV encodes a viral protease, Vpg and an RNA-dependent RNA polymerase (RdRp). It is translated from two overlapping open reading frames, ORF 2a and 2b, by a –1 PRF mechanism (Mäkinen et al., 1995a) (Fig. 1). The consensus signals for –1 PRF were identified at the beginning of the overlap region: the slippery sequence U UUA AAC (nucleotides 1634–1640, preframeshift codons are indicated) and a predicted stem-loop structure (nucleotides 1648–1676) starting seven nucleotides...
downstream. Both mutating the heptanucleotide sequence and deleting the putative secondary structure were shown to completely abolish the frameshifting activity, indicating that these cis-acting elements are absolutely required for −1 PRF to proceed (Lucchesi et al., 2000). The minimal frameshift sequence required for efficient in vitro frameshifting was mapped at the nucleotides 1621–1690 (Lucchesi et al., 2000) (Fig. 1). This 70 nucleotide sequence, containing both the slippery sequence and the predicted downstream secondary structure, was shown to direct −1 PRF in wheat germ extract (WGE) with an efficiency of 12.7 ± 1.4%. Similar in vitro frameshifting efficiency, 10.6 ± 1.4%, was determined for the entire ORF2a–2b encoding region (Lucchesi et al., 2000; Tamm et al., 1999).

In this study, we report that the −1 PRF signal of CMV indeed includes a stem-loop structure as a downstream element. We have mapped the structure of the frameshifting site of CMV by chemical probing. Several mutations were introduced to study the importance of particular structural elements in determining the frameshifting efficiency. The impact of these mutations was quantitatively analyzed by measuring the frameshifting efficiencies in a WGE in vitro translation system. The effects of mutating the frameshifting region on the local and systemic infection of CMV was examined in oat plants.

2. Materials and methods

2.1. Plasmid construction

The base numbering used in this study refers to the genome of the CMV Norwegian isolate as in Mäkinen et al. (1995b). The construction of pAB-21, containing CMV polyprotein region (nucleotides 418–3265), has been described earlier (Lucchesi et al., 2000). This plasmid was used as a template to create all the other constructs for in vitro studies.

For the construction of pRF2, a fragment containing the slippery sequence and the stem-loop region (nucleotides 1604–1898) was amplified by PCR and cloned into pGEM-T Easy (Promega) under the control of T7 RNA polymerase promoter. This plasmid was used for the in vitro transcription experiments.

The following mutations in pAB-21 were introduced by PCR-based mutagenesis (Fig. 3A). pAB(ΔCUU) contains a deletion of a conserved CUU triplet (nucleotides 1685–1687). In the case of pAB(ΔC), the C nucleotide at position 1667 was deleted and a restriction site for Apal introduced (nucleotides 1679–1684) to retain the reading frame. In pAB(+G), an additional G nucleotide was inserted at position 1657 and nucleotides 1683–1799 were deleted in order to restore the reading frame. Plasmids pAB(ΔCUU), pAB(ΔC) and pAB(+G) were obtained using Excise site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. pAB(+UUU) was generated by inserting UUU at position 1662–1664. In pAB(G → U), the G nucleotide at position 1649 was mutated to U. In pAB(C → A), the C nucleotide at position 1875 was mutated to A. In pAB(G → U, C → A), the G at position 1649 and C at position 1675 were changed to U and A, respectively. These mutations were introduced as described by Meier et al. (2006). The PCR fragments carrying the mutations were cut with Kpn1 and Cfr42I and inserted into pAB-21 cut with the same enzymes.

The construction of the infectious cDNA of CMV (CMV icDNA) and replicase-deficient CMV cDNA clone (CMV RdRp(−)) have been described earlier (Meier et al., 2006). Three CMV cDNA clones were constructed carrying the following mutations: in CMV mRF(C → A), the C nucleotide at position 1675 was mutated to A; in CMV mRF(+UUU), the UUU were inserted at position 1662–1664; in CMV mRF(ΔC,+C), the C nucleotide at position 1667 was deleted and one extra C nucleotide inserted at position 1677 (Fig. 4A). The mutations were introduced by PCR-based mutagenesis (Meier et al., 2006). The obtained PCR fragments were cut with Kpn1 and Eco147I and inserted into an icDNA construct cut with the same enzymes.

All mutations were verified by sequencing.

2.2. RNA in vitro transcription, chemical probing and primer extension analysis

pRF2 was linearized with SalI for run-off transcription with bacteriophage T7 RNA polymerase. In vitro transcribed RNA was purified on a Sephadex S400 (GE Healthcare) spin column as described (Liiv et al., 1998). Modification reactions with dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMCT) or kethoxal were done in a 50 μl reaction volume containing 20 pmol of RNA according to Liiv and Remme (2004). The unpaired bases are accessible to alklylation by these agents. DMS modifies the N3 position of cytosine and the N1 position of adenine. CMCT modifies the N3 group of uracil and N1 of guanine. Kethoxal modifies guanine residues at positions N1 and N2. Modification sites were determined by primer extension using reverse primer 117,864 −32P]dCTP labelling was done as described earlier (Maivälä et al., 2002; Stern et al., 1988). Control experiments with untreated RNA were carried out to detect natural pauses of reverse transcription. Primer extension products were separated by 7% urea–polyacrylamide gel electrophoresis (PAGE).
2.3. Coupled wheat germ extract (WGE) translation

*In vitro* translation was carried out using TNT T7 Coupled Wheat Germ Extract System (Promega) in the presence of \([^{35}S]\)methionine. The plasmids were linearized with NdeI before adding to the reaction mixture. Translation products were separated by 10% SDS–PAGE and detected on dried gels using Bio-Rad Molecular Imager System GS-525. The images were analyzed using Molecular Analyst Software (Bio-Rad). The relative amount of frameshifted product versus P2a was calculated from five independent translations by quantifying the radioactive signal in specific areas and correcting for background and different methionine content of products.

2.4. Virus inoculation and infection analysis

CfMV icDNA and mutated cDNA constructs were linearized with Sall and used as templates for 5′ capped RNA synthesis. *In vitro* transcription was carried out as described earlier (Meier et al., 2006). Gold particles (diameter 0.3-3.0 μm, ChemPur) were coated with transcribed RNA. Two-weeks old oat plants (cv. Jaak) were infected biolistically using Helios Gene Gun (Bio-Rad). In one experimen-

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Fig. 2. Chemical probing of the RNA structure at CfMV minimal frameshift site. (A) A pRF2-derived transcript was modified by 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-\(p\)-toluene sulfonate (CMCT), kethoxal (Ket) or dimethyl sulfate (DMS), and sites of modification were mapped by primer extension. Unmodified RNA (UM) was used as a control. Lanes C, A, U, C represent the corresponding sequencing ladder of pRF2. Arrows on the left side of the panels indicate transcriptional stops from primer extension representing the chemically modified bases in the treated RNA molecule. The positions of the slippery sequence and secondary structure elements are indicated on the right side of the panels. (B) Summary of the analysis of primer extension results. The bases accessible to CMCT, kethoxal and DMS are shown by filled square, triangle and circle, respectively.
tal series, 16 oat plants were infected with one construct and the experiments were repeated at least twice.

Total RNA was extracted from infected leaves (10 dpi) and upper leaves (27 dpi) as described earlier (Meier et al., 2006). For Northern blot analysis, total RNA was separated on 1% agarose/formaldehyde gel and transferred to Hybond N+ membrane (GE Healthcare). Viral RNA was detected by hybridization with [α-32P]dCTP-labelled probe specific for the CfMV coat protein region (nucleotides 3093–3857). The signal was detected with Personal Molecular Imager FX (Bio-Rad).

RT-PCR with primers detecting the positive strand of viral RNA was performed according to Meier et al. (2006). To verify the presence of the mutation in viral RNA, the region containing the mutation site was amplified from total RNA isolated from infected or upper leaves by RT-PCR. The obtained RT-PCR products were purified from the gel and the region containing the signals for −1 PRF was sequenced.

3. Results

3.1. A stem–loop structure constitutes the structural element for CfMV −1 ribosomal frameshifting

Computer-based analysis of CfMV ORF2a–2b overlapping region (nucleotides 1604–1710) predicted a stem-loop structure seven nucleotides downstream from the slippery sequence (Mäkinen et al., 1995a). To experimentally define the structure, the conformation of the CfMV RNA in the frameshift region was investigated using chemical modification approach. Results from chemical probing and primer extension experiments of the in vitro transcript of pRF2 are shown in Fig. 2A. These results are summarized on the secondary structure model in Fig. 2B.

Chemical probing experiments showed that the spacer region 1 between the slippery sequence and the stem structure (nucleotides 1641–1647) is single-stranded. Positions A (1645) and C (1647) were accessible to DMS, and G (1646) to kethoxal, respectively. Stems 1 and 2 were protected from chemical modifications as expected for base-paired stem regions. Also the G (1654)–U (1670) pair in stem 2 was inaccessible, indicating the formation of an intrahelical wobble pair. The proposed tetraloop (loop, nucleotides 1660–1663) was completely exposed, as was a bulged C at position 1667. In the loop, U (1667) was hit by DMS. The accessibility of the tetraloop rules out the potential involvement of 5′-UACG-3′ nucleotides in forming a pseudoknot. The spacer region 2 (1677–1690) appears to be single-stranded. Several positions were chemically modified beginning with a CMCT modification of U (1677).

3.2. Mutations that shorten or destabilize the stem in the stem–loop structure decrease the efficiency of −1 ribosomal frameshifting

To further evaluate the role of various secondary structure elements in frameshifting, we performed extensive site-directed mutagenesis of the frameshift region (Fig. 3A). The CfMV polyprotein encoding construct pAB-21 and individual mutants were analyzed for their ability to promote −1 frameshifting in vitro in WGE system (Fig. 3B). For pAB-21, the frameshifting efficiency in WGE was calculated to be 11.1 ± 1.3% (Fig. 3C), value similar to those seen in previous studies (Lucchesi et al., 2000; Tamm et al., 1999).

![Fig. 3](https://example.com/fi.png)

**Fig. 3.** The effect of different mutations in the CfMV minimal frameshift region on the in vitro frameshift efficiency. (A) The schematic representation of CfMV frameshift site. The slippery sequence is underlined, the downstream stem-loop structure and the introduced mutations are indicated. (B) Results of 10% SDS-PAGE analysis of [35S]methionine-labeled translation products from the in vitro frameshifting assay of pAB21 construct and different mutants. The positions of the non-frameshifted P2a and frameshifted P2a–b products are indicated by arrows. The calculated frameshift efficiencies are shown in the bottom of the gel. (C) Summary of the mutations made and the resulting frameshift efficiencies in WGE. The quoted frameshift efficiencies are the average of five independent translation reactions.
Based on the structure mapping data, stem 1 of the stem-loop structure contains 6-bp G–C/C–G double-stranded stretch. To test the necessity of this paired region in frameshifting, two variants of mutants were generated (Fig. 3A). Firstly, the nucleotide on one side of the second GC-pair was mutated to disrupt the base pairing. The substitution of G (1649) with uridine or C (1675) with adenosine, both resulted in twofold reduction (from 11.1 ± 1.3% to 4.7 ± 0.8% or 4.5 ± 0.9%, respectively) of the frameshifting efficiency (Fig. 3B and C). Secondly, the second G–C base pair was replaced with the U–A base pair. The compensatory mutations (G1649 → U, C1675 → A) that should restore the base-paired structure of stem 1 did not restore wild type levels of frameshifting efficiency (5.1 ± 0.6%; Fig. 3B and C), demonstrating the necessity of an intact G–C/G–C-base-paired segment during −1 PRF.

We also investigated whether the 5′-UACG-3′ tetraloop participates in the frameshifting event. The structure mapping data already ruled out the possibility of an RNA pseudoknot formation. To assess whether the size of the loop is important, three extra U nucleotides were inserted at the position 1662–1664 (Fig. 3A). We found that this mutation did not noticeably affect the frameshifting efficiency (13.1 ± 1.5%; Fig. 3B and C).

Two constructs were generated to examine the requirement of the bulged C residue at position 1667 for an efficient −1 PRF (Fig. 3A). In mutant pAB(ΔC), the C at position 1667 was deleted, whereas in construct pAB(+C), one extra G was introduced to the position 1657 to form the G–C base pair. In both constructs, additional mutations were introduced after the minimal frameshifting region to maintain translational reading frame. Deletion of the bulged C caused a moderate but significant reduction (from 11.1 ± 1.3% to 7.4 ± 0.8%) in the −1 PRF efficiency (Fig. 3B and C). However, the insertion of one extra G did not affect the frameshifting efficiency noticeably (efficiency 10.4 ± 0.9%; Fig. 3B and C).

The analysis of putative sobemoviral frameshifting signals showed that the motif CUU, located eight nucleotides downstream from stem-loop structure, is conserved in nearly all sequenced sobemoviruses (Fig. S1). The beginning of the stem 1 is G–C rich, containing 6-bp G–C/C–G double-stranded stretch. To test the relevance of this motif for frameshifting, two variants of mutants were generated (Fig. 3A). Firstly, the nucleotide on one side of the second GC-pair was mutated to disrupt the base pairing. The substitution of G (1649) with uridine or C (1675) with adenosine, both resulted in twofold reduction (from 11.1 ± 1.3% to 4.7 ± 0.8% or 4.5 ± 0.9%, respectively) of the frameshifting efficiency (Fig. 3B and C). Secondly, the second G–C base pair was replaced with the U–A base pair. The compensatory mutations (G1649 → U, C1675 → A) that should restore the base-paired structure of stem 1 did not restore wild type levels of frameshifting efficiency (5.1 ± 0.6%; Fig. 3B and C), demonstrating the necessity of an intact G–C/G–C-base-paired segment during −1 PRF.

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3.3. The effect of mutations in stem-loop structure on virus infectivity

To examine the role of the stem-loop structure in viral infection, we constructed three mutant CfMV cDNAs. Based on the data from in vitro frameshifting assays, the substitution of C (1675) with adenosine in the stem 1 and deletion of bulged C (1667), both reduced frameshifting efficiency. In contrast, the insertion of three extra U nucleotides in the loop (to position 1662–1664) did not have a noticeable effect on −1 PRF, a construct pAB(ΔCUU) was created in which the conserved CUU (nucleotides 1685–1687) was deleted (Fig. 3A). The resulting frameshifting efficiency was similar to that of wild type (efficiency 10.4 ± 1.1%; Fig. 3B and C).

The cryo-electron microscopy images (∼16Å) of a mammalian 80S ribosome-mRNA pseudoknot complex that is stalled in the process of −1 frameshifting has allowed to propose a mechanistic explanation for −1 PRF (Namy et al., 2006). According to this model, the pseudoknot blocks the mRNA entrance channel and the ribosome becomes stalled during the transcription phase of the elongation cycle. The P- and A-sites of stopped ribosome must locate over the slippery heptameric sequence. Should the mutation extend the spacer region, the ribosome will pause downstream of the slippery sequence and the probability for it to slip back by 1 nucleotide is reduced. In most cases where −1 PRF is proposed or confirmed to occur, the distance between the slippery sequence and downstream RNA structure is 5–9 nucleotides (Giedroc et al., 2000).

The significance of the optimal spacer length has been systematically investigated using Infectious bronchitis virus (IBV)-derived frameshift signal (Kontos et al., 2001; Naphine et al., 1999). The alteration of the 6-nucleotide spacer as little as a single nucleotide either way reduced frameshifting efficiency in vitro indicating that the precise distance is important. The proposed −1 PRF model also indicates the importance of the strength of the downstream element.
Based on published studies, most viral pseudoknots are G–C rich at the bottom of stem 1 (Giedroc et al., 2000). Replacing G–C pair(s) with A–U pair(s) at the beginning of stem 1 of the IBV pseudoknot reduced the frameshifting efficiency several fold (Napthine et al., 1999). As shown in Fig. 3, the stem 1 of CMV consists of six G–C/C–G pairs. The change of the second G–C pair with U–A pair reduced the CMV \textit{in vitro} frameshifting efficiency twofold. These results indicate that not only the formation of the stem with a certain length, but also the strength and stability of the formed structure are important features for inducing ribosomal frameshifting.

The structure prediction analysis of the sobemoviral frameshift-promoting structures revealed that 8 out of 11 analyzed structures contain at least one bulged nucleotide after the G–C rich stem 1 region (Fig. S1). The structure of \textit{Mouse mammary tumour virus} pseudoknot determined by NMR demonstrated that the bulged adenine is creating a hinge between the stems 1 and 2 and therefore direct coaxial stacking of the stems is not possible (Shen and Tinoco, 1995). Instead, the pseudoknot stem 1 bends towards the major groove of stem 2 resulting in relieving the strain caused by the short loop 1. The presence of similar bent conformation has been shown...
for Beet western yellow virus (BWVVY), Potato leaf roll virus (PLRV), Feline immunodeficiency virus and HIV-1 frameshift-inducing structures (Pallan et al., 2005; Staple and Butcher, 2005; Su et al., 1999; Yu et al., 2005). The mutational analysis demonstrated that the deletion of this bulged nucleotide either causes slight reduction of the frameshifting efficiency in vitro in the case of BWVVY, or even abolishes the frameshifting phenomenon as shown for the PLRV pseudoknot structure (Kim et al., 1999; Kim et al., 2000). However, the structure of the Simian retrovirus type-1 (SRV-1) pseudoknot, determined by NMR, showed that in SRV-1 the stems 1 and 2 are stacked upon each other and for that reason the intercalated base at the junction is not needed for efficient frameshifting (Michiels et al., 2001). The structure mapping data for the CfMV stem-loop structure indicates that the bulged C at position 1667 is accessible to modifiers (Fig. 2). To study the importance of the potential bent conformation, C 1667 was deleted, or a single G was inserted into the opposite stem to promote the formation of G–C base pair. The in vitro measured frameshifting efficiencies revealed that the stem length is important, rather than the bending. The formation of a new G–C pair by inserting one extra G did not affect the frameshifting efficiency while the deletion of the bulged C only moderately reduced it (Fig. 3). However, the deletion of bulged C in the CfMV cDNA clone abolished the viral replication in the infected leaves reduced it (Fig. 3). Nevertheless, we cannot rule out the possibility that the amino acid implicating the importance of the length of the stem region. Nevertheless, we cannot rule out the possibility that the amino acid

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