Basic Amino Acid Mutations in the Nuclear Localization Signal of Hibiscus Chlorotic Ringspot Virus p23 Inhibit Virus Long Distance Movement

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Abstract
The p23 is a unique protein in the Hibiscus chlorotic ringspot virus which belongs to Family Tombusviridae Genus Carmovirus. Our previous results showed that the p23 is indispensable for host-specific replication and is localized in the nucleus with a novel nuclear localization signal. To investigate additional function(s) of p23, mutations of basic amino acids lysine (K), arginine (R) and histidine (H) that abolish its nuclear localization, were introduced into a biologically active full-length cDNA clone p223 of HCRSV for testing its effects on virus replication and virus movement in vivo. Primer-specific reverse transcription-PCR was conducted to detect gene transcript level of p23 and viral coat protein separately. Virus replication and its coat protein expression were detected by fluorescent in situ hybridization and Western blot, respectively. The effect of p23 was further confirmed by using artificial microRNA inoculation-mediated silencing. Results showed that the two mutants were able to replicate in protoplasts but unable to move from inoculated leaves to newly emerged leaves. Both the p23 and the CP genes of HCRSV were detected in the newly emerged leaves of infected plants but CP was not detected by Western blot and no symptom was observed on those leaves at 19 days post inoculation. This study demonstrates that when p23 is prevented from entering the nucleus, it results in restriction of virus long distance movement which in turn abrogates symptom expression in the newly emerged leaves. We conclude that the p23 protein of HCRSV is required for virus long distance movement.

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Introduction
Among positive-sense single-stranded plant RNA viruses, there are 19 reported members in the genus Carmovirus, family Tombusviridae (International Committee on Taxonomy of Viruses, http://ictvonline.org/virusTaxonomy.asp?version = 2012). A few of them are studied more extensively. These include Carnation mottle virus [1], Cowpea mottle virus [2], Hibiscus chlorotic ringspot virus (HCRSV) [3], Melon necrotic spot virus [4], Pelargonium flower break virus [5], Saguaro cactus virus [6] and Turnip crinkle virus [7]. In general, the two 5’ proximal open reading frames (ORFs) of Carmoviruses encode a p28 and a readthrough p81, which are thought to be involved in virus replication [8-10]. The p8 and p9, which are translated from subgenomic (sg) RNA1, are involved in cell-to-cell movement [8,11]. In addition, coat protein (CP) is also involved in virus movement for TCV [8].

HCRSV genome contains 3911 nucleotides with seven ORFs (Figure 1). A biologically active cDNA clone of HCRSV p223 has been obtained previously [3]. The HCRSV CP (p38) [3] is a gene silencing suppressor [12]. In addition, p27 and its other in-frame isoforms (p25 and p22.5) affect symptom expression and potentiates Carmoviruses movement in kenaf (Hibiscus cannabinus L.) [13]. Different from other Carmoviruses, HCRSV contains a novel ORF (p23) which is a putative transcription factor and it is indispensable for host-specific replication [3,14]. In addition, the p23 possesses a novel nuclear localization signal (NLS) which interacts with importin α and facilitates HCRSV RNA genome to enter nucleus [15].

For the p23 NLS, any mutation to the three basic amino acids lysine (K), arginine (R) and histidine (H) (Figure 1) will abolish its nuclear localization. Since p23 is essential for HCRSV replication and it is a putative transcription factor, whether HCRSV infection can be affected by mutations of the basic amino acids is not known. This study is aimed to address this question and to uncover any additional function(s) of p23, based on mutations of its basic amino acids. This will also contribute to the understanding of virus long distance movement and symptom development.

Materials and Methods
Plant Materials and Plasmid Construction
Kenaf seeds (cultivar Everglades 41) were obtained from Mississippi State University, U.S.A. and germinated in potting mixture (Universalerde Universal Potting Soil, The Netherlands) for 7 days. Kenaf seedlings were transferred into potting mixture after emergence of their first true leaves. All plants were grown under 16 h light and 8 h dark conditions at 25 °C.
Mutations in the basic amino acids of the p23 NLS, which includes mutants p223 (H to A) and p223 (K, R to A, A), respectively, were introduced into the biologically active full-length cDNA clone of HCRSV p223 [14] using appropriate primers (Table 1). Enzyme DPnI was used to remove the original template after amplification using high fidelity enzyme (KAPA Biosystems) and PCR products were transformed into *Escherichia coli*. Single colonies were picked individually for plasmid preparation and sequence verification.

Artificial-microRNA (amiRNA) amiRp23 or amiRSO was engineered into the miR319a precursor (plasmid pRS300) by site-directed mutagenesis (overlapping PCR) (Table 1), following the protocol described by Rebecca Scheab of Max-Plank Institute for Developmental Biology, Tuebingen, Germany (2005) (http://wmd.weigelworld.org/cgi-bin/mirnatools.pl. Ossowski Stephan, Fitz Joffrey, Schwab Rebecca, Riester Markus and Weigel Detlef, personal communication). The amiRp23 or amiRSO fragment was inserted into pGreen vector with EcoRI and BamHI restriction enzyme sites. The verified plasmid was transformed into

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**Figure 1. Organization of HCRSV genomic RNA and its corresponding open reading frames and predicted proteins (not drawn to scale).** The upper rectangles represent open reading frames. Untranslated region (UTR); putative transcription factor (PTF); RNA-dependent RNA polymerases (RdRps); movement protein (mp); pathogenesis-related gene (prg); coat protein (CP). The dotted vertical line represents a readthrough codon UAG. The 3-dimensional rectangles below represent the corresponding predicted mature proteins. Basic amino acids K, R, and H represent the mutation sites in the nuclear localization signal (NLS) of p23.

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**Table 1.** Primers used in this study.

| Primer          | Sequence (5’ to 3’) |
|-----------------|---------------------|
| I. amiR-p23 F   | GATTTAATTGACTGCACGTCCTTTTCTCTTTTTTTGATTCC |
| II. amiR-p23 R  | GAAAGACGTGCAGTCAATTAAAATCAAAGAGAATCAATAA |
| III. amiR-p23* F | GAAAAGTGCACGTCTATTAAATTCACAGGTCGTGATATG |
| IV. amiR-p23* R | GAATTTAATAGACTGCACGTTTTTCTCACATATATATTCCT |
| I. amiR-HcSO F  | GATTTAATTGACTGCACGTCCTTTTCTCTTTTTTTGATTCC |
| II. amiR-HcSO R | GAAAGACGTGCAGTCAATTAAAATCAAAGAGAATCAATAA |
| III. amiR-HcSO* F | GAAAAGTGCACGTCTATTAAATTCACAGGTCGTGATATG |
| IV. amiR-HcSO* R | GAATTTAATAGACTGCACGTTTTTCTCACATATATATTCCT |
| A               | CTGCAAGGCCGATTTAAGTTGGTAC |
| B               | GCGGATACAAATTTCCACAGAAGACAG |
| P23F            | CCGGAATTCTAGCTTTTCTCAATTTGCTTTC |
| P23R            | CCGGAATTCTAGCTTTTCTCAATTTGCTTTC |
| H-CP-F          | CTTGAATTTGGATGCGCTGAGAACATG |
| H-CP-R          | GTGCTAGATTTGGATGCGCTGAGAACATG |
| P23(K,R-A,A)58–123F | CGCACTGTTGGATTTAACATACAGAACCCACCCTGTCGAGCGATAGA |
| p23(K,R-A,A)58–123R | CGGCGGATATCGTGTAAATCCAAAGTCGGTCGCGGAAAGTGCGC |
| p23 (H-A)58–123F | AATTATACGCACCCCCCTGCCTGACGAGATCCCTTGAGATCCTG |
| p23 (H-A)58–123R | AAGGATCTGCGAGCGATACCCGGGCGCGCTGTGATAAAATTTCAACAGCTG |
| HcAct-qf603     | ACGAGCAAGAGACGACT |
| HcAct-qr734     | TGAGGTAGCCTGGAAGAGA |

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**Plant Inoculation with in Vitro Transcripts of p223 and its Two Mutants**

Plasmids (p223, p223 (H to A) and p223 (K, R to A, A) were linearized with Smal and transcribed accordingly using the in vitro transcription kit (Ambion, mMESSAGE mMACHINE). In vitro transcribed RNA was verified for integrity by gel electrophoresis. One μg of the transcribed RNA was mixed with equal volume of 2xGKP buffer (50 mM glycine, 30 mM K₂HPO₄, pH 9.2, 1% bentonite and 1% celite). Two fully expanded true leaves from healthy kenaf plant were inoculated. Twelve individual plants were used for each treatment. In addition to plants treated with in vitro transcribed RNAs of the wild type (wt) HCRSV (positive control) and two mutant viruses, plants were also inoculated with 1% GKP buffer as mock (negative control). The experiments were repeated twice.

**Preparation of Kenaf Protoplasts for Fluorescent in Situ Hybridization (FISH)**

Protoplasts were isolated following previously described protocol [16]. Briefly, the collected kenaf leaves were sterilized for 10 min with 0.8% (v/v) Clorox® and cut into 1 mm × 1 mm stripes and then digested with filter-sterile cellulase (0.8%) and macerase (0.25%) for 16 h. The isolated protoplasts were transfected with in vitro transcribed RNA from full-length HCRSV wt and its basic amino acid mutants. At 72 h post transfection, protoplasts were concentrated by 100× centrifugation and fixed with the fixation buffer 4% (w/v) paraformaldehyde, 2.5% sucrose (w/v) in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. The FISH procedures were adopted from a protocol described by Zenklusen and Singer [17,18] with slight modifications. Briefly, 50 ng Cy3-labeled HCRSV cDNA probe [bind to genomic RNA (gRNA) nt 330 to 301 which is located in the region of the p23 sequence]. The probe sequence 5’-GGTTTGGGAGTGGCCCCCCAAAAATG-GTAGCTTTGTGCTGCCTGGCGCATGGAGGC-3’ was used to localize HCRSV RNA. All fixed protoplasts were attached to poly-L-lysine coated coverslips and stored in 70% ethanol (v/v) at −20°C before hybridization. Cover slips were inverted onto 25 g of the transcribed RNA was mixed with equal volume of 2xGKP buffer (50 mM glycine, 30 mM K₂HPO₄, pH 9.2, 1% bentonite and 1% celite). Two fully expanded true leaves from healthy kenaf plant were inoculated. Twelve individual plants were used for each treatment. In addition to plants treated with in vitro transcribed RNAs of the wild type (wt) HCRSV (positive control) and two mutant viruses, plants were also inoculated with 1×GKP buffer as mock (negative control). The experiments were repeated twice.

**RNA Extraction and cDNA Synthesis for RT-PCR and qRT-PCR**

Both inoculated and newly emerged leaves were harvested for RNA extraction with TRIzol® reagent (Invitrogen) at 19 days post inoculation (dpi). Total RNA (~2 μg) were used to generate cDNAs through reverse transcription, using oligo(dT)₁₅ as primer and SuperScript® III Reverse Transcriptase kit (Invitrogen). Reverse transcription PCR (RT-PCR) (Table 1) was used to analyze the gene transcript levels of p23 and CP. The quantitative real-time reverse transcription PCR (qRT-PCR) was used to analyze amiRp23 expression with the CFX96™ real-time PCR detection system (Bio-Rad). Actin gene was amplified using appropriate primers (Table 1) as an internal control for all qRT-PCR. Samples of mock, HCRSV wt and two mutants included three biological repeats and each qRT-PCR sample provided three technical repeats. The qRT-PCR results from different treatments were subjected to the Student’s t-test for statistical analysis.

**Western Blot Analysis of HCRSV CP**

Western blot was carried out according to previously published protocol [19]. Briefly, inoculated and newly emerged leaves (0.1 g) from p223, p223 (H to A) and p223 (K, R to A, A) were collected for protein extraction, with 0.2 ml of protein extraction buffer [220 mM Tris–HCl, pH 7.4, 250 mM sucrose, 50 mM KCl, 1 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and 1×complete EDTA-free protease inhibitor (Sigma, St. Louis, USA)]. The denatured protein samples were separated on the 12% SDS PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad), followed by incubation with anti-HCRSV antibody and visualized using nitroblue tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (Merck).

**Agrobacterium tumefaciens-Mediated Transient Expression of amiRp23 and amiRSO**

Agrobacteria liquid culture containing each of the pGreen-amiRp23, pGreen-amiRSO (a negative control, it targets a host factor sulfite oxidase) and 35SppGreen plasmids (a second negative control), was grown to OD reading (at 600 nm) between 1.0–1.5 and harvested. The cell pellet was resuspended in infiltration buffer (pH 7) containing 10 mM each of MgCl₂ and 2-(N-morpholino) ethanesulfonic acid (MES), and 100 μM acetosyringone and infiltrated into kenaf leaves using a syringe without needle [13]. The experiment was carried out thrice.

**Inoculation of amiRp23 and amiRSO into Apical Meristems of HCRSV-infected Kenaf Leaves**

Inoculation procedures followed the published protocols [20,21] with slight modifications. Briefly, Agrobacteria were resuspended in the infiltration buffer (1×10⁶ cell/ml) instead of water and used as inoculum. After pricked with a needle (Φ 0.71 mm), the apical meristems of 10-day-old seedlings (10 cm height) were inoculated with a cotton applicator drenched with the inocula or infiltration buffer alone (as a mock treatment). The inoculated seedlings were kept at 22°C in the dark for 3 days and subsequently grown under 16 h light and 8 h dark conditions at 25°C. After one month, the newly emerged leaves were observed for symptom expression.

**Results**

**Viral Replication was Unaffected in the Two HCRSV Mutants**

The three basic amino acid mutations of p23 also resulted in changes of both amino acids tryptophan (T) and proline (P) to arginine (R) in the p28 and thus its readthrough protein p81 which encode the putative RNA-dependent RNA polymerases. Therefore, we first investigated if the replication of these two mutants were affected due to changes in the p28/p81. Using the Cy3-labeled probe with the FISH method, we monitored the viral replication of the HCRSV wt and the two mutants in kenaf protoplasts. RT-PCR results showed that basic amino acids lysine (K), arginine (R) and histidine (H) remained in their mutated status as alanine (A) within 72 hours post transfection. A representative section for each of the three dimensional images of protoplasts transfected with HCRSV wt and two mutants was shown.
The replicase of RNA viruses, except retroviruses, is highly error-prone [22]. Generally artificially introduced mutations in a virus genome will be reverted back under selection pressures. As a result, RNA viruses can rapidly eliminate genetic mutations in the virus genome will be reverted back under selection pressures. As a result, RNA viruses can rapidly eliminate genetic mutations. In this study, two representative mutants p223 (H to A) and p223 (K, R to A, A) were chosen to test the effects of basic amino acids on virus movement. From these results, we extrapolate the same effects to other basic amino acids within the NLS of p23 mutants on virus movement. In order to determine the subcellular localization of p23 protein, a GFP fused protein driven by the 3SS CaMV promoter was used [15]. The GFP signal in the fused protein can be traced using confocal laser microscopy. However, in the virus mutants, localization of p23 protein is not possible due to its minute amount produced. Since p23 is an individual ORF expressed in the virus mutant with the same amino acids as in the GFP fused protein, it is reasonable to believe that mutations in the p23 region will also yield similar results when there are expressed as part of the virus mutants.

The replicase of RNA viruses, except retroviruses, is highly error-prone [22]. Generally artificially introduced mutations in a virus genome will be reverted back under selection pressures. As a result, RNA viruses can rapidly eliminate genetic mutations introduced into their genomes. However, mutations may induce certain phenotypes on the infected plants. In this study, no symptom was observed in the newly emerged leaves of kenaf plants infected with the two mutants at 19 dpi.
Figure 2. Comparative replication of HCRSV wild type (wt) and two p23 mutants. (A) A single molecule fluorescent in situ hybridization (FISH) method was used to detect virus replication, using a Cy3-labeled cDNA probe (corresponding to the HCRSV p23 coding region at nt 350–301). DAPI stained nuclei (blue-color foci) were superimposed with the differential interference contrast to form a merged image. Kenaf protoplasts were fixed with 4% paraformaldehyde. Cy3 signals were not detected in mock transfected protoplasts. Representative sections for each of the three dimensional images of protoplasts transfected with HCRSV wt and two mutants were shown (mock, wt, H to A and K, R to A, A). Single RNA molecules (red dots) were detected in the protoplasts transfected with in vitro transcript of full-length cDNA clone of HCRSV wt or mutants H to A (H A) or K, R to A, A (K, R A, A) mutant at 72 hours post transfection. The average density of Cy3 signals in the protoplasts quantified using Velocity 6.1.1 software (PerkinElmer) showed that the replication level was similar for the HCRSV wt and its two mutants. Bar = 2 μm. (B) Comparison of the average Cy3 density measured from 10 protoplasts for mock, HCRSV wt, HCRSV basic amino acid mutants H to A and K,R to A,A, respectively. Standard deviations were shown.

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In addition to the NLS of p23, we have also identified the HCRSV gRNA in the nucleus where one of the predicted viral miRNAs (vir-miRNAs) hcrsv-miR-H1-5p is thought to be generated from the p23 sequence [15]. Although the two mutations in the basic amino acids in p23 did not affect virus replication (Figure 2), they could disrupt the nuclear localization of p23 and prevent the entry of HCRSV genome into the nucleus. It is believed that vir-miRNAs play essential roles in overcoming host resistance for efficient infection of viruses [23]. When the p23 is unable to enter nucleus, it will not be able to interact with host genomes and thus interferes with host transcription that confers resistance. In addition, the lack of vir-miRNAs generated by the p23 sequence would not be made available to target host sequences to counteract host defence.

In this study, we have adopted two approaches to uncover additional function(s) of p23. First, a transgenic approach was used to study the function of amiRp23 to silence its target p23 gene. The amiRp23 was designed specifically to cleave p23 messenger RNA or to inhibit p23 translation. It may also downregulate viral gRNA in the p23 region, but not other regions of the viral genome. The sgRNAs and viral genes located outside the p23 gene region are not affected. Therefore, the downregulation of p23 using amiRp23 (Figure 5D) caused silencing of p23 and inhibition of long distance virus movement (Figure 3) and resulted in reduced symptom severity in infected leaves (Figure 5E). Since the kenaf plants grown under our laboratory conditions did not produce...
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Flowers, we were unable to generate transgenic progeny plants. However, using the apical meristem inoculation method, we have introduced amiRNA into the plants to silence p23. Due to these limitations, we were also unable to generate a reporter transgene to monitor the silencing efficiency of amiRp23. Another approach is the use of the single molecule detection technique FISH to study virus replication. It is a direct visualization technique which tracks signals in a single cell. It is a more specific and sensitive method for study of viral replication, as compared to Northern blot which requires relatively larger amount of RNA samples. We chose this method over northern blot due to the lack of sufficient amount of RNAs present in some of our test samples. Therefore, the FISH technique can be broadly applied to other virus replication studies.

In conclusion, this study has uncovered an additional function of p23 of HCRSV. Although the basic amino acid mutants of p23 are prevented from entering the nucleus, those mutants are still able to replicate but unable to be moved long distance. This indicates yet another example of multi-functional roles of plant viral genes.

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Author Contributions

Conceived and designed the experiments: RMG SMW. Performed the experiments: RMG. Analyzed the data: RMG SMW. Contributed reagents/materials/analysis tools: RMG SMW. Wrote the paper: RMG SMW.
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