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Short communication

Tandem repeat sequence of duck circovirus serves as downstream sequence element to regulate viral gene expression

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ABSTRACT

Duck circovirus (DuCV) has a small, single-stranded circular DNA genome of approximately 1.99 kb. Through a genome sequence analysis using the dottup program, we found that a quadruple tandem repeat sequence (QTR) in the intergenic region between the *rep* and *cap* genes of the DuCV genome, but not in other circoviruses. The QTR was also substantially different and evolutionarily conserved in the genotype 1 and 2 DuCV strains. Furthermore, a luciferase reporter assay demonstrated that QTR functioned as a downstream sequence element (DSE) of polyadenylation signals to enhance mRNA stability, which was dependent on four copies but not the QTR direction. Cap and Rep expression derived by subgenomic constructs also revealed a critical role of QTR in regulating viral gene expression. Finally, a reverse genetic study of a DuCV-based minicircle DNA technique found that a deletion of QTR induced a significant deficiency in viral genes transcription and replication. Our findings were the first to report that QTR only exists in the DuCV genome and serves as a novel molecular marker of DuCV genotyping, and has revealed its crucial biological function in regulating viral gene expression.

1. Introduction

Duck circovirus (DuCV) has been reported in many countries, including Germany, Hungary, Korea, USA and China (Banda et al., 2007; Cha et al., 2013; Chen et al., 2006; Fringuelli et al., 2005; Hattermann et al., 2003). DuCV can infect most duck species and causes feathering disorders and poor body condition (Soike et al., 2004). Lymphocyte apoptosis in bursa of Fabricius induced by DuCV is considered as vital pathogenesis for the immunosuppressive lesion and secondary infection by other pathogens (Soike et al., 2004; Zhang et al., 2009). Recently, DuCV has been reported to induce beak atrophy and dwarfism syndrome in duck together with goose parvovirus-related virus (Li et al., 2018). However, DuCV infection and replication regulatory mechanism are still not clear yet.

The genome of DuCV is a short single-stranded circular DNA of approximately 1.99 kb, which contains a stem loop and three major open reading frames (ORFs): ORF1, ORF2 and ORF3. ORF1 and ORF2 encode the replication-associated replicase (Rep) and the major immunogenic capsid protein (CP), respectively. ORF3 is located in the complementary strand of ORF1 and encodes the ORF3 protein with apoptotic activity (Xiang et al., 2012). Stem loop is considered as the site of viral DNA rolling circle replication initiation (Steinfeldt et al., 2001). Basing on the homology of full genomic sequence, DuCV is divided into two genotypes: genotypes 1 and 2 (Wang et al., 2011). We previously found the additional C-terminal 20 residues of genotype 2 DuCV ORF3 protein induce its nuclear localization and inhibit its apoptotic activity (Wu et al., 2018). However, whether there are other important function units in DuCV genome and their function difference between two genotypes, it is still unclear and needs further investigation.

In eukaryotes and prokaryotes, repeat sequences include tandem repeats and interspersed repeats, which are important for gene expression and genome replication (Gymrek et al., 2016; Jeffreys et al., 1985; van Belkum et al., 1998). Repeat sequences in retrovirus, Marek's disease virus (MDV), adeno-associated virus, Epstein-Barr virus and herpes virus also play a role in regulating viral gene expression, genome replication, life cycle and pathogenicity (Gendelman et al., 1986; DesGroseillers and Jolicoeur, 1984; Heller et al., 1982; Jenkins and Martin, 1990; Xiao et al., 1997). However, up to now, the mechanism how repeat sequences participate into viral replication is still less

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known.

In this study, we found that a quadruple tandem repeat sequence (QTR) specifically exists in the intergenic region of the *rep* and *cap* genes of the DuCV genome, but not in other members of the circovirus genus. Moreover, the QTR was obviously different and evolutionarily conserved in genotype 1 and 2 DuCV strains. The QTR served as a downstream sequence element (DSE) of polyadenylation signals to enhance viral mRNA stability and gene expression. Our findings are the first to report that the QTR only exists in the DuCV genome and reveal its biological function in viral replication.

2. Materials and methods

2.1. Cells culture and reagents

Genotype 2 *FJ0601* strain (GenBank no. EF370476) was previously isolated from the livers of sick Muscovy duck in Fujian province of China in 2006 (Jiang et al., 2008). Genotype 1 *DuCV* strain (GenBank no. AY228555) was firstly reported by Hattermann in 2003 (Hattermann et al., 2003) and its genomic DNA was synthesized from GENEWIZ Inc., Suzhou, China. The duck embryo fibroblast cells (DEF) were grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 6% fetal bovine serum (FBS) at 37 °C in an atmosphere containing 5% CO2. The mouse anti-HA monoclonal antibody (1:5000; HA-7; Sigma, St. Louis, MO, USA) and anti-β-actin monoclonal antibody (1:5000; 66009-I-Ig; Proteintech) were used for western blot. FJ0601 infectious clone was generated basing on minicircle DNA system containing MN0501 vector and ZYCY10P3S2T E. coli producer strain (System Bioscience, Mountain View, CA, USA).

2.2. Plasmids construction

QTR luciferase reporter plasmids and subgenome plasmids were constructed using Infusion Cloning Kit (Clontech, Mountain View, CA, USA) according its instruction. Briefly, QTR with 15 bp homologous arm at 5' and 3'-ends with vector was amplified, and then ligated with pGL3-promoter digested with indicated restricted endonucleases using infusion enzyme at 50℃ for 15 min. DuCV subgenome plasmids were constructed by inserting Rep or Cap genes with QTR into the pCMV-HA vector (Clontech). Parent FJ-0601 minicirle plasmid (pmini-FJ0601) was generated by ligating FJ0601 genome and basic MN0501 vector using Infusion Cloning Kit. QTR truncation luciferase reporter mutants and MN-FJ0601-ΔQTR were generated by KOD-Plus-Mutagenesis Kit (TOYOBO CO., LTD, Japan). MC-FJ0601 and MC-FJ0601ΔQTR were generated through arabinose induced pmini-FJ0601 recombination.

2.3. Luciferase assay

DEF cells cultured on 48-well plates were transfected with a combination of luciferase reporter plasmids and control renilla luciferase plasmid pRL-TK (Promega, Madison, WI, USA) at 50:1 ratio. The cells were lysed with passive lysis buffer at 36 h post-transfection, and collected to analyze relative firefly luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol and normalized to Renilla luciferase activity.

2.4. mRNA half-life

Cells pre-cultured on 24-well plates were transfected with indicated plasmids for 36 h, then treated with actinomycin D (ActD, 10 μg/ml) for indicated periods of time from 0 to 12 h. Total RNA was extracted from cells using TRIzol reagent, and 1 μg RNA was digested with DNase I and used to synthesize cDNA (Thermo Fisher Scientific). qPCR analysis was performed using SYBR Premix Ex Taq™ (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. Relative gene expression was normalized to the internal control GAPDH by the $2^{-\Delta\Delta Ct}$ method. The half-life of luciferase and rep mRNA was determined using GraphPad Prism 7.01 (two-phase decay model). The data represents mean values ± SEM from two independent experiments.

2.5. Progeny DuCV DNA detection

DEF cells were transfected with MC-FJ0601 or MC-FJ0601ΔQTR and culture supernatant was collected at indicated times. 200 μl supernatant was firstly digested with 5 U DNase I (Thermo Fisher Scientific) for 30 min at 37℃, viral DNA was then extracted with QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to to the manufacturer's protocol. Relative dynamic change of DuCV DNA level was normalized to DNA level at 12 h post transfection. The primers sequences used for qPCR detection of DuCV DNA are DuCV-qF: TGTTATCTTTGGGCGTGG, DuCV-qR: CATTTCCCGAGTAACCGTC.

2.6. Statistical analysis

Data are presented as mean ± standard error (SEM) from triplicate experiments. Significance was determined using Student's t-tests or one-way ANOVA. A value of $p < 0.05$ was considered as significant.

3. Results

3.1. Repeat sequence analysis of DuCV and other circoviruses

To date, the regulatory mechanism of DuCV replication remains unclear. Repeated sequences have been shown to regulate viral gene expression, life cycle, and pathogenicity. Thus, we first analyzed the repetitive element in the DuCV genome using the dottup tool of the EMBOSS program. Using the genotype 1 strain *DuCV* genome (genotype 1-DuCV) and genotype 2 strain *FJ0601* genome (genotype 2-DuCV) as the representative genomes of genotypes 1 and 2 DuCV, respectively, we discovered a QTR in both the genotype 1-DuCV and genotype 2-DuCV genomes (Fig. 1A and B), that was not present in psittacine beak and feather disease virus (PBFDV), goose circovirus (GoCV), genotype 1 and 2 pig circovirus (PCV) (Fig. 1C), and other circoviruses, including pigeon circovirus (PiCV), mink circovirus (MiCV), gull circovirus (GuCV) and chicken anemia virus (CAV) (data not shown). Furthermore, two copies of short interior repeats specifically existed within the QTR longer repeat unit in genotype 1-DuCV, but not in genotype 2-DuCV (Fig. 1A and B, right panel).

Further DuCV genome analysis confirmed that the QTR was localized in the intergenic region of the *rep* and *cap* genes (Fig. 2A). In genotype 2-DuCV, the QTR was 168 nt comprised of four 42 nt tandem repeat units (Fig. 2B). However, the QTR length in genotype 1-DuCV was 180 nt and contained four 45 nt long tandem repeat units and eight 7 nt regularly interspaced repeats (Fig. 2B). The sequence alignment of more representative genotype 1 and 2 DuCV strains found that the QTR sequence identity was 97.2–100% in genotypes 1 DuCV and 98.2–100% in genotypes 2 DuCV, but it was only 79.1–81.6% between genotypes 1 and 2 DuCV (Fig. 2C and D), which revealed that the QTR was highly evolutionarily conserved in each genotype. Taken together, these data indicate that the QTR uniquely existed in the DuCV genome and could serve as an accurate molecular marker to distinguish between DuCV genotypes.

3.2. QTR functions as a DSE of the transcription termination signal

As a cis-acting element, the DNA repeat sequence has been shown to regulate gene expression as an enhancer or other elements (Qian and Adhya, 2017). Thus, we first investigated the cis-acting role of the QTR *via* a dual luciferase reporter assay. When genotype 2-DuCV QTR (G2-QTR) and genotype 1-DuCV QTR (G1-QTR) were inserted downstream of polyA, luciferase activity did not obviously change, but dramatically

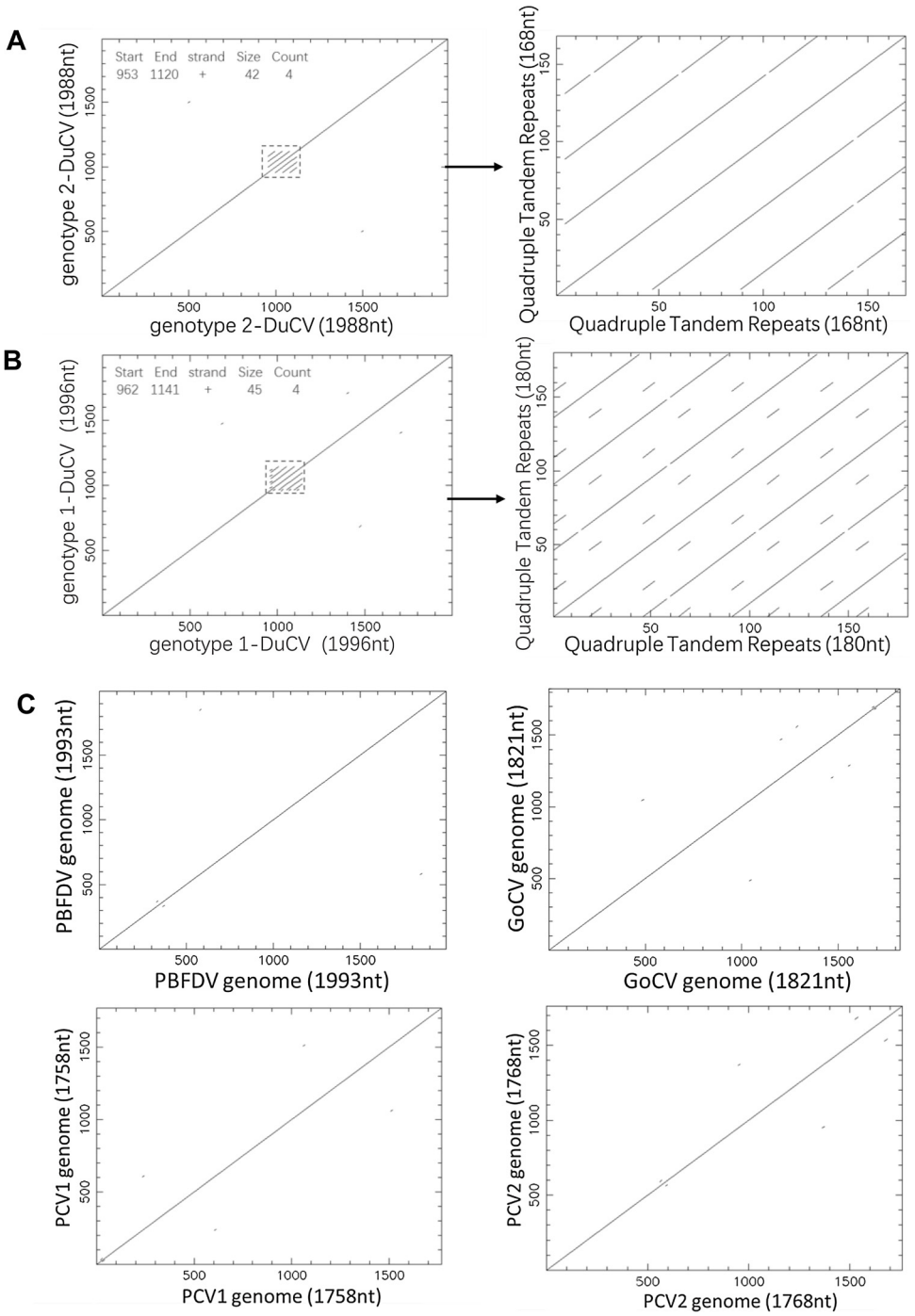

Fig. 1. Repeat sequence analysis of DuCV and other circoviruses. (A and B) Repeat sequence analysis of genotype 2 *DuCV* strain and genotype 1 *FJ0601* strain genome (left panel) and predicated tandem repeats using dottup tool of EMBOSS Program (right panel). (C) Repeat sequence analysis of PBFDV, GoCV, PCV1 and PCV2 genome with dottup tool.

increased in the SV40 enhancer construct (Fig. 3A), which excluded the role of QTR as an enhancer. When the QTR was inserted into the spacer of the CMV promoter and luciferase gene, the relative luciferase activity sharply decreased to 30% and 10% in the G2-QTR and G1-QTR groups, respectively (Fig. 3A). Furthermore, inversal of the QTR insertion also obviously reduced the level of luciferase activity (Fig. 3A), which indicated that the inhibitory role of QTR on luciferase expression was bi-directional.

Since the QTR is located downstream of the *cap* and *rep* genes, we next investigated its role as a polyA signal. SV40 polyA deletion almost completely silenced the luciferase activity, which was not recovered after replacement with G2-QTR or G1-QTR (Fig. 3B). The complete polyA signal is comprised of upstream sequence element (USE), AAUAAA, CA, and downstream sequence element (DSE) (Proudfoot, 2011). An upstream sequence analysis of the QTR found an AATAAA and CA element located upstream of the QTR (Fig. 4A), and the QTR contained a higher proportion of GU nucleotides (Fig. 2B). Hairpin and stem-loop structures at the 3' end of the mRNA have been found to be essential for destructing the elongation complex to induce transcription termination *via* a factor-independent mechanism (Santangelo and Artsimovitch, 2011). Further RNA secondary structure prediction with the RNAfold Web Server also found that the G2-QTR possessed two

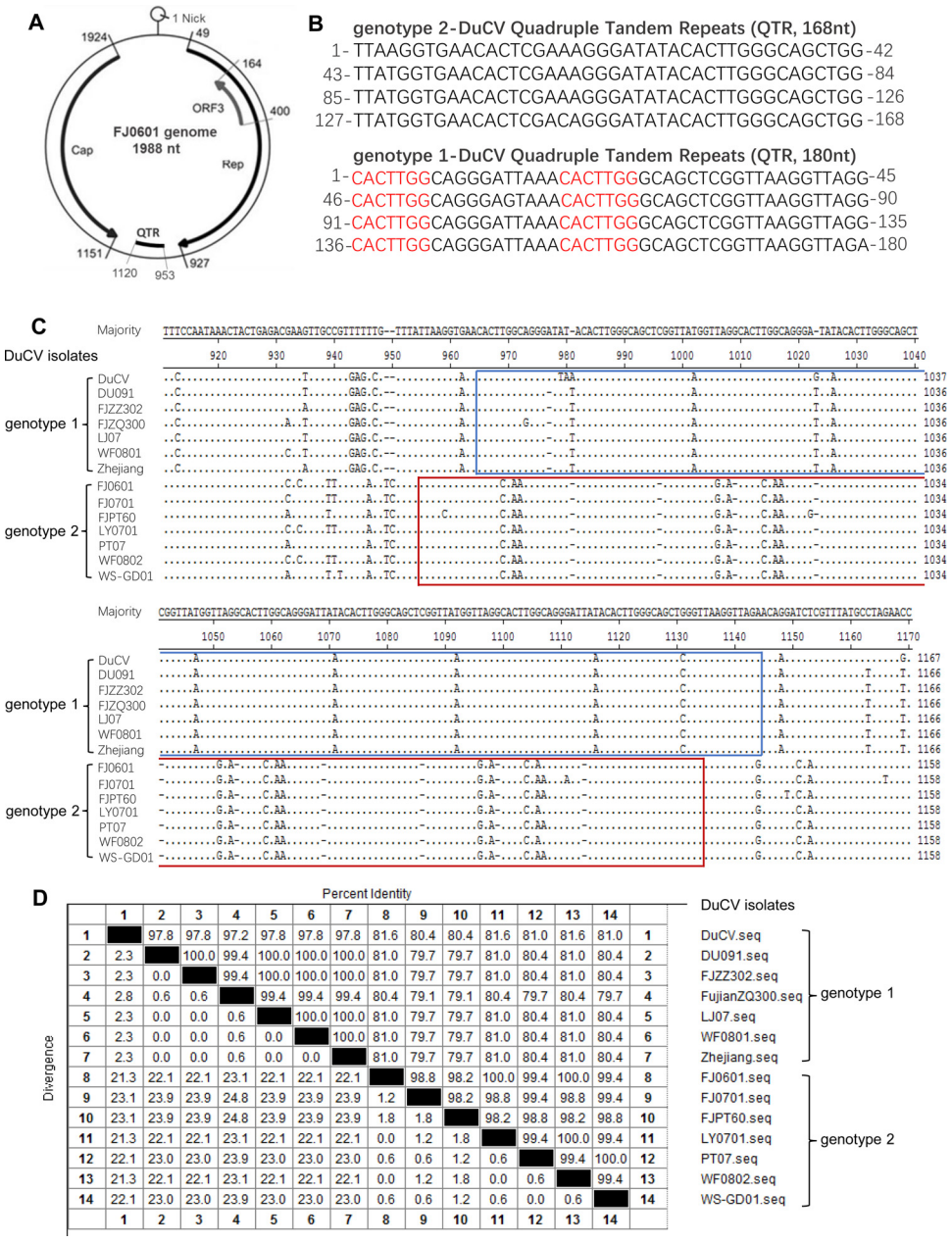

Fig. 2. Sequence characteristic and alignment of QTR from different DuCV strains. (A) QTR localization in FJ0601 genome. (B) QTR sequence characteristic of genotype 2 *DuCV* strain and genotype 1 *FJ0601* strain, red bold was short repeats in the long repeat units. (C) Sequence alignment of QTR from representative genotype 1 and 2 DuCV strains using Clustal W algorithm of DNAstar Megalign software. Dot represents the identical nucleotide; blue box indicates QTR in genotype 1 strains; red box indicates QTR in genotype 2 strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

hairpins and G1-QTR possessed one long hairpin structure (Fig. 4B). These data suggest that the QTR may act as a DSE. As shown in Fig. 4C, DSE deletion of the SV40 polyA signal substantially reduced the level of luciferase activity, which was fully recovered by G2-QTR or G1-QTR substitution. DSE replacement by an inversal of the G2-QTR and G1-QTR also displayed a similar recovery effect (Fig. 4C). These data demonstrate that the QTR functioned as a DSE to promote gene expression. Moreover, a comparison of the luciferase activity among G2-QTR mutants with different copies indicated that the DSE role of the QTR was dependent on four tandem repeat copies being intact (Fig. 4D).

Since a polyA signal is required for mRNA maturity and stability, we next investigated the role of the QTR in regulating mRNA stability. A DSE deficiency of SV40 polyA (pGL-ΔDSE) resulted in rapid luciferase mRNA degradation, in which the half-life was reduced to 4.2 h compared with 9 h in an intact SV40 polyA (pGL3-promoter) (Fig. 4E). The insertion of the G2-QTR into pGL-ΔDSE significantly recovered the half-life of luciferase mRNA to 8 h, similar to that of the pGL3-promoter transfectants (Fig. 4E). Taken together, these results demonstrate that the QTR functions as a DSE of the polyA signal to promote mRNA stability, which was similar in both genotype 1/2 DuCV and dependent on four intact copies.

3.3. QTR regulates viral gene expression

In light of the bidirectional characteristics of the QTR as a DSE, as well as the interspace localization between the *cap* and *rep* genes, we sought to further confirm the role of the QTR from the FJ0601 strain in regulating Cap and Rep expression derived by the SV40 promoter in a subgenomic model. The level of Rep protein expression in the pRep-QTR was similar to that of pRep-polyA (Fig. 5A, upper panel). Further QTR deletion in the Rep-QTR subgenome resulted in a deficiency in Rep expression (Fig. 5A, upper panel). Similar results were also found in the Cap-QTR subgenome (Fig. 5A, lower panel). These data demonstrate a critical role of the QTR in regulating viral gene expression.

To date, there has been no appropriate cell culture system available for DuCV infection. To better understand the role of the QTR in the DuCV life cycle, we first established a novel DuCV reverse genetic model, MC-FJ0601, and its QTR deletion mutant, MC-FJ0601ΔQTR,

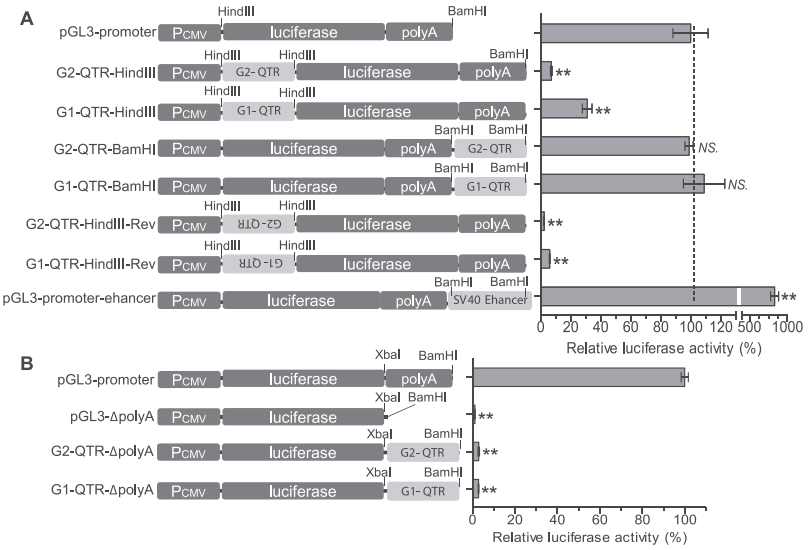

Fig. 3. QTR possessed transcription inhibitory role. (A) Transcriptional enhance and silence activity analysis of G2-QTR and G1-QTR by luciferase reporter assay. (B) PolyA signal activity analysis of G2-QTR and G1-QTR by luciferase reporter assay.

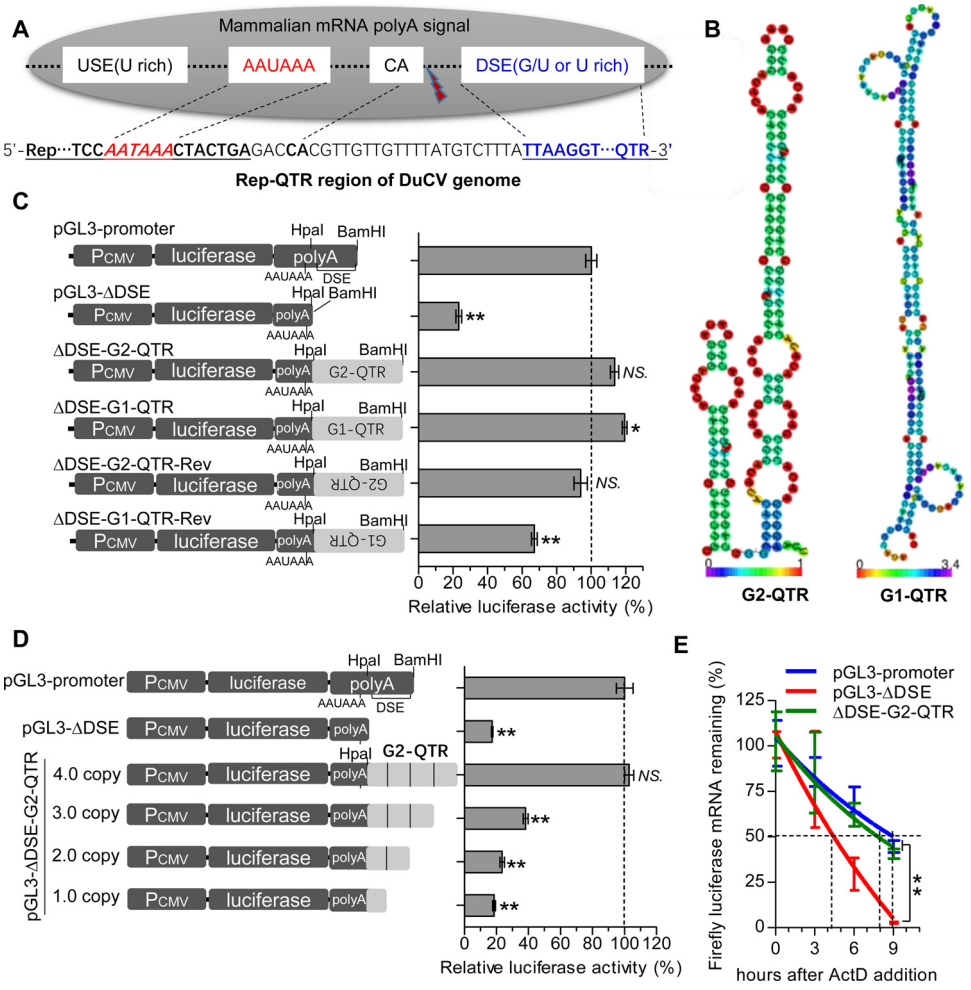

Fig. 4. QTR functions as a downstream element (DSE) of transcription termination signal. (A) Comparison of classical polyA signal composition and upstream sequence feature of G2-QTR. (B) Analysis of RNA secondary structure of G2-QTR and G1-QTR by RNAfold program. (C) DSE activity of G2-QTR and G1-QTR was analyzed by luciferase reporter assay. (D) The role of QTR copies was estimated by luciferase reporter assay. (E) Half-life of firefly luciferase mRNA was measured with qPCR.

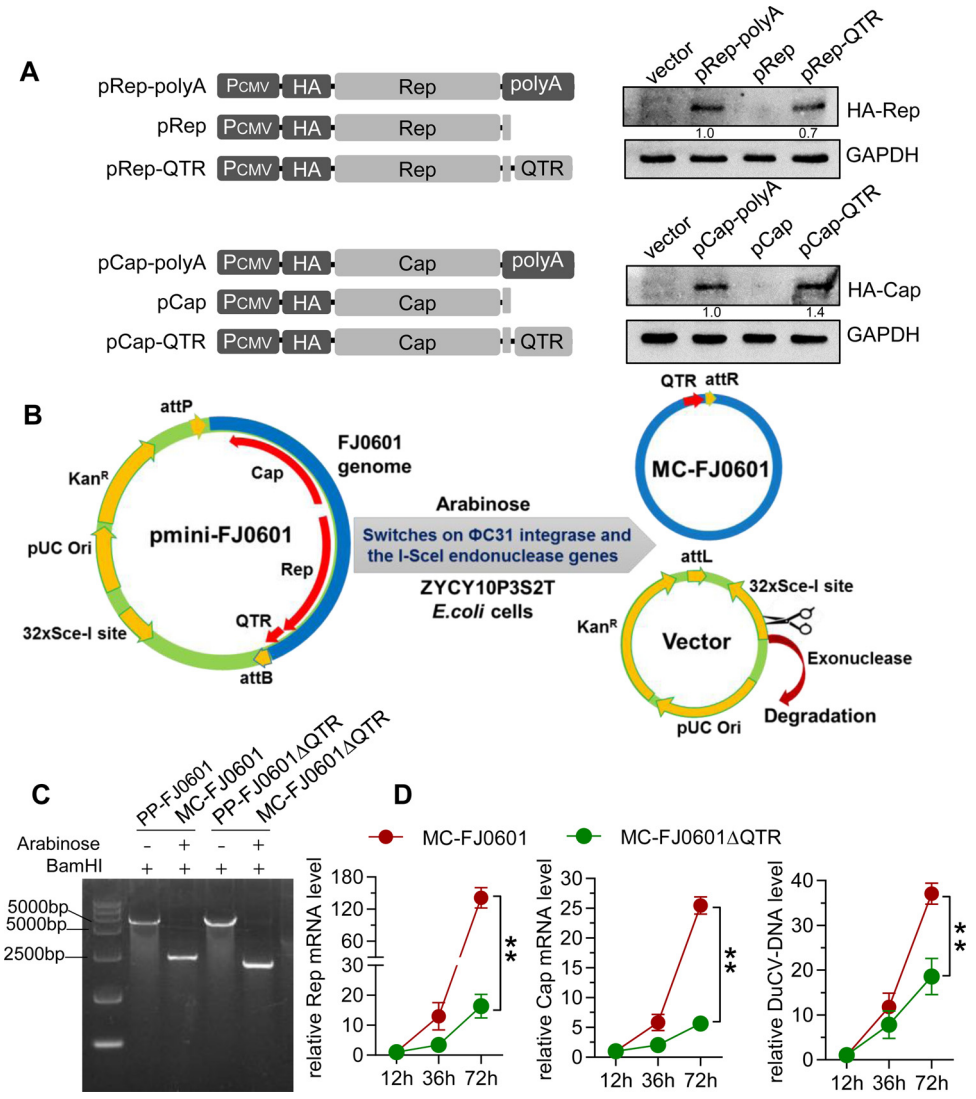

Fig. 5. QTR regulates viral gene expression in subgenome and MC-FJ0601 models. (A) Rep and Cap expression regulated by QTR was analyzed by western blot. (B) Schematic diagram of FJ0601 reversed genetic system construction basing minicircle DNA vector technique. (C) Identification of induction efficiency of MN-FJ0601 and MC-FJ0601ΔQTR with BamHI digestion and gel electrophoresis analysis. (D) The dynamic change of Rep/Cap mRNA and DuCV DNA level in DEF transfected with MN-FJ0601 and MC-FJ0601ΔQTR was measured by qPCR.

based on the minicircle DNA technique, which was highly authentic to the covalently closed circular DNA intermediate in the viral genome replication process (Fig. 5B). MC-FJ0601 was generated from pmini-FJ0601 recombination in the ZYCY10P3S2T strain following arabinose-induced ΦC31 integrase and endonuclease I-SceI expression (Fig. 5B). The length of MC-FJ0601, MC-FJ0601ΔQTR, and their parent plasmids were verified by BamHI digestion and agarose gel electrophoresis (Fig. 5C). In the MC-FJ0601-transfected DEF model, we firstly monitored the dynamic change in Rep and Cap mRNA. qRT-PCR analysis indicated that the level of Rep mRNA gradually increased and peaked at 72 h post-transfection with MC-FJ0601. QTR deletion in MC-FJ0601ΔQTR resulted in significantly slower Rep mRNA accumulation, which was approximately one-tenth that of the abundance of Rep mRNA in MC-FJ0601 at 72 h (Fig. 5D, left panel). Similar phenomenon was also found in Cap mRNA transcription (Fig. 5D, middle panel). Since both Rep and Cap are essential for viral genome replication and assembly, we further detected the role of QTR for the DuCV progeny virion production in the supernatant. In accordance with viral gene transcription, DuCV DNA level in the supernatant exhibited a significantly dynamic increase in the MC-FJ0601 than MC-FJ0601ΔQTR group (Fig. 5D, right panel). These data fully demonstrate a vital role of the QTR as a DSE in regulating Rep/Cap expression and DuCV replication.

4. Discussion

Duck circovirus (DuCV) can infect most duck species and cause immunosuppression lesions (Hattermann et al., 2003; Soike et al., 2004), however, the regulatory mechanism associated with viral replication is still less known. Repeat sequences play an important role in the regulation of viral gene expression, genome replication, and pathogenicity (Gendelman et al., 1986; Jenkins and Martin, 1990; Xiao et al., 1997). DuCV can be further divided into two genotypes: genotypes 1 and 2 (Wang et al., 2011). Through dottup program analysis, we first found that a QTR specifically existed and was evolutionarily conserved in genotype 1 and 2 DuCV strains but was not found in other circoviruses. Furthermore, the QTR functioned as a DSE of transcription termination signals to promote viral genes expression and replication.

In both eukaryotes and prokaryotes, repetitive DNA, including highly repetitive, tandem repeats and interspersed retrotransposon repeats, are important for gene expression and genome replication (Gymrek et al., 2016; Qian and Adhya, 2017; van Belkum et al., 1998).

For example, telomeres protect genome stability, and a deficiency will result in cellular senescence and apoptosis (O'Sullivan and Karlseder, 2010). However, the frequency of repeat sequences in the viral genome is relatively rare, and has been primarily reported in retrovirus, Epstein-Barr virus, and herpes virus (DesGroseillers and Jolicoeur, 1984; Heller et al., 1982; Jenkins and Martin, 1990). In circoviruses, an 11-bp inverted repeat and two copies of 6-bp tandem direct repeats are located in the origin of DNA replication (Ori), and have been shown to be important for viral genome replication (Cheung, 2012). A QTR is a longer tandem repeat located in the intergenic region of the *rep* and *cap* genes and functioned as a DSE element to regulate Rep and Cap expression (Fig. 4). These findings indicate that the QTR may play a dual role in regulating both early viral genome replication and late virion assembly. Interestingly, the longer QTR element found in our study was unique to the DuCV genome, and was not identified in PBFDV, GoCV, PCV1, PCV2 (Fig. 1), or other circoviruses (data not shown). Moreover, QTR was not found in the genomes of duck or other avian species through nucleotide blast analysis (data not shown). This suggests that QTR emergence maybe a relatively late and critical molecular event in the DuCV evolution process.

Based on full genome or cap gene homology, DuCV can be divided into two distinct branches: genotype 1 and 2 (Wang et al., 2011), however, whether there are other distinctive molecular features remains unclear. We previously found a point mutation of T236A in the ORF3 gene of the DuCV genotype 1 strains results in a premature stop codon (from TTA to TAG) and 20 aa truncation at the C terminus of the ORF3 protein compared with the genotype 2 strain (Wu et al., 2018). In this study, we found that the QTR in genotypes 1 and 2 was substantially different and highly conserved, which also could serve as an easier and more accurate molecular marker for genetic typing.

To date, there are no appropriate cell lines for DuCV propagation *in vitro*. It is also unclear whether a DuCV infectious clone can rescue complete viral genome replication and mature progeny virions. Full-length DNA clones of DuCV with two copies has been reported to generate viruses *in vivo* (Li et al., 2015), which provides a good indicator for investigating DuCV replication with a reverse genetic model. Recently, the minicircle vector technique has exhibited a substantial advantage in gene delivery and the HBV reverse genetic model (Chabot et al., 2013; Yan et al., 2017). To better illustrate the role of the QTR in viral replication, we firstly constructed both a DuCV cccDNA and viral replication model in DEF. In this system, we demonstrated a critical role for the QTR in regulating Rep and Cap transcription, otherwise, we also detect a gradual increase of viral DNA in the supernatant, which indicated that the MC-FJ0601/DEF model could support viral replication lifecycle and serve as a great model for future DuCV genomic biology study.

In conclusion, this is the first report of a novel tandem repeat element, QTR, that uniquely exists in the DuCV genome and is not present in other circoviruses. The QTR is obviously distinctive and conserved between genotype 1 and 2 DuCV strains and may serve as a molecular marker for genetic typing. Moreover, the QTR functioned as a DSE element for regulating Rep and Cap expression by maintaining mRNA stability; however, although the sequence composition of the QTR in genotypes 1 and 2 was markedly different, the functional capacity of the QTR as a DSE was similar. Since there may be other diverse regulatory roles of the QTR in genotypes 1 and 2, further investigation is required in future studies. Taken together, our findings reveal a novel genetic typing marker and provide insight into the biological characteristics of the QTR in the DuCV life cycle.

Acknowledgments

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