**Quadruplex DNA in long terminal repeats in maize LTR retrotransposons inhibits the expression of a reporter gene in yeast**

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**Abstract**

**Background:** Many studies have shown that guanine-rich DNA sequences form quadruplex structures *in vitro* but there is scarce evidence of guanine quadruplexes (G4) *in vivo*. The majority of potential quadruplex-forming sequences (PQS) are located in transposable elements (TEs), especially close to promoters within long terminal repeats of plant LTR retrotransposons.

**Results:** In order to test the potential effect of G4s on retrotransposon expression, we cloned the long terminal repeats of selected maize LTR retrotransposons upstream of the lacZ reporter gene and measured its transcription and translation in yeast. We found that G4s had an inhibitory effect on translation *in vivo* since “mutants” (where guanines were replaced by adenines in PQS) showed higher expression levels than wild-types. In parallel, we confirmed by circular dichroism measurements that the selected sequences can indeed adopt G4 conformation *in vitro*. Analysis of RNA-Seq of polyA RNA in maize seedlings grown in the presence of a G4-stabilizing ligand (NMM) showed both inhibitory as well as stimulatory effects on the transcription of LTR retrotransposons.

**Conclusions:** Our results demonstrate that quadruplex DNA located within long terminal repeats of LTR retrotransposons can be formed *in vivo* and that it plays a regulatory role in the LTR retrotransposon life-cycle, thus also affecting genome dynamics.

**Keywords**

**Background**

Guanine-rich sequence motifs with four closely spaced runs of Gsare able to form a four-stranded structure known as a **G-quadruplex** (G4, for review see [1]). Quadruplexes can be formed by both DNA and RNA molecules, are stabilized by potassium or sodium ions and can adopt various conformations involving one, two or four molecules [2]. Recent genome-wide *in silico* studies revealed that genomes contain thousands of G4 motifs which are enriched in certain loci, as seen in the human [3,4] and maize [5]. The highest occurrences of G4 motifs have been observed at the telomeres, origins of replication, promoters, translational start sites, 5’ and 3’ UTRs, and intron-exon boundaries, thus suggesting specific/molecular/biological functions.

Many studies have shown that guanine-rich sequences form quadruplex DNA or RNA *in vitro* but solid experimental evidence of quadruplex formation ***in vivo* has been gathered only** recently (for review see [6]) although many quadruplexes that are formed *in vitro* are unfolded in living cells [7]. This research was greatly aided by the development and use of small chemical ligands to stabilize the G4s [8] as well as a single chain antibody specific for G4s [9].

While in general the biggest focus is on genic and telomeric G4 motifs, the majority of **G4 motifs are** however **localized in the repetitive fraction** of genomes. For example, in the maize genome, mostly composed of LTR retrotransposons, 71% of non-telomeric G4 motifs are located in repetitive genomic regions [5]. Lexa *et* al. [10] analysed 18,377 LTR retrotransposons from 21 plant species and found that PQS are frequently present within LTRs, more often at specific distances from other regulatory elements such as transcription start sites. Moreover, evolutionarily younger and active elements of plants and human had more PQS, altogether indicating that G4s can play a role in the LTR retrotransposon life cycle [10,11]. In addition, recent study has shown that quadruplexes localized within the 3’UTR of LINE-1 elements can stimulate retrotransposition [12].

**Here we show** that the presence of G4 motifs within maize LTRs results in a markedly reduced expression of the downstream located lacZ gene in yeast compared to a similar sequence with mutations preventing quadruplex formation. Additionally, our results suggest that G4 formation affects translation rather than transcription, in a strand-specific manner.

**Methods**

**TE reference sequence annotation**

All LTR retroelement sequences were downloaded from Maize Transposable Element Database (<http://maizetedb.org/~maize/>) and searched for G4 motifs using the R/Bioconductor [13] package *pqsfinder* [14]. Default settings were used, except for the minimum score value. A value of 65 was used when fewer false positive results were desirable. LTRs were predicted by LTR finder [15]. BLASTX [16] was used against a collection of TE protein sequences downloaded from GypsyDB [17] with e-value threshold set to 0.01 to generate annotations in the Additional file 1: Figure S2. For LTR amplification BAC clones containing selected elements were ordered from the Arizona Genomics Institute. Table S1 (Additional file 2) shows selected elements used for the yeast *in vivo* assay.

**CD measurements and polyakrylamid gel electrophoresis**

Circular dichroism and polyakrylamid gel electrophoresis were performed as described in Lexa *et* al. [10] but with the temperature at 27 °C in accordance with to yeast growth conditions.

**Cloning and Mutagenesis**

We used the pESC-URA plasmid (Agilent) as the backbone for our constructs. The Gal1 promoter was excised through SpeI/XhoI digestion and a p424 SpeI/XhoI fragment containing MCS was cloned in [18]. We used the following primers and Q5 polymerase (NEB) for lacZ coding sequence amplification from *E. coli* (K12) genomic DNA:

lacZ\_F ATCGTCGACATGACCATGATTACGGATTCACTGG and lacZ\_R CCTGTCGACTTATTTTTGACACCAGACCAACTGG. Both primers have SalI extension which was used for lacZ cloning, with the orientation being verified by PCR and sequencing. A list of primers used for LTRs amplification is in Table S1. LTRs were amplified using Q5 polymerase under the recommended conditions and blunt cloned into the SmaI site of pBC. Again the insertions were verified by PCR and sequencing. Mutations in G4 forming sequences in cloned LTRs were introduced using single mutagenic primers for each LTR and Q5 polymerase (recommended conditions). The products were treated with DpnI (Neb) and 1 μl was used for XL-1 blue electrocompetent cell (Agilent) transformation. Mutations were verified by sequencing.

**Yeast lacZ assay**

We used the *S. cerevisiae*  strain CM100 (MATα, can1-100 oc, his3, leu2, trp1, ura3-52) for the lacZ expression assay. Vectors containing lacZ under control of LTR promoter were transformed into yeast using S.C. Easy Comp Transformation Kit (Invitrogen). Transformed cells were plated on selective media without Uracil. For each construct we measured lacZ expression as follows. Six positive primary transformant colonies were inoculated into 500 μl liquid media in a deep-well plate and grown overnight (cca 20 hours) at 28 °C / 250 rpm. The next day 150 μl culture was transferred into 1500 μl new media and cultivated overnight at 28 °C / 250 rpm. The following morning the OD600 of the culture was about 1. We transferred 200 μl of the culture into a 96-well microplate and centrifuged to collect the cells, discarded 190 μl of the supernatant, resuspended the cells and permeabilized them for 15 min at 30 °C / 250 rpm in 110 μl modified Z-buffer (100 mM Na2HPO4, 40 mM Na2H2PO4, 10 mM KCl, 2 mM MgSO4, 0.1% SDS). Next 25 μl of 4,17 nM ONPG was added and the plate incubated at 30 °C/ 250 rpm. When a pale yellow colour developed the reaction was ceased using 135 μl stop solution (1M Na2CO3). The plate was centrifuged and clear supernatant was used for reading Abs420 (both Abs420 and OD600 were measured using a Tecan Sunrise microplate reader with Rainbow filter). For the starting value of Abs420 we used a well where no cells were added and so autolysis of ONPG was included. LacZ units were calculated using the formula: lacZ units = 1000 \* (Abs420 / (OD600 \* volume [ml] \* time [min]). Each plasmid was tested in triplicate. We averaged measurements for each colony and used ANOVA (p > 0.001) and post-hoc Tukey HSD to compare lacZ units in different construct pairs (wt vs mutant).

**Yeast RNA isolation and Q-PCR**

Yeast for RNA isolation were grown the same way as for lacZ assay but for the final day the whole volume was used. RNA was prepared by extraction with hot acidic phenol [19] and then treated with TURBO DNase (Ambion). Reverse transcription was carried out using a High-Capacity RNA-to-cDNA kit (Applied Biosystems) and Q-PCR was performed using a SensiFAST SYBR Hi-ROX kit (Bioline). We used 2 pairs of primers, first for lacZ as gene of interest (qlacZ\_F GAAAGCTGGCTACAGGAAG; qlacZ\_R GCAGCAACGAGACGTCA) and second for URA marker as reference gene (qURA3\_FGGATGTTCGTACCACCAAGG; qURA3\_R TGTCTGCCCATTCTGCTATT).

**Transcription start sites prediction and rapid cDNA ends amplification (RACE)**

Transcriptional start sites (TSS) were predicted using TSSPlant [20]. Experimental verification of TSS was performed with SMARTer™ RACE cDNA Amplification Kit (Clontech) using total RNA from yeast and maize (B73) respectively, which were isolated as described herein. Primers used for RACE are listed in Table S1 (Additional file X). Products were cloned into pCR™II Vector (Invitrogen) and transformed into One Shot™ TOP10 *E. coli* electrocompetent cells (Invitrogen), 8 colonies were sequenced.

**Plant material preparation**

*Zea mays* B73 seeds were obtained from the U.S. National Plant Germplasm System (<https://npgsweb.ars-grin.gov>). Seeds were sterilized and germinated in moisturized filter papers for 5 days at room temperature. 5th day seedlings were transferred to ¼ concentration of aerated Reid-York solution [21] in a greenhouse. Each seedling was secured by plastic foam strip in separate 50 ml falcon tubes and positions of NMM treated and non-treated plants were randomized, solution was changed on daily basis. After 2 and 4 days the solution was replaced by ½ and full concentration, respectively. Treatment by 16 μM NMM (Frontier Scientific) commenced after one day growth in full Reid-York solution concentration and continued for 3 days. After 3 days of NMM treatment, the roots of 4 treated and 4 non-treated plants were used for RNA isolation by NucleoSpin® RNA Plant kit (Machery-Nagel).

**cDNA library preparation and RNA sequencing**

In total eight RNA samples (2 μg each) were provided to the Genomics Core Facility Center (EMBL Heidelberg) for the construction of cDNA libraries with poly(A)+ selection and sequencing. Sequencing libraries were prepared using an ILMN truseq stranded mRNA Kit (Illumina, San Diego, CA, USA) according to manufacturer's protocol. Sequencing libraries were pooled in equimolar concentration and sequenced on an Illumina NextSeq 500, producing 2x80-nucleotide paired-end reads.

**RNA-Seq quality control and preprocessing**

Raw RNA-Seq libraries contained 47-56 million paired-end reads for treated samples and 47-62 million paired-end reads for control samples. Reads were checked for quality using FastQC ([22], available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads with low-quality, containing adaptor sequences, unpaired reads, containing rRNA contamination (18S rRNA - GenBank: AF168884.1, 26S rRNA - GenBank: NR\_028022.2, 5.8S rRNA - GenBank: U46603.1) and reads containing poly-G runs, which are a typical error for NextSeq platform, were removed using Trimmomatic 0.36 [23] and trimmed to 75 bp length. After preprocessing, read libraries ranged between 17-35 million paired-end reads for treated samples and 14-45 million paired-end reads for control samples. In order to obtain more consistent results, the smallest libraries were discarded from both groups, giving libraries ranging from 30 to 35 million paired-end reads for treated samples and from 33 to 45 million paired-end reads control samples. RNA-Seq data was deposited in the European Nucleotide Archive ENA under primary accession number: PRJEB23390. To find out if there was any contamination in the reads, they were mapped onto the maize reference genome B73 RefGen\_v3 (ftp://ftp.ensemblgenomes.org/pub/plants/release-31/fasta/zea\_mays/dna/) using STAR aligner v2.5.2b [24] with default settings. For all libraries, more than 95% of reads mapped onto the reference genome, indicating that there was no significant contamination.

**Mapping RNA-Seq on library of transposable elements and their differential expression analysis**

To estimate the expression of individual maize transposable elements, RNA-Seq reads were mapped using STAR aligner v2.5.2b [24] on the Maize transposable elements database (<http://maizetedb.org>). Due to a differences in mapping reads onto transposable elements (multiple copies in genome, sequence variability of transposons falling into same family/subfamily, less variable length) compared to onto genes, we adjusted mapping settings to allow multimaps and a higher number of mismatches in mapped reads to reflect transposon variability: *--winAnchorMultimapNmax* 1000, *--outFilterMultimapNmax* 1000, *--outFilterMismatchNmax* 15, *--alignIntronMin* 5 *--alignIntronMax* 20000. The number of mapped reads with these settings varied from 234 to 360 thousand, corresponding to 0.68-1.05 percent of library sizes. Subsequently, to obtain raw counts of mapped reads per transposable element, the *featureCounts* [25] tool with *--fraction* option was used to assign counts of multi-mapped reads to transposons correctly and to avoid multiple counts of the same sequence. These raw counts were used for differential expression analysis performed with the *EdgeR* package [26], which is recommended to use for smaller numbers of biological replicates [27]. Poorly expressed transposons which had count-per-million (CPM) figures of less than 45 in at least three samples (corresponding to 10-12 reads mapped onto transposons) were removed from further analysis. The statistical values (log fold change (LFC), p-value) were estimated using the *exactTest* function and adjusted p-values (FDR) with the *p.adjust* function. Transposons with LFC > | 1.5 | and FDR < 0.05 were considered as differentially expressed. Such transposons were annotated as described above in the TE reference sequence annotation section. Elements with inconsistecies in annotation, e.g. wrong order of protein domains, were excluded from the analysis. To correlate RNA-Seq coverage with position of quadruplexes in differentially expressed LTR retrotransposons, RNA-Seq coverage was estimated by *bedtools genomecov* [28] with settings *-d -split -scale $norm\_factor*, where *$norm\_factor* represents normalization factor calculated for each library by the *EdgeR* package. RNA-Seq coverage for all control and treatment samples was aggregated to average coverage and plotted by using custom R script together with annotation of LTR retrotransposons.

**Results**

**1. Selection of maize LTR retrotransposons with PQS and confirmation of quadruplex formation by circular dichroism**

We **searched for maize LTR retrotransposons** having potential quadruplex-forming sequences (PQS) using *pqsfinder* (**Figure 1, S1**). We found that about 27% of all families contained at least one PQS (Figure 1a) with a tendency to have a higher number of PQS in the same element - on average more than 3 PQS per family. This trend is even more pronounced when we take into account only long terminal repeats (LTRs) and their immediate neighborhood (less than 350bp from the end of detected LTR), since only 8% (34) of families harbour in their LTRs 43% of all predicted PQS, on average almost 5 PQS per family (**Figure 1d**). This also indicates that LTRs are enriched for G4 motifs compared to other regions of the elements.

Surprisingly, the majority (79%) of all high-scoring PQSs in maize elements were accumulated **in the minus strand** (**Figure 1b**). The prevalence of PQS in the minus strand was also seen in Copia retrotransposons but these elements tend to harbour PQS in the plus strand particularly inside LTRs (**Figure 1c**). It suggests that if a PQS is located in plus strand of a Copia element then it is preferentially located within the LTRs. Notably in Gypsy retrotransposons it is evident that while 5’-LTRs tend to contain more PQS in the minus strand, 3’-LTRs contain more PQS in the plus strand, with a small peak on the opposite strand present in the immediate vicinity, presumably in the untranslated region (UTR) (Figure S1).

Although LTR retrotransposons tend to harbour more than one PQS in their LTRs, for clarity and convenience we **selected 10 elements** possessing only one PQS within their LTRs. Since even sequences with very long central loop can form G4s, our selection included five elements with PQS having short loops (up to 8 nucleotides) and five elements with PQS possessing a central loop of 27-49 nucleotides.

To confirm the ability of selected PQS to adopt G4 structures *in vitro* we measured **circular dichroism (CD) spectra** using synthetic oligonucleotides (**Figure 2a**). We performed UV melting analysis for short loop G4 motifs to determine Tm and to confirm the results obtained by CD (in all cases UV melting was in agreement), and also on oligonucleotides with long loops as they are difficult to assess for G4 formation by CD measurement. Out of five tested oligonucleotides with short loops, four formed G4 in vitro (Table 1) - one oligonucleotide corresponding to the Gyma Gypsy LTR retrotransposon formed a parallel stranded quadruplex as indicated by a high peak at 265 nm. The other three oligonucleotides corresponding to the Huck, Tekay and Dagaf Gypsy LTR retrotransposons formed a 3+1 arrangement as indicated by a high peak at 265 nm and a secondary peak at 290 nm (**Figure 2a**). Tm values varied from 55 °C to 62 °C. Five oligonucleotides, having long loop PQS, did not form G4s under the tested conditions (not shown).

The ability of tested oligonucleotides to form quadruplexes was also confirmed by native **PAGE** providing information about on molecularity (**Figure 2d**). All oligonucleotides formed monomolecular G4s at 27 °C since these migrated faster (they are more compact) than oligonucleotides of the same length.

We tested the **effect of mutations** on G4s formation by substituting some guanines with adenines with the aim to disrupt G4 formation. The substitutions were carried out on two inner runs of guanines, since we had previously observed that this had a greater effect on G4 formation than in outer G runs ([10], **Table S1**). Our CD spectra measurements as well as native PAGE confirmed that these mutations did indeed disrupt G4 formation (**Figure 2b**). For yeast *in vivo* experiments we chose G4 disruption by mutations rather than stabilization by ligands because (i) the G4s with ligands could behave differently from “ligand-free” G4s and (ii) ligands have large-scale biological effects that could lead to artefacts. The control substitution we introduced in the loop of the Huck G4 sequence verified that the effect was not sequence-specific but correlated with G4 structure as it did not disrupt G4 formation (**Figure 2c**).

**2. Effect of G4 formation on the expression of the lacZ reporter gene in yeast and the detrimental effect of mutations on G4 formation**

The *in vitro* CD measurements of short oligonucleotides possessing PQS were followed by an *in vivo* study of G4 formation contained within longer LTR sequences and its effect on downstream located reporter gene. We cloned selected LTRs amplified from BAC clones upstream of the lacZ reporter gene to create a **plasmid construct (Figure3a)** which was used to transform *Saccharomyces cerevisiae* (CM100). LTRs originated from four LTR retrotransposons: the Huck, Gyma, Dagaf and Tekay families belonging to a Gypsy superfamily and were 1.3-3.5 kb long (Figure 3b). Gyma, Dagaf and Tekay harboured the G4 motifs on the minus strand closer to the 5´ end of the LTR whereas in the Huck element the G4 motif was situated near the 3´ end of the LTR and was located on the plus strand.

Next we used site-directed mutagenesis on **G4 motifs** to produce the same PQS mutations as in the CD measurement. The constructs with mutated PQS were used for yeast transformation. Then we compared the LTR driven lacZ expression of wild-type and mutant LTRs *in vivo* on both protein and mRNA levels.

All tested constructs exhibited low **lacZ protein** levels under the LTR control, the highest expression was observed in the LTR of the Dagaf element that reached up to 20 lacZ units. In three constructs (Gyma, Dagaf and Tekay) lacZ expression was not affected by G4 disruption while in the Huck element the lacZ protein level was more than twice the amount in G4 mutants than in the wild-type and control mutant LTRs (mutation in G4 motif loop) that both harbored stable G4s (p < 0,001; Figure 4a). Contrastingly, there was no difference between wild-type and control mutant LTRs. However, it remained to be determined whether DNA or RNA quadruplex affects lacZ expression.

**3. Effect of G4 on transcription and the mapping of transcription start sites by RACE**

We isolated RNA and performed **qRT-PCR** in order to assess the effect of G4 formation on transcription and/or translation. We used a URA marker as a reference gene, which was also located on the plasmid construct. No differences were observed in lacZ mRNA levels between wild-type and mutant LTRs (not shown). Increases in lacZ protein levels in mutants disrupting G4s inside Huck LTRs in contrast with unaffected levels of mRNA suggest that G4 hampered translation rather than transcription and that quadruplex formation occurs at the RNA level.

In order to determine whether transcription is specific for LTR retrotransposons i.e. being initiated at a promoter located within LTR, and is not a result of read-through (co-transcription), we estimated transcription start sites (TSS) using the Strawberry TSSPlant prediction tool and then performed Rapid Amplification of cDNA Ends (**RACE**) on both yeast and maize total RNA. We found that the transcription start site of the Huck element is located within the LTR and upstream of the G4 sequence both in yeast and maize although the position of specific TSS differed slightly (**Figure 4b**). Notably, the yeast experimentally determined TSS by RACE was in the same position as the one predicted by TSSPlant.

**4. Stabilization of quadruplexes in maize seedlings grown in the presence of G4-stabilizing ligand NMM and the effect of NMM on LTR retrotransposon expression**

In yeast we used mutations of PQS and tested the effect of G4 formation on a very limited number of elements, however, the potential effect of G4 on gene expression *in vivo* can also be studied by using a G4-stabilizing ligand. Therefore, to know more about the genome-wide G4 stabilization effect on retrotransposons transcription, **maize seedlings** were grown in the presence of the **NMM** ligand and polyA RNA sequencing was performed using Illumina. The subsequent analysis of RNA-Seq data revealed that the elements studied above had low transcription and were not differentially expressed. On the other hand, several LTR retrotransposons showed high transcription and were differentially transcribed in the presence/absence of NMM. ~~The effect of NMM (downregulation or upregulation) was both inhibitory and stimulatory and was dependent on the LTR retrotransposon family~~. The Gypsy retrotransposons of Grande and Uvet showed lower transcription in the presence of NMM while in the Guhis and Maro families NMM had stimulatory effect on transcription (**Figure 5**).

**Discussion**

In this study **we showed** that the G4 motif, previously confirmed to adopt quadruplex conformation *in vitro*, located downstream of TSS within the long terminal repeat of LTR retrotransposons, affects the LTR driven expression of the lacZ reporter gene by regulating translation. The translation repression by G4s located in the 5´UTR of the firefly luciferase reporter gene has been well-documented in both cell-free and *in cellulo* systems [29,30]. Our work belongs to several rare studies, emerging only during recent years, determinating the biological role of quadruplexes *in vivo* and indicating the importance of non-B DNA conformation in the life cycle of LTR retrotransposons.

Our work on **prediction of G4 motifs**, revealed that central loop length is an important determinant of *in vivo* G4 formation. Four out of five tested oligonucleotides with shorter loops readily formed G4s *in vitro*. Contrastingly, the motifs with longer central loops (27-49 nt) did not readily adopt quadruplex conformation under tested conditions and G4 formation was rather an exception here. Although our study was focused only on the maize LTR retrotransposons, our results are in agreement with previous analyses from 21 plant species that revealed enrichment of G4 motifs within the LTRs of retrotransposons [10]. The difference in PQS number and location (on plus or minus strands) in Copia and Gypsy retrotransposons may be connected with differences in their regulation, mode of amplification and/or the age of families where younger families have more PQS than older ones [10,11].

The **prevalence** of PQS in the **minus strand** suggests that there is selection pressure against the presence of G4 in the plus strand where G4s inhibit the translation and subsequent amplification of retrotransposons. This is consistent with our results showing that the translation of the Huck retrotransposon (possessing G4 in the plus strand) was inhibited while the translation of the Gyma, Tekay and Dagas retrotransposons (possessing a G4 motif in the minus strand) was not affected. Strand specificity in G4-affected processes has also been observed in other systems and organisms. For example, Smestad and Maher [31] demonstrated strand differences in PQS presence in human genes differentially-transcribed in Bloom Syndrome and Werner Syndrome, two disorders resulting in the loss of PQS-interacting RecQ helicases.

Although we demonstrated the effect of the G4-stabilizing drug NMM on the transcription of LTR retrotransposons, irrespective of their subsequent impact on translation, the elucidation of the **role of G4s in transcription** and other steps of the LTR retrotransposon life-cycle needs further research. It remains a question to what extent does the positive or negative effect of G4 on transcription depend on the LTR retrotransposon family and its mode of regulation. Moreover, when assessing the differences between the G4 effect on transcription and translation in yeast and maize, we should keep in mind that there are different cellular factors binding the G4s in each case.

The inhibitory or stimulatory effect of G4s on LTR retrotransposons expression can also be explained by the formation of quadruplex structures within only a specific genomic context and/or in particular cellular (ionic and protein) environments. Such an explanation is consistent with the finding that quadruplexes are globally unfolded in eukaryotic cells [7]. The abundance and strand-location (plus or minus) of G4 motifs within retrotransposons is probably the result of an **interplay between** the propensity of mobile elements to amplify and the demand of the cell to suppress retrotransposon activity in order to maintain genome and cell integrity.

We have demonstrated the effect of G4s on the transcription of LTR retrotransposons in maize and on their translation in yeast but we cannot exclude that G4s affect also **other steps of LTR retrotransposon life cycle**. The effect of G4 on other life cycles has previously been shown in closely related retroviruses, e.g. in HIV-1, nucleocapsid proteins are bound to the G4 structure of the preintegration genome leading to the initiation of the virion assembly [32]. In addition, sequences near the central polypurine tract that form bi-molecular quadruplex also facilitate strand transfer and promote template switching during reverse transcription of HIV-1 [33,34]. Moreover, the formation of bi-molecular quadruplex is believed to stabilize the pairing of the two RNA genome molecules which ensures the encapsulation of both genome copies in virion [35,36].

It is also possible that in some cases G4s take part in retrotransposon stress activation. RNA quadruplexes are essential for **cap-independent translation** initiation [37] during which the 40S subunit of the ribosome is recruited into a position upstream or directly at the initiation codon via a specific internal ribosome entry site (IRES) element located in the 5'UTR. In plants, stress conditions (drought, high salinity and cold) lead to dehydration and thus increase molecular crowding in the cell favouring G4 formation [38]. Furthermore, cap-independent translation is often related to **stress** **states** and diseases such as cancer [39] and, remarkably, stress also activates transposable elements that in turn, by inserting their new copies, probably spread new G4 motifs throughout genomes [40]. In this way, quadruplex DNA can participate both in short-term (physiological) and long-term (evolutionarily) responses to stress.

Our finding that all four tested G4s adopted **intramolecular** (monomolecular) quadruplex agrees with its regulatory role during translation or transcription where a single RNA/DNA molecule participates. Moreover, all our G4s show parallel strand orientation prevalence supporting their potential role during transcription since promoter-associated quadruplexes tend to be parallel-stranded [3].

**Conclusions**

Our study provides, to our knowledge, the first experimental evidence that quadruplex DNA located within the long terminal repeat of LTR retrotransposons can affect the expression of plant LTR retrotransposons *in vivo*: (i) mutation disrupting G4 in the LTR resulted in a higher translation level of a downstream located reporter gene in yeast compared to the wild-type the G4 motif and (ii) the G4 stabilizing drug NMM affected transcription of LTR retrotransposons in maize. This demonstrates that quadruplex DNA plays a regulatory role in the maize LTR retrotransposon life-cycle. Therefore, stabilization of quadruplexes present in LTR retrotransposons under specific cellular conditions can, thanks to the multicopy character of LTR retrotransposons, influence whole genome dynamics as well as represent the abundant barriers for DNA replication.

**List of abbreviations**

ANOVA - Analysis of variance

BAC - Bacterial artificial chromosome

CD - Circular dichroism

cDNA - Complementary DNA

CPM - Counts per million

DNA - Deoxyribonucleic acid

G4 - G-quadruplex

HSD - Honest significant difference

LFC - Log fold change

LTR - long terminal repeat

mRNA - messenger RNA

NMM - N-methylmesoporphyrin IX

PCR - Polymerase chain reaction

PQS - Potential Quadruplex-forming Sequence

RACE - Rapid amplification of cDNA ends

RNA - Ribonucleic acid

UTR - Untranslated region

TE - Transposable element

TSS - Transcription start site

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and material**

RNA-Seq data generated and analyzed during the current study are available in the European Nucleotide Archive (<http://www.ebi.ac.uk/ena>) under primary accession number: PRJEB23390.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

VT performed all experiments, ML and JP performed bioinformatic analysis, EK conceived the study, EK, ML, JP and VT wrote the manuscript. All authors read and approved the final manuscript.

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**Figure Legends:**

**Figure 1: Comparison of different retrotransposon superfamilies and *in silico*  predicted potential quadruplex-forming sequences (PQS)**

**(a)** Chart shows proportion of families that possess at least one PQS (blue) PQS located on plus (PQS+; red) minus (PQS-; green) and both strands (purple). **(b)** Shows absolute numbers of PQSs predicted in different superfamilies with respect to LTRs. **(c)** Proportion of PQS found within superfamiles present in the LTR region (e.g. out of all PQS+ found in Copia superfamily 73% are located in LTRs) **(d)** Density of predicted PQS in LTR regions and full elements in main superfamilies.

**Figure 2. CD spectra of selected oligonucleotides representing the parts of LTRs with wild-type and mutant PQS. (a)** CD spectra of oligonucleotides representing wild-type PQS within LTRs from various LTR retrotransposons obtained at different concentrations of potassium ions (orange: 0 mM K+; blue: 150 mM K+ and red: 150 mM K+ after annealing). The peak at 265 nm corresponds to a parallel-stranded quadruplex. Sketches correspond to the most probable folding of the dominating quadruplex structure according to CD and electrophoretic results. **(b)** CD spectra of oligonucleotides representing mutant PQS within LTRs of various LTR retrotransposons. **(c)** CD spectra of Huck LTR retrotransposon having a control CG to TC substitution in G4 loop. **(d)** Native gel electrophoresis of oligonucleotides in the presence of 150 mM KCl at 28°C, length of oligonucleotides is indicated below names.

**Figure 3. Scheme of plasmid constructs possessing LTRs with PQS. (a)** Scheme of pBC in which LTRs (green) were cloned into *Sma*I restriction site. **(b)** Overview of cloned LTRs. Length is shown on the bottom scale. G4 position and orientation is indicated by red (coding strand) and blue (template strand) rectangles. Predicted transcriptional start sites (TSS) are also shown, both with TATA box (orange) and TATA-less TSS (purple triangles).

**Figure 4. Effect of G4 on expression of lacZ gene in yeast. (a)** Comparison of protein expression of lacZ reporter gene cloned downstream of LTR with mutated PQS and wild-type PQS. Red column is comparison of control mutation against wild-type. **(b)** Transcription start sites (TSS) determined by RACE. Both yeast and maize TSSs located upstream of G4 sequence are shown by blue and red arrows, respectively. The G4 sequence in DNA plus strand (green cube) is transcribed into mRNA (blue or red cubes).

**Figure 5. Effect of NMM on transcription of LTR retrotransposons in maize seedlings.** Graph of coverage of LTR retrotransposon families by RNA-Seq reads obtained from plants treated with G4 stabilizing drug NMM (red) and from control plants not treated with NMM (blue). Positions of G4 motifs are displayed by purple ticks, G4 motifs on plus strand are above the element, G4 motifs on minus are below the element.

**Table 1. Selected oligonucleotides with PQS, their stability and conformation determined by circular dichroism and thermal denaturation.**

**Figure S1. Occurrence of high-scoring PQS along maize LTR retrotransposons.** The distribution of PQS containing a minimum of four adequately spaced G runs in the sense strand (PQS3+, upper row) and antisense strand (PQS3-, lower row) as identified by *pqsfinder* where **(a)** score > 64 and **(b)** score > 25. Gypsy (RLG), Copia (RLC) and other (RLX) superfamilies are shown in separate columns. Frequency (vertical axis) represents the number of PQS present in a window covering 2% of TE length. 75% of LTRs fall within the black rectangles shown below the horizontal axis (3rd quartile = 0.125; mean LTR length = 0.100; maximum length = 0.427).

**Table S1. Overview of oligonucleotides, BAC clones and mutagenic primers used in study.**

**File S1. Annotation of all 579 maize TEs included in this study showing the presence and position of detectable LTRs, PBS and PPT sequences (LTR Finder), protein-coding domains (BLASTX) and potential quadruplex sequences (PQS; pqsfinder). White rectangles represent LTRs, blue rectangles are common TE domains (labelled) or other domains detected in Uniprot (unlabelled). Small blue bars are PQS with score >24 (>64 larger bar).**