UNDERSTANDING A TELOMERASE RNA INTRON SEQUENCE AND STRUCTURE REQUIREMENT FOR TELOMERASE NEGATIVE REGULATION IN *ARABIDOPSIS THALIANA*

An Undergraduate Research Scholars Thesis

By

ADITYA PANTA

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Dorothy Shippen

May 2013

Major: Molecular Cell Biology

TABLE OF CONTENTS

Page

ABSTRACT

Understanding a telomerase RNA intron sequence and structure requirement for telomerase negative regulation in *Arabidopsis thaliana*. (May 2013)

Aditya Panta
Department of
Biology
Texas A & M University

Research Advisor: Dr. Dorothy Shippen
Department of
Biochemistry

TER2 is a telomeric RNA subunit in Arabidopsis thaliana that has been suggested to be working as a negative regulator of the telomerase complex. When the TER2 is over-expressed, a significant decrease in the telomerase activity has been observed in Arabidopsis. Interestingly, the TER2 gets spliced into a different form of RNA, TER2s. However, the mechanism of splicing is totally different with what we observe with the pre-mRNA in the eukaryotic cells. The intron region that is being spliced out might itself be responsible for the splicing mechanism. For this, various mutations were constructed in which certain specific sequences in the intron region were deleted. By transforming the construct back into the plants, any differences in splicing of TER2 and negative regulation on the telomerase activity will be checked.

DEDICATION

This is dedicated to my beloved father, Mr. Deepak Upadhyay Pant, mother, Mrs. Ambika Pant Chapagain, and brother, Ayush Pant.

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Dorothy Shippen, for having faith in me and providing me with such a wonderful opportunity to start my research career. I would also like to thank my mentor Hengyi Xu, for teaching me valuable research skills and for helping me prepare various presentations and this thesis.

Last but not the least; I would like to thank the Department of Biology at Texas A & M University for awarding me a very generous scholarship to pursue my undergraduate studies.

CHAPTER I

INTRODUCTION

Telomeres, G-rich sequences found at the end of most eukaryotic chromosomes (1), are important for two reasons. They protect the ends of chromosomes from being recognized by DNA repair machinery as a break in linear DNA. This would be disastrous for a cell, and deficiencies in telomere protection lead to many genetic diseases. Chromosome end deprotection, as it is known, can result from the loss of function of myriad telomere binding proteins. In addition, chromosome end deprotection can arise from telomeres becoming too short. Maintaining a stable telomere length leads to the second function of telomeres, which is to serve against the chromosome end replication problem, first described by James Watson (2) and Alexey Olovnikov (3) in the 1970s. Due to the semi-conservative nature of DNA replication, a segment of DNA corresponding to the final RNA primer on the most terminal Okazaki fragment is lost each cell division. In order to overcome this loss of genetic material, and transmit a complete genome between parent and progeny, a method for the extension of telomeres must be present in stem and germ-line cells. The method for telomere extension, and thereby genome maintenance, was discovered by Elizabeth Blackburn and her graduate student, Carol Greider in the 1980s.

Telomere extension is performed by a ribonucleoprotein complex aptly named telomerase (4). Telomerase is comprised of an enzymatic reverse transcriptase, TERT, and an RNA subunit called TER. TER not only provides the template for telomere addition, but also serves as a scaffold for telomerase accessory proteins to bind. Using the telomere template found within TER, telomerase binds to a short, single-stranded extension of the telomere region. Once bound,

telomerase adds telomere repeats in the 5’ to 3’ direction, extending the single-stranded extension.

One unique characteristic of Arabidopsis is that it has undergone several whole genome duplication events, resulting in multiple copies of certain telomere-associated genes. One gene that has duplicated in Arabidopsis is the TER locus, resulting in two full-length copies of TER, TER1 and TER2. TER1 serves as the canonical telomerase RNA subunit. It is critical for telomere length maintenance, and is the template for telomere addition. Interestingly, TER2 acts as a novel negative regulator of telomerase activity in response to DNA double-strand break (5). TER2 levels spike upon the addition of DNA damaging drugs, resulting in diminished telomerase activity. This regulatory pathway is hypothesized to prevent telomerase from acting on DNA substrates that are not telomeres (double-stand DNA breaks).

Another interesting feature about TER2 is that it gets processed, or spliced in vivo into a smaller RNA, called TER2s. TER2s results from the removal of about 529 nucleotides “intron” and part of the 3’ end of TER2 (5). These two events result in a much smaller RNA (219 nts for TER2s versus 784 nts for TER2).

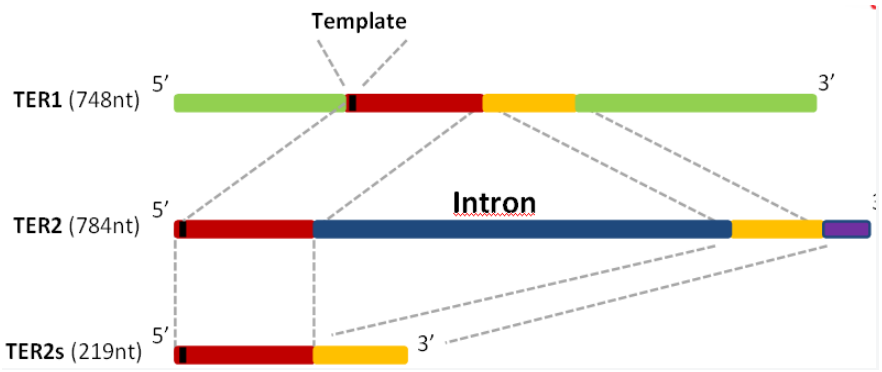

Fig 1: TER1, TER2 and TER2s in *Arabidopsis thaliana.*

We have been unable to identify sites of conventional mRNA processing, and therefore we think that TER2s maturation depends on a completely different process altogether. Also, we are unable to identify sites in TER2 that contribute in the negative regulation of the telomerase activity. In order to determine what sequences in the RNA are important for processing and also for negative regulation, I have been taking a targeted mutagenesis approach, using both structure predictions and bioinformatics to guide the mutagenesis. My hypothesis is that the sequences within and next to the intron in the full-length TER2 would be necessary for the removal of the intron as well as the negative regulation of telomerase. These elements were the main targets I was trying to disrupt.

CHAPTER II

METHODS

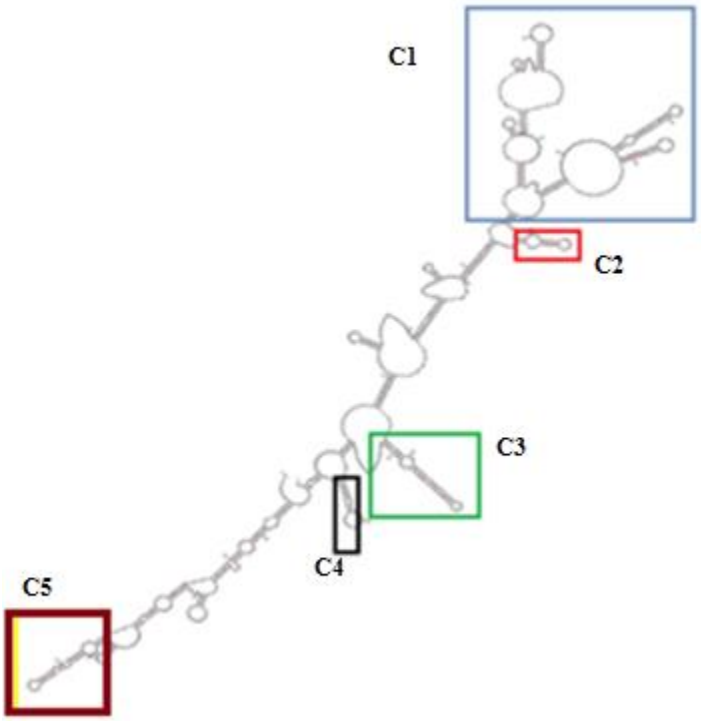

Fig 2: Region to be deleted in the TER2.

TER2 assembles with proteins, Ku and POT1b, to form one more RNP (ribonucleoprotein) complexes distinct from TER1 RNP (6). Since a stem-loop motif in RNA is typically required for RNA-protein interaction, these structures were chosen for constructing mutations. C1, C2, C3 and C4 each have a deletion of stem-loop region in the intron region. C5, a stem-loop region outside but closer to the intron region in the 3' end, was also chosen for mutation.

Overlapping PCR and TA cloning

After extracting DNA using small scale DNA Extraction Protocol, I set out to delete some sequence from the TER2 coding segment on the basis of RNA secondary structure. In an attempt to perturb the overall folding of the RNA as little as possible, secondary structure of TER2 was analyzed in Silico. Overlapping PCR was used to perform the deletion, since the segment of DNA to be removed was too large for conventional mutagenesis.

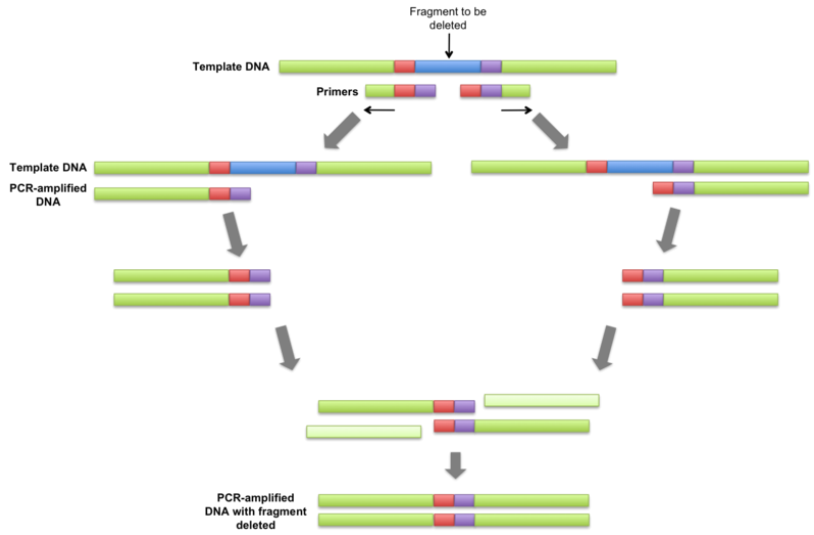

Fig 3: Principle of overlapping PCR (Source: commons.wikimedia,org).

Primers were designed that amplified two fragments that we wanted to join together as part of the mutation. The primers had a flanking segment that was complementary to the other fragment of our interest. Primers that were designed for each construct are listed below.

Construct 1:
Fragment 1:
Forward Primer: 5’- GTGCTAGACATAAGATGACAGTCAACTGAGTTT – 3’
Reverse Primer: 5’-TAC TTC TAC GAA TGG GCC CTC GAT ACA GTA GCT GCT CC-3’

Fragment 2:
Forward Primer: 5’-CTA CTG TAT CGA GGG CCC ATT CGT AGA AGT AGC TTA TTC TAG CTA -3’
Reverse Primer: 5’- ACGACGTCTCTTTTTTTTTTCTTTTTTTTTTTTCTTTCTTCTTCTT – 3’

Construct 2:
Fragment 1:
Forward Primer: 5’- GTGCTAGACATAAGATGACAGTCAACTGAGTTT – 3’
Reverse Primer: 5’-TAC TCT ACC TCA AGT ACG AAT GGG CCA CAA CAA AAC ATA C -3’

Fragment 2:
Forward Primer: 5’- TTG TGG CCC ATT CGT ACT TGA GGT AGA GTA GTT GGG GTT G – 3’
Reverse Primer: 5’- ACGACGTCTCTTTTTTTTTTCTTTTTTTTTTTTCTTTCTTCTTCTT – 3’

Construct 3:
Fragment 1:
Forward Primer: 5’- GTGCTAGACATAAGATGACAGTCAACTGAGTTT – 3’
Reverse Primer: 5’-TTA GAC CTT AAA CTT TTA CAC AAC CTT ACC TTA GCC ACT A-3’

Fragment 2:
Forward Primer: 5’-GGT AAG GTT GTG TAA AAG TTT AAG GTC TAA GAG AGT GGG T-3’
Reverse Primer: 5’- ACGACGTCTCTTTTTTTTTTCTTTTTTTTTTTTCTTTCTTCTTCTT – 3’

Construct 4:
Fragment 1:
Forward Primer: 5’- GTGCTAGACATAAGATGACAGTCAACTGAGTTT – 3’
Reverse Primer: 5’-ACA CAT TAC ACA TAT CCA CTC TCT TAG ACC TTA AAC TTA GTC ACT -3’

Fragment 2:
Forward Primer: 5’- GTC TAA GAG AGT GGA TAT GTG TAA TGT GTG TAA ACT TAT GAG TGT GTG A -3’
Reverse Primer: 5’- ACGACGTCTCTTTTTTTTTTCTTTTTTTTTTTTCTTTCTTCTTCTT – 3’

Construct 5:
Fragment 1:
Forward Primer: 5'- GTGCTAGACATAAGATGACAGTCAACTGAGTTT – 3'
Reverse Primer: 5'- CTG ATT CCT GAA GTT CCA TCT CCG ACG ATG TTG TTT TTC T-3'

Fragment 2:
Forward Primer: 5'- CAT CGT CGG AGA TGG AAC TTC AGG AAT CAG TTT GCC TTA TG-3'
Reverse Primer: 5'- ACGACGTCTCTTTTTTTTTTCTTTTTTTTTTTTCTTTCTTCTTCTT – 3'

High fidelity Phusion-polymerase was used for amplification as it would not introduce any adenine nucleotide onto the fragments. PCR Protocol for Phusion High-Fidelity DNA polymerase was followed. After carrying out the PCR, the expected fragments were amplified, and then an extension PCR was performed in order to join the two fragments. For the extension PCR, 1 µL each of fragment 1 and fragment 2 were added with 2 µL of 10X Extaq Buffer, 0.4 µL of dNTPs and 14.6 µL of nuclease free water. The thermocycling conditions for this PCR were: 95°C - 5 min, 60°C - 1min, 72°C - 10 min.

After the extension reaction, one more PCR reaction was performed to amplify the extended product. Protocol for Ex Taq DNA Polymerase was followed. In the primers, the restriction sites for EcoRV and SacI were introduced. In this way, five different TER2 constructs were prepared. All of these constructs were TA cloned following the protocol from TOPO TA Cloning Kit from Invitrogen, and then colonies were screened by blue/white screening on antibiotic plates containing IPTG/XGAL and sent for sequencing to Eton Bioscience.

PBA002 cloning

Once sequencing confirmed the mutation, the constructs were cloned in a binary vector, PBA002 (designed in Dr. Xiuren Zhang's lab, TAMU), which can replicate in bacteria (*E. coli* and *Agrobacterium tumefaciens)* and can exist in Arabidopsis. PBA002 contains Spectinomycin antibiotic resistance and BASTA resistance genes. By using restriction enzymes EcoRV and SacI, the constructs were inserted in the vector. A volume of 0.5 µL EcoRV and SacI, 3 µL of 10X Buffer4, 0.3 µL BSA, 5.7 µL of water and 20 µL (about 1000ng) of DNA were used for the restriction enzyme digestion. The cloned constructs were transformed into competent Agrobacteria cells by electroporation.

Floral dipping and herbicide selection

After transforming constructs into Agro competent cells, agro transformants were selected on LB plate with Spec antibiotics. Three colonies from each construct plate were picked to set up a 5mL LB overnight culture with the same antibiotic. Next, this culture was transferred into a 500 mL LB media for another overnight culture and cells were collected by centrifuge. The cell pellets were re-suspended in a 500mL buffer containing 5% sucrose and 1/2000(V/V) of surfactant Silwet L-77 and poured in a beaker. Flowers were then dipped into the solution for more than 1 min. The transformed plant was then wrapped and stored in dark for overnight. The next day, the plant was unwrapped and let to grow.

After the transformed plant turned yellow, its seeds were then harvested and selected on MS-agar plates containing herbicide called BASTA. The positive transformants were then grown in soil.

TRAP assay

To measure telomerase enzyme activity, Telomere repeat amplification protocol (TRAP) assay was used. This assay has multiple steps: protein extraction, protein concentration determination, telomerase activity by primer extension and qPCR determination for newly synthesized telomeric DNA (to measure relative telomerase activity).

TRAP assay was performed as described (7). Since only C1 construct was successfully selected, protein was extracted from the C1 seedling. The entire extraction process was done in ice and VRC and DTT was used to prevent any protein degradation. PEG8000 was used to enrich telomerase RNP. Bradford assay was then performed to obtain a calibration curve. The concentration of the protein extracted was determined. The protein was then diluted and telomerase activity test was performed. For the activity test, SYBR Green Hot start PCR master mix (Finnzymes) of 12.5 µL and a 10uM Forward TRAP primer was added to 10.5 µL of Protein (50ng) and water. The reaction was set up in the Bio-Rad low profile qPCR plates along with the wild type telomerase protein. The plate was covered with foil and incubated at 37°C for 45 min to let the telomerase add telomeric sequence onto the provided forward primer. A volume of 1 µL of Reverse TRAP primer was then added to the reaction. The plate was sealed with microfilm B and after a spin down, the reaction was run on Bio-Rad CFX qRT machine with qTRAP protocol. The telomerase activity was reflected by newly synthesized telomeric DNA and the amount of this DNA was determined by qPCR. The telomerase activity of the C1 construct was normalized to wild type Col-0. Δ Ct method was used to calculate relative activity levels.

CHAPTER III

RESULTS

Overlapping PCR and TA cloning

Five constructs were made with the help of overlapping PCR.

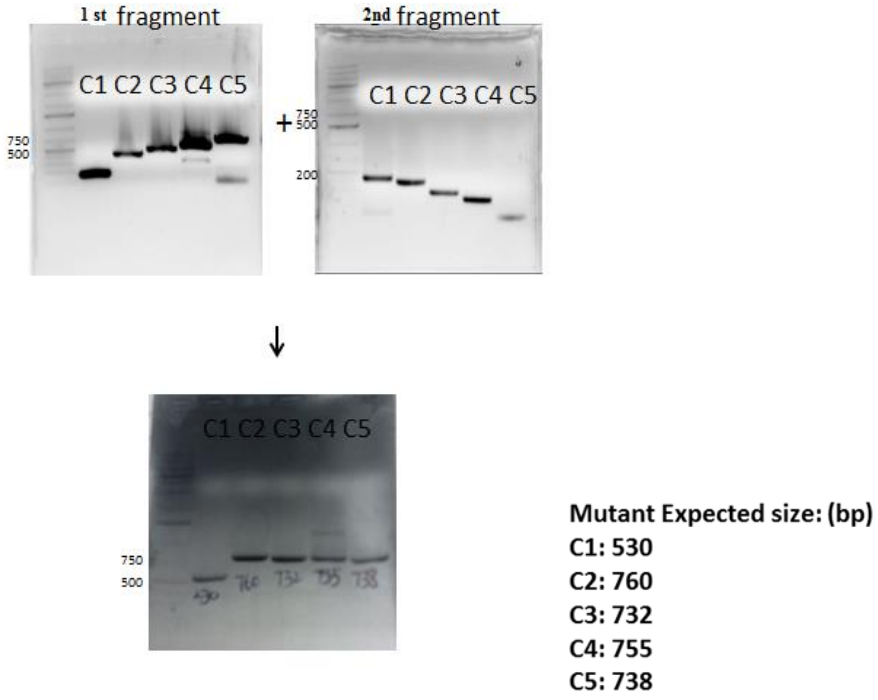

Fig 4: Overlapping PCR forming the constructs of expected size.

The length of TER2 is 784bp in *Arabidopsis thaliana*. Five constructs were prepared by deleting five regions in the TER2 structure that is within or near the intron region. First, fragments on

both sides of the expected deletion were amplified using the designed primer. First fragments for C1, C2, C3, C4, and C5 were 411bp, 412bp, 487bp, 562bp and 701bp respectively. Second fragments for C1, C2. C3, C4, C5 were 150bp, 378bp, 275bp, 222bp and 67bp respectively. The overlap PCR annealed the two fragments of each construct together and, thus, the expected sizes for the five constructs were obtained: 530bp for C1, 760bp for C2, 732bp for C3, 755bp for C4 and 738bp for C5.

TA cloning also was successful. Positive transformants were screened using the blue white screening on XGAL IPTG plates. Sequencing further confirmed the mutation in the constructs.

PBA002 clone

PBA002 cloning was successful. Colonies were isolated from the LB-Spec agar plate and sent for sequencing. Sequencing result confirmed the presence of the constructs in the vector.

Herbicide selection after floral dipping

The C1 construct was selected in the MS-BASTA plate. Only C1 seedlings developed true leaves on the selection plate.

TRAP assay

For the TRAP assay, a standard calibration curve was obtained in order to determine the concentration of the protein extracted (Fig 5). Relative telomerase activity was then obtained for the wild type construct (Col) and C1 construct in Col (Fig 6). µL

Table 1: BSA concentration and their absorbance

BSA concentration (µg/ml)	0	5	10	15	25	50	100
Absorbance at 595 nm	0	0.072	0.111	0.186	0.253	0.464	0.807

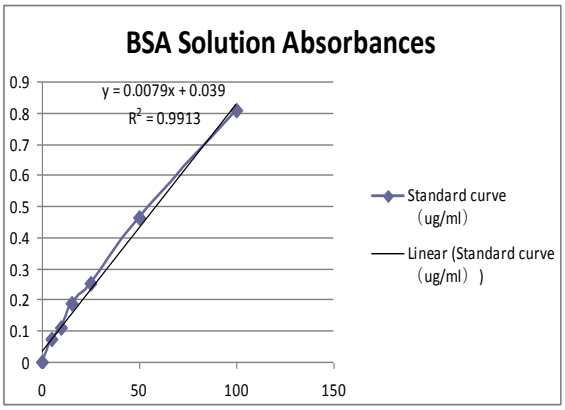

Fig 5: Table of BSA concentration versus absorbance and the Calibration Curve.

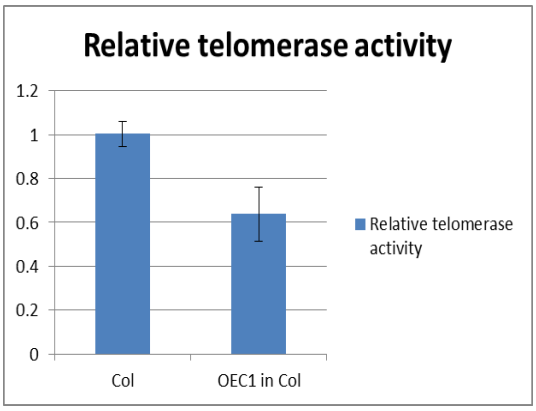

Fig 6: Telomerase activity of C1 and the wild type.

Fig 6 shows the comparison of telomerase in wild type plants and the over expressed C1 construct plant. C1 had lower telomerase activity than the wild type. The decrease in the telomerase activity was about 35 percent.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The main goal of this research was to construct different mutations in the TER2 intron region and check for their effect on telomerase activity regulation. The other goal was to determine which intron element is required for TER2 splicing and its regulation, especially upon DNA damage. Mutations were successfully constructed. We were successful in selecting over expression C1 (construct that had a deletion of the first step-loop region of about 254 nucleotides in the TER2 structure) in Col-0 wild type plants. The TRAP assay showed about 35% lower telomerase activity in C1 compared to the wild type lines. Previous work shows that TER2 is a negative regulator to telomerase (3). From our result, the reduction of telomerase activity by over expression of C1 construct suggests that the C1 construct can still compete with endogenous TER2 or TER1 with TERT binding, and the negative regulation by TER2 C1 is retained even with this mutation. Although we do not know the extent to which this construct gets over expressed, the negative regulation effect is minimal. Therefore, our construct may affect telomerase negative regulation after deletion of a 25 nucleotide long intron because we have observed only 2.5 fold TER2 induction after zeocin, which caused 70% telomerase activity down regulation.

The C1 result also indicates that other intron sequence remained in intron in this construct help to keep its negative regulatory role. Because we are also interested to know if our intron sequence deletion will affect TER2 splicing, qRT-PCR will be performed to check for the telomerase RNA level. Together with our telomerase activity assay result, we have gained a better idea about how

intron structure and intron splicing is involved in TER2 mediated telomerase negative regulation. In the future, TRAP assay and qRT-PCR should be performed on the remaining constructs once they are selected on the BASTA plates. Since these five constructs were over expressed in wild type plants, there is endogenous TER2 RNA which will affect our analysis in our qRT-PCR for determining the splicing capability of our constructs. To get a better idea about how splicing is affected by these mutations, we are also working on getting these constructs cloned into a different vector with a native promoter and terminator sequence. By transforming these new constructs into TER2-/- plants, we will be able to compare the splicing and telomerase activity of the TER2. C2, C3, C4 and C5 constructs are still being selected on the BASTA plate. Overall, the goal of constructing different mutation was achieved. The results from construct C1 support the hypothesis that certain sequences in the intron region are important for negative regulation of telomerase. Further tests will also be performed to check the effect of the designed mutations on the splicing of TER2.

REFERENCES

1) Shampay, J., J.W. Szostak, and E.H. Blackburn, *DNA sequences of telomeres maintained in yeast.* Nature, 1984. **310**(5973): p. 154-7.

2) Watson, J.D., *Origin of concatemeric T7 DNA.* Nat New Biol, 1972. **239**(94): p. 197-201.

3) Olovnikov, A.M., *A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon.* J Theor Biol, 1973. **41**(1): p. 181-90.

4) Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.

5) Cifuentes-Rojas, C., et al., *An alternative telomerase RNA in Arabidopsis modulates enzyme activity in response to DNA damage.* Genes & Development, 2012. **26**(22): p. 2512-2523.

6) Cifuentes-Rojas, C., et al., *Two RNA subunits and POT1a are components of Arabidopsis telomerase.* Proc Natl Acad Sci U S A, 2011. **108**(1): p. 73-78.

7) Fitzgerald, M.S., T.D. McKnight, and D.E. Shippen, *Characterization and developmental patterns of telomerase expression in plants.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(25): p. 14422-14427.