INVESTIGATING REPLICATION PROTEIN A's ROLE IN TELOMERE MAINTENANCE IN *ARABIDOPSIS THALIANA*

An Undergraduate Research Scholars Thesis

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CATHERINE ELIZABETH SMITHSON

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ABSTRACT

Protein interaction studies between individual Replication Protein A subunits and CST subunits

Catherine Smithson
Department of Biochemistry and Biophysics
Texas A&M University

Research Advisor: Dr. Dorothy Shippen
Department of Biochemistry and Biophysics
Texas A&M University

Replication protein A (RPA) and CST are two highly similar heterotrimeric telomere complexes involved in various aspects of DNA metabolism. RPA functions in genome-wide DNA metabolism (including DNA replication, repair and recombination) while CST has been shown to play a specific role in telomere protection and maintenance. Recent studies have implicated RPA in telomere maintenance. Two CST subunits, STN1 and TEN1, have been found to form a sub-complex independent of the CST complex. Given the high level of structural conservation between RPA and CST, I hypothesize that individual CST and RPA subunits form alternative complexes. The aim of this study is to test the hypothesis by determining if RPA and CST subunits associate with each other using an *in vitro* co-immunoprecipitation assay. Expression vectors have been constructed with RPA2A, RPA2B, RPA3A, RPA3B, STN1, and TEN1 and have been tested in rabbit reticulocyte lysate for protein expression. If association between CST and RPA subunits is found, further studies could investigate how and why the CST and RPA complexes work together to maintain telomeres and to promote aspects of genome stability. Another goal of this study is to construct fluorescent protein tagged RPA1B (GFP) and RPA1D (YFP) for use in subcellular localization studies in *Arabidopsis thaliana*.

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CHAPTER I

INTRODUCTION

Replication protein A (RPA) is a highly conserved single-stranded DNA (ssDNA) binding protein complex involved in DNA metabolism (Wobbe et al., 1987; Wold, 1997). The complex can also bind double-stranded DNA (dsDNA) and RNA with less affinity than for ssDNA (Wold and Kelly, 1988; Wold et al., 1989). Like bacterial SSBs, RPA binds to ssDNA during processes such as DNA replication to prevent the DNA from re-annealing or forming secondary structures (Wold, 1997). RPA also interacts with other proteins to help regulate and recruit proteins during various DNA metabolic activities (Wold, 1997).

RPA is a heterotrimeric complex consisting of: RPA1, RPA2, and RPA3. These three subunits have molecular masses of 70, 30, and 14 kDa, respectively (Wold, 1997). The largest subunit, RPA1, contains DNA-binding domains and is involved in ssDNA-binding. RPA2 regulates the complex's function (Wold, 1997). RPA2 is phosphorylated by the kinases ATM, ATR, and DNA-PK following DNA damage and during DNA replication and mitosis (Aklilu et al., 2014; Binz et al., 2004; Din et al., 1990). RPA3 may serve a structural role within the RPA complex (Bochkareva et al., 2002). All three subunits contain DNA binding domains, but RPA1 has the highest affinity for ssDNA (Eschbach and Kobbe, 2014). The formation of the RPA complex is proposed to occur in two steps; RPA2 and RPA3 form a stable complex after which RPA1 binds to RPA2 (He et al., 1993; Henricksen et al., 1994; Wold, 1997). While animal and fungal RPA complexes generally have one form of each subunit, plants often have multiple subunits of RPA1, RPA2 and RPA3 (Aklilu and Culligan, 2016). *Arabidopsis thaliana*, for

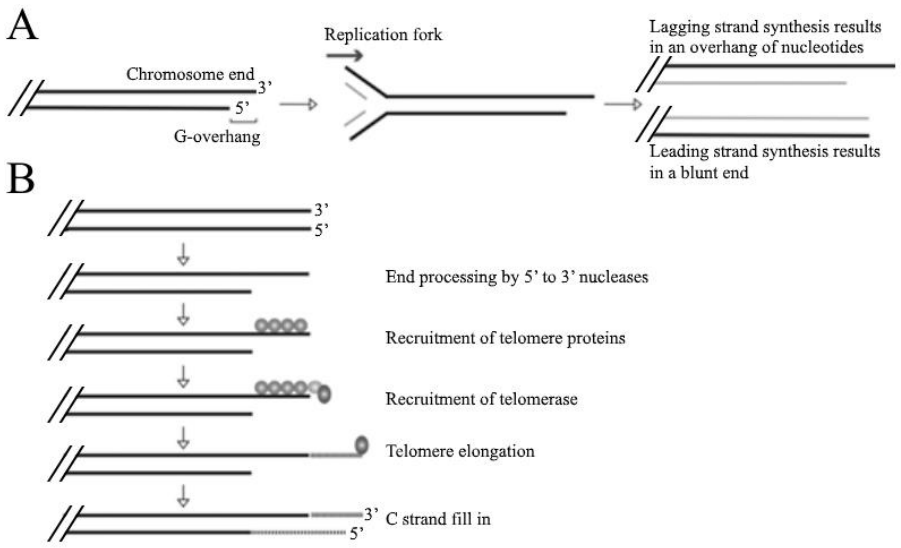

Figure 1. Schematic of telomere replication. A) Products of DNA replication. B) Processing of blunt telomere ends. Adapted from Matsui et al.

example, has several paralogues of each of the three subunits: five of RPA1 (named RPA1A-E), two of RPA2 (named RPA2A-B), and two of RPA3 (named RPA3A-B) (Aklilu et al., 2014).

Telomeres are repeated nucleotide sequences at the ends of linear chromosomes that associate with proteins to cap and protect the ends of linear chromosomes from being recognized by the DNA double-strand break repair pathway, homologous recombination and non-homologous end joining mechanisms (Biessmann and Mason, 1992; Levy et al., 1992). Additionally, telomeres ensure the complete replication of chromosomes by overcoming the end replication problem that results from normal linear DNA replication. DNA polymerases can only synthesize DNA in a 5'-3' direction and require an RNA primer to initiate synthesis (Figure 1A). The leading strand of the DNA is replicated continuously, while the lagging strand must be replicated discontinuously. The entire length of the leading strand is replicated resulting in blunt end on the chromosome. However, on the lagging strand, an overhang is left when the most terminal RNA primers are removed after DNA replication (the end replication problem) (Figure 1A) (Levy et al., 1992).

Telomerase, an important telomere-associated enzyme, overcomes the end replication problem by extending telomeres independent of conventional DNA polymerases (Figure 1B) (Cerone et al., 2001; Matsui, 2011).

Recent studies have implicated RPA in the regulation of telomeres in yeast and mammals (Cohen et al., 2004; Schramke et al., 2004; Smith et al., 2000). Mutations in RFA1 (replication factor A1, a RPA homologue in *Schizosaccharomyces pombe* (*S. pombe*) and *Saccharomyces cerevisiae* (*S. cerevisiae*), caused defects in telomere length homeostasis (Sun et al., 2009). Yeast RPA was shown to be localized on the telomeres of *S. cerevisiae* (Schramke et al., 2004). Additionally, RFA2 (the RPA2 homologue in yeast) regulates the extension of telomeres by telomerase (Schramke et al., 2004). The yeast RPA3 homologue binds stably to the G-rich strand of telomeres (Gao et al., 2007). Human RPA has been shown to participate with BLM (Bloom Syndrome) and WRN (Werner Syndrome) helicases, to unwind long double-stranded telomere regions (Opresko et al., 2002).

The existence of multiple paralogues of each RPA subunit in Arabidopsis simplifies the dissection of RPA's overall function in telomere maintenance. T-DNA insertion mutants have been used to deduce each paralogue's function. Double mutants for *rpa1c* and *rpa1e* are hypersensitive to ionizing radiation (Aklilu et al., 2014). Additionally, *rpa1c* mutants are hypersensitive to the double-strand break inducing agent camptothecin (Aklilu et al., 2014). These results suggest that RPAC and RPA1E are involved in DNA repair (Table 2) (Aklilu et al., 2014). *rpa1a* mutants have

Table 2. Summary of the role of RPA subunits in DNA replication and telomere maintenance in Arabidopsis.

RPA Subunit	Phenotype observed in T-DNA insertion mutants	Function	Source
RPA1A	low pollen viability due to erroneous pairing of homologous chromosomes during prophase I	Involved in meiosis	Osman et al. 2009
	Lengthened telomeres	Negative regulation of telomere length	Takashi et al. 2009
RPA1B and RPA1D	Delayed root and shoot development	DNA Replication	Aklilu et al. 2014
	Telomere dysfunction	Telomere maintenance	Aklilu et al. unpublished data
RPA1C and RPA1E	Hypersensitivity to ionizing radiation	DNA Repair	Aklilu et al. 2014
RPA1C	Hypersensitivity to the double-strand break inducing agent camptothecin		

low pollen viability due to erroneous pairing of homologous chromosomes during prophase I and thus RPA1A is likely required for meiosis (Osman et al., 2009). Although the results have not been replicated in our lab, Takashi et al. found *rpa1a* mutants to have lengthened telomeres, suggesting that RPA1A functions in negatively regulating telomere length (Takashi et al., 2009). Double mutants for *rpa1b* and *rpa1d* display defective root and shoot growth and development as well as delayed DNA synthesis (Aklilu et al., 2014). In addition, they show a defect in telomere maintenance (Aklilu et al. unpublished data). These findings suggest that the RPA complex containing RPA1B and RPA1D is involved in both DNA replication and telomere biology (Table 2) (Aklilu et al. unpublished data; Aklilu et al., 2014).

CST is a RPA-like complex of three proteins (CTC1, STN1 and TEN1) that have a high level of structural and functional similarity to RPA subunits (Price et al., 2010; Sun et al., 2009). STN1 and TEN1 are structurally similar to RPA2 and RPA3, respectively. CTC1, although similar to RPA1, is the least conserved of the three subunit homologues (Sun et al., 2009). Like RPA, CST binds ssDNA. However, the CST complex specifically binds telomeric ssDNA sequences to either inhibit telomerase and recruit polα-primase to complete fill-in synthesis of C-strands, or to

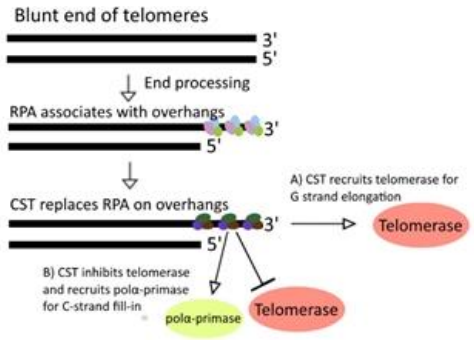

Figure 2. Summary of RPA's possible role in telomere maintenance. The C-strands of telomeres with blunt ends are processed by nucleases to leave G rich overhangs. RPA first binds these overhangs and then is replaced by the CST. CST binding either recruits telomerase for G strand elongation or inhibits telomerase and recruits pot1α for C strand fill-in. Adapted from Chen and Lingner, 2013.

recruit telomerase to elongate the G-strand overhang (Chen and Lingner, 2013; Price et al., 2010).

RPA's role in telomere maintenance could involve a stepwise process of RPA binding and subsequent CST binding to single-stranded telomere sequences. After replication, RPA could bind the G-rich overhangs left after the C-strands of blunt telomere ends are removed by nucleases (Figure 1B). The CST complex could then replace RPA and either recruit telomerase to extend the G overhangs or inhibit telomerase and recruit polα-primase to complete C-strand fill-in synthesis (Figure 2) (Chen and Lingner, 2013). Interestingly, STN1 and TEN1 have been found as a heterodimer independent of CTC1 (Sun et al., 2009). It is unknown if these subunits participate in other non-CST complexes. The high level of similarity between RPA and CST subunits suggests that the complexes serve similar functions, perhaps even interacting with each other.

Arabidopsis thaliana is an ideal model organism for the study of telomere biology as plants have a higher tolerance for telomere dysfunction than yeast or mammalian cells (Riha et al., 2001). Additionally, because RPA is a highly-conserved complex, research on Arabidopsis RPA could apply to RPA homologues in other organisms. Research on RPA's possible role in telomere

maintenance is valuable as telomere dysfunction has been implicated in several cancers and degenerative stem cell diseases such as Dyskeratosis Congenita (DKC) and idiopathic pulmonary fibrosis (IPF) (Blasco, 2005; Garcia et al., 2007).

The major goal of this research is to test the hypothesis that the homologous CST and RPA subunits associate with each other *in vitro* to form heterologous complexes. The hypothesis will be tested using an *in vitro* co-immunoprecipitation (Co-IP) assay. Another goal of this research is to construct fluorescent protein tagged RPA1B (GFP) and RPA1D (YFP) for use in subcellular localization studies in *Arabidopsis thaliana*.

CHAPTER II

METHODS

Plasmids, strains, enzymes, and reagents

Wild type (WT) *Arabidopsis thaliana* plants were donated by Dr. Behailu Aklilu in the Shippen lab. TOP10 *E. coli* was used for cloning and maintaining recombinant plasmids. pCITE-4a+_STN1, pET28a+_STN1, pCITE-4a+_TEN1, and pET28a+_TEN1 constructs were donated by Gabrielle Lessen in the Shippen lab. PCR product and gel purification was conducted using the Nucleospin Gel and PCR cleanup (Machery Nagel). Plasmids were purified using a QIAprep spin miniprep kit (Qiagen). Oligonucleotide primers were purchased from Thermofisher Scientific**.** All other reagents were of analytical grade or higher. *Arabidopsis thaliana* genomic DNA (gDNA), the pCAMBIA2300 plasmid, and enhanced green and yellow fluorescent protein (GFP and YFP, respectively) DNA were kindly provided by the Shippen lab, Texas A&M University

Isolation of total RNA and cDNA synthesis

Total RNA from WT *A. thaliana* was isolated using Zymo Research's Direct-Zol RNA Miniprep kit. cDNA was synthesized using Quantabio's qScript cDNA SuperMix.

Co-Immunoprecipitation

A co-immunoprecipitation assay was used to determine if RPA3 subunits interact with the CST subunit STN1.

Construction of recombinant vectors for Co-IP

RPA subunits were amplified using the primers described in Table 2. All forward primers contained a *BamH*I site (bold) and all reverse primers contained an *Eag*I site (underlined) (Table 2). The 50 µl PCR reactions contained 5 µl TaKaRa ExTaq 10X buffer, 4 µl 2.5 mM dNTPs, 0.3

Table 2. Primers and PCR conditions used for the amplification of RPA2 and RPA 3 subunits

RPA Subunit		Primer Sequence	PCR Annealing Temperature	PCR Extension Time
RPA2A	Forward Primer	GGCTGATATC**GGATCC**ATGTTCTCCAGCAGCCAATTCGAG	56	1 min
	Reverse Primer	TGCTCGAGTGCGGCCGTCAAAGCTCCACGTGCTTGAAG		
RPA2B	Forward Primer	GGCTGATATC**GGATCC**TAATCAGATTGATATTTGAAAAAAAAT GCTTCTG	55	1 min
	Reverse Primer	TGCTCGAGTGCGGCCGTCAAGCGTTAGCAGTCGATTTGAAG		
RPA3A	Forward Primer	GGCTGATATC**GGATCC**ATGGATACTTCAAGTCCTTCAGC	55	30 sec.
	Reverse Primer	TGCTCGAGTGCGGCCGTTAGATGAACAAGTGCTTGAACTCC		
RPA3B	Forward Primer	GGCTGATATC**GGATCC**ATGGATACATCAAGTCCTGCTG	55	30 sec.
	Reverse Primer	TGCTCGAGTGCGGCCGTTAGATGAACAAGTGTCTAAACTCAC		

Primers and PCR conditions used to amplify RPA subunits. *BamH*I restriction sites added to forward primers and *Eag*I restriction sites added to reverse primers are bold and underlined, respectively.

µl 10 mM forward primer (Table 2), 0.3 µl 10 mM reverse primer (Table 2), 0.25 µl TaKaRa ExTaq DNA polymerase, and 3 µl *A. thaliana* cDNA. PCR reactions were carried out using a thermal cycler under the following conditions: an initial denaturing step at 94˚C for 30 sec; 30 cycles at 94˚C for 10 sec (denaturing), annealing at the temperatures described in Table 2 for 30 sec, and extension at 72˚C for the time described in Table 2; a final extension cycle of 72˚C for 2 min, and preservation at 4˚C. The amplicons were gel purified and subjected to a double digestion with *BamH*I and *Eag*I (New England Biolabs) to render sticky ends needed for cloning. The products were gel purified and each gene was ligated using QuickLigase (NEB) into pCITE-4a+ and pET28a+ plasmids (Novagen) linearized with *BamH*I and *Eag*I. *E. coli* were transformed with the ligation reactions and plated on solid LB agar + ampicillin (pCITE-4a+) and LB agar + kanamycin (pET28a+) for selection of the recombinant plasmids. DNA sequencing (Eton Bioscience) was used to verify that no mutations were generated during the cloning process. Proteins expressed from the pET28a+ constructs contained a T7-tag and would serve as the "bait" proteins bound to T7 antibodies on the magnetic beads. Proteins expressed from the pCITE-4a+ constructs would serve as the "prey" proteins that are expected to bind to the bait proteins on the magnetic beads conjugated with T7 antibodies.

Preparation of magnetic beads

For each protein interaction reaction, the supernatant from 9 µl of Dynabeads Protein A magnetic beads (Invitrogen) was removed using a magnetic rack and then the beads incubated on a lab quake with 0.26 µg T7 Antibody (Thermo Fisher Scientific) and 4 µl of PBS+0.1% Tween for 10 min at room temperature. The supernatant was removed and the beads washed with 4 µl W100 buffer (Bryan et al., 2000). The beads were then incubated for 30 min at 4°C on a lab quake in 4 µl of blocking buffer (Bryan et al., 2000). The supernatant was removed and an additional 4 µl blocking buffer added and incubated under the same conditions for 30 min (Bryan et al., 2000).

In vitro expression of proteins

In vitro transcription and translation was carried out using the Promega TnT® T7 Coupled Reticulocyte Lysate (RRL) System according to the manufacturer's instructions. Each protein expression reaction of the pCITE-4a+ vector contained 65 µl of RRL, 5.2 µl Reaction Buffer, 2.6 µl T7 RNA Polymerase, 5.2 µl [^{35}S]methionine (^{35}S-Met) (PerkinElmer), 2.6 µl RNasin Plus RNase Inhibitor (Promega), 2.6 µg plasmid DNA and water to a final volume of 130 µl. The expression reactions of pET28a+ constructs contained 25 µl of RRL, 2 µl Reaction Buffer, 1 µl T7 RNA Polymerase, 2 µl ^{35}S-Met, 1 µl RNasin Plus RNase Inhibitor (Promega), 1 µg plasmid DNA and water to a final volume of 47 µl. Transcription and translation was carried out at 30°C for 90 min after which 7.8 µl and 2.5 µl of cycloheximide was added to the pCITE-4a and pET28a reactions, respectively, to quench the reaction.

Protein interaction reactions

Protein interaction reactions consisted of 60 µl of the bait protein (pET28a+ constructs) reaction and 20 µl of prey protein (pCITE-4a+) reactions. Interaction reactions were incubated at 30°C for 30 min and then incubated on a lab quake at 4°C for 20 min. All incubations were

performed at 4°C with constant rotation to ensure adequate mixing. To each 80 µl interaction reaction, 240 µl blocking buffer was added and incubated 30 min. Reactions were centrifuged at 1400 RPM for 15 min at 4°C and the supernatant decanted. 6 µl of the supernatant was saved to be used as a protein loading control. 36 µl of prepared magnetic beads were added to each reaction and incubated 13 h. The supernatant was removed and the beads washed three times with W300 buffer, twice using 750 µl PBS incubating 10 min during each wash (Bryan et al., 2000). Beads were then washed twice using 750 µl TMG (Bryan et al., 2000). 20 µl of TMG and 7.5 µl of 6X SDS loading buffer was added to the bead reactions (Bryan et al., 2000). 6 µl of 3X SDS loading buffer was added the saved supernatant from centrifugation of original protein interaction reactions. Reactions were boiled for 3 min, centrifuged to spin down, and 10 µl were subjected to 15% SDS-PAGE. The gels were dried for autoradiography.

Molecular cloning of RPA1B, RPA1D, GFP and YFP

Because the RPA1B and RPA1D subunits may play a role in telomere maintenance, they are predicted to localize on the telomeres. To test this hypothesis, vectors containing fluorescent protein tagged RPA1B and RPA1D were constructed for use in protein localization studies. The coding sequences (CDS) and native promoters (promo) of RPA1B and RPA1D along with either a GFP tag (RPA1B) or a YFP tag (RPA1D) were cloned into pCAMBIA2300 (Marker Gene

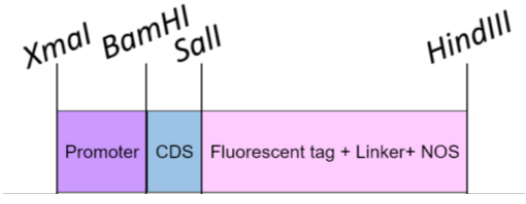

Figure 3. Strategy for the construction of the pCAMBIA2300 vectors containing the fluorescent protein tagged RPA1B and RPA1D proteins. Key: Promo- native promoter, CDS-coding sequence.

Technologies) using the strategy described in Figure 3. Further detail on the methods used for cloning are described below.

Molecular cloning of GFP and YFP

GFP, along with a N-terminal glycine-rich linker (GGTGGAGGTTCTGGAGGTGGAGG TTCTGGAA) and a C-terminal NOS terminator (NOS) sequence were amplified from the DNA provided by the Shippen Lab at Texas A&M University using the primers detailed in Table 3. YFP, along with the same glycine rich linker and C-terminal NOS sequence were amplified from the DNA provided by the Shippen Lab at Texas A&M University using the same primers used for the amplification of GFP. The 50 µl PCR reactions contained 0.5 µl Phusion Taq DNA polymerase (NEB), 10 µl 10X Phusion HF buffer, 1 µl 10 mM dNTPs, 2.5 µl 10 mM forward primer (Table 3), 2.5 µl 10 mM reverse primer (Table 3), 32.5 µl water, and 1 µl DNA template (250 ng/µl). PCR reactions were carried out using a thermal cycler under the following conditions: an initial denaturing step at 98˚C for 30 sec; 39 cycles at 98˚C for 10 sec (denaturing), 59°C for 30 sec (annealing), 72˚C for 30 sec (extension); a final extension at 72˚C for 5 min, and preservation at 4˚C. PCR products were gel purified. Purified products and the pCAMBIA300 vector were

Table 3. Primers used for the amplification of fragments used in the construction of fluorescent protein tagged RPA1B and RPA1D expression vectors

DNA fragment		Primer Sequence	Restriction Site
RPA1B promoter	Forward Primer	GCGACA**GGATC**CATGGAGAACTCAGTGACCCAAGATG	XmaI
	Reverse Primer	ACTACA**GTCGAC**CTGAGATGTCTTGTTCTTGGAAATGTC	*BamH*I
RPA1B coding sequence	Forward Primer	TACATA**CCCGGG**AAGAAACCATTGTCAAGGATTTGGG	*BamH*I
	Reverse Primer	TACATA**GGATCC**CTCTATCTTAATCTCTAGCTCTATC	*Sal*I
RPA1D promoter	Forward Primer	ACATA**CCCGGG**AAGAAGAAAAGTTGTGGTGCACG	XmaI
	Reverse Primer	TACATA**GGATCC**CTTTCTCTCTAGATATTGAAAAATGAAACG	*BamH*I
RPA1D coding sequence	Forward Primer	GCGACA**GGATCC**ATGCAGACTTCGGTGACCCC	*BamH*I
	Reverse Primer	ACTACA**GTCGAC**CTGAGATGTTTTGTTCTTGTTGGAGATG	*Sal*I
YFP + flexible linker + NOS	Forward Primer	CCGACG**GTCGAC**GGTGGAGGTTCTGGAGGTGG	*Sal*I
	Reverse Primer	GCGGCC**AAGCTT**CCGATCTAGTAACATAGATGACACCG	*Hind*III

subjected to a restriction digestion, with the restriction enzymes detailed in Table 3 at 37°C for 1 hour and 80°C for 20 minutes. The restriction products were gel purified and ligated using QuickLigase using a 1:3 insert to vector ratio to produce pCAMBIA2300_GFP and pCAMBIA2300_YFP. CaCl2-competent cells of *E. coli* Top10 were transformed with the ligated reaction and plated on solid LB agar media containing kanamycin (50 μg/ml) for selection and IPTG and blue/white screening of pCAMBIA2300_GFP and pCAMBIA2300_YFP.

Molecular cloning of RPA1B and RPA1D coding sequences

The RPA1B and RPA1D coding sequence (RPA1B_CDS and RPA1D_CDS, respectively) were amplified from cDNA using the primers detailed in Table 3. pCAMBIA2300_ RPA1B_CDS_GFP and pCAMBIA2300_RPA1D_CDS_YFP cloning was performed using the same method described in the cloning of GFP and YFP with the following modifications: an annealing temperature of 56˚C was used for PCR amplification, and LB plates used for selection lacked IPTG.

Molecular cloning of RPA1B and RPA1D promoters

The RPA1B and RPA1D promoters (RPA1B_promo and RPA1D_promo, respectively) were amplified from gDNA using the using the primers detailed in Table 3. pCAMBIA2300_ RPA1B_promo_RPA1B_CDS_GFP and pCAMBIA2300_ RPA1D_promo_ RPA1D_CDS_YFP cloning was performed using the method described for the cloning of the coding sequences with the following modifications: annealing at 53˚C (RPA1B_promo) and 55˚C (RPA1D_promo) for the PCR amplification of the promoters. DNA Sequencing (Eton Biosciences) was performed to verify that no mutations were generated during the cloning process. Final constructs were renamed pCAMBIA2300_RPA1B_GFP and pCAMBIA2300_RPA1D_YFP.

CHAPTER III

RESULTS

RPA3 subunits do not interact with STN1 *in vitro*

RPA subunits RPA 2 and RPA3 and structurally similar to STN1 and TEN1, respectively (Sun et al., 2009). Because CST subunits STN1 and TEN1 form sub-complexes independent of the CST complex, perhaps they also participate in other heterologous complexes (Sun et al., 2009). Interactions between RPA subunits and the CST paralogue of the RPA binding partner are the most likely participants in heterologous complexes. For this reason, Co-IP experiments testing the interaction between STN1 and RPA3 subunits were performed (Figure 4).

All proteins expressed from the pCITE-4a+ and pET28a constructs (Figure 5). The interactions between both T7-tagged RPA3 subunits with radiolabeled STN1 and T7-tagged STN1 with radiolabeled RPA3 subunits were tested (Figure 6A-C). Co-IP experiments showed that

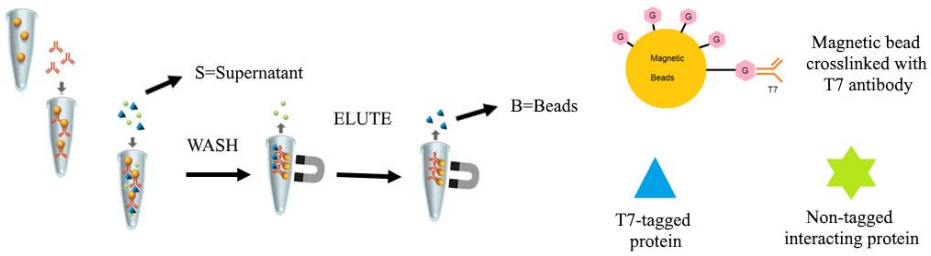

Figure 4. Schematic of Schematic of protocol for protein expression in RRL and Co-IP using magnetic beads.

	STN1	TEN1	RPA3A	RPA3B
T7 Tagged (pET28a construct)				
Untagged (pCITE-4a+ construct)				

Figure 5. SDS-PAGE of expression of RPA3 and CST subunits from pCITE-4a+ and pET28a vector constructs.

neither RPA3A nor RPA3B associates with STN1 *in vitro* under the conditions tested (Figure 6A-C). However, because the positive control interaction between STN1 and TEN1 was observed it is likely that absence of interaction is not due to experimental conditions (Figure 6A-C). Because the T7-tag was moved from one hypothesized interacting protein to the other, it is unlikely that the T7-tag was responsible for the lack of interaction. These data suggest that RPA3 subunits do not interact with STN1.

Molecular cloning of RPA1B, RPA1D, GFP and YFP

The vectors containing fluorescent protein tagged RPA1B and RPA1D (pCAMBIA2300_ RPA1B_GFP and pCAMBIA2300_RPA1D_YFP) were successfully constructed. The next step in this project is to use these constructs for subcellular localization studies in *A. thaliana.*

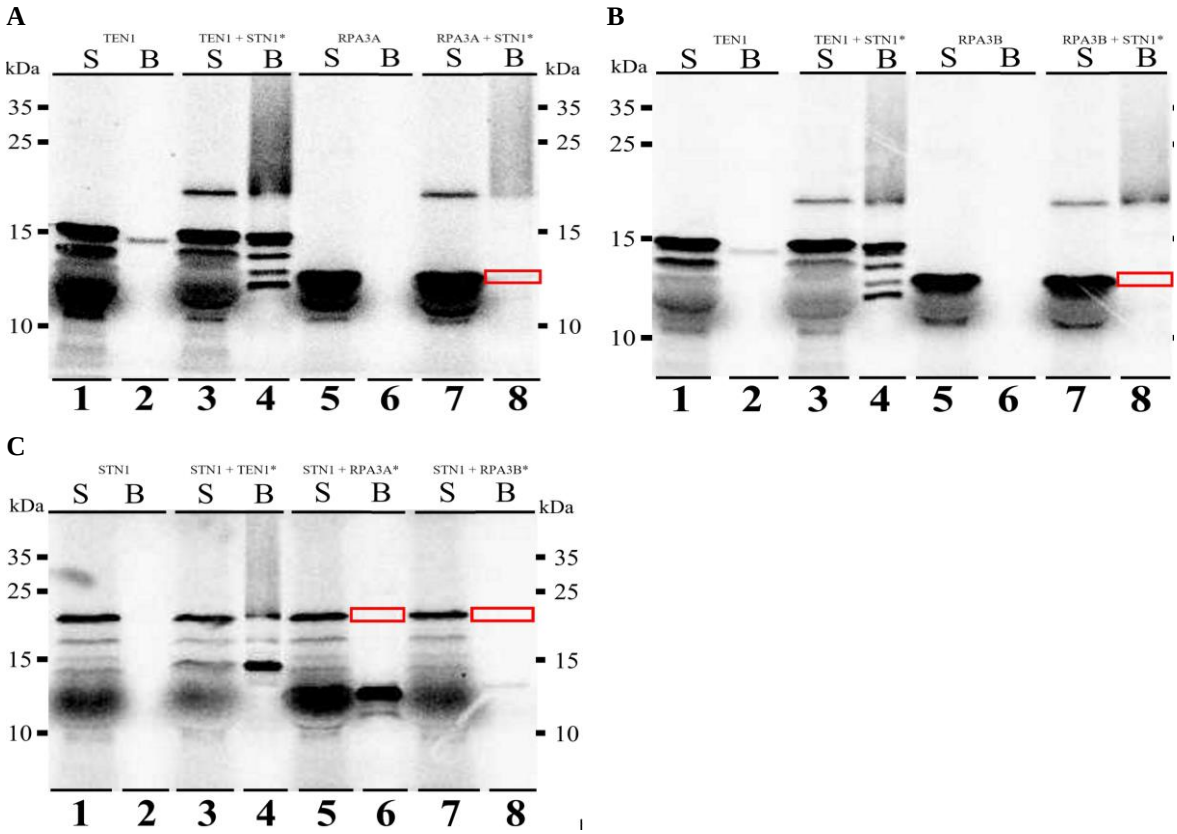

Figure 6. *In vitro* co-immunoprecipitation (Co-IP) results for RRL-expressed RPA3 subunits and TEN1 with STN1. The supernatant from each interaction reaction was run as a loading control (S). Reactions incubated with beads conjugated with T7-tag antibody are labeled "B". The symbol "*" indicates that a protein has a T7-tag. Negative controls (beads conjugated with T7-tag antibody) for A and B were performed using either radiolabeled TEN1 without STN1* (A2, B2) or radiolabeled STN1 without TEN1* (C2). Positive controls for A and B were performed with either STN1* and radiolabeled TEN1 (A4, B4) or TEN1* and radiolabeled STN1 (C4). A) Co-IP results for RRL-expressed radiolabeled TEN1 and RPA3A interactions with radiolabeled STN1*. The expected band for RPA3A is boxed in red (Lanes 6 and 8). No interaction between radiolabeled RPA3A and STN1* was observed (Lane 8). B) Co-IP results for RRL-expressed radiolabeled TEN1 and RPA3B interactions with radiolabeled STN1*. The expected band for RPA3B is boxed in red (Lanes 6 and 8). No interaction between radiolabeled RPA3B and T7-tagged STN1 was observed (Lane 8). C) *In vitro* Co-IP results for RRL-expressed radiolabeled STN1 interactions with radiolabeled T7-tagged RPA3 subunits. Expected bands for RPA3A and RPA3B are boxed in red (Lanes 6 and 8). No interactions between RPA3A* or RPA3B* and radiolabeled STN1 were observed (Lanes 6 and 8).

CHAPTER IV

DISCUSSION

RPA3 subunits do not interact with STN1 *in vitro*

Although RPA seems to function in plant and human telomere maintenance, little is known about what specific function the complex performs. From this study, we have shown that RPA3 subunits do not associate with the CST subunit STN1 *in vitro* under the conditions tested. This finding suggests that heterologous complexes consisting of RPA3 and STN1 may not form *in vivo*. Further studies are needed to determine if other individual RPA subunits interact with other CST subunits. The next course of action is to test for an interaction between RPA2 and TEN1 because TEN1 is the CST homologue of RPA3, RPA2's typical binding partner (Sun et al., 2009; Wold, 1997). If an association between other CST and RPA subunits is found, further studies could investigate how and why the subunits of CST and RPA complexes work together to maintain telomeres and to promote aspects of genome stability.

Protein localization studies

The pCAMBIA2300_RPA1B_GFP and pCAMBIA2300_RPA1D_YFP vectors were successfully constructed. The next step for this project is transient expression of the constructs in protoplasts to confirm that the proteins are expressed and localizing in the nucleus. Once expression and localization in protoplasts is confirmed, *Agrobacterium tumefaciens* will be transformed with each construct and used to create transgenic Arabidopsis plants. These plants will be crossed to create double mutants for both tagged genes. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) and *in vivo* fluorescence in situ hybridization (FISH) will be used to will

to stain the nuclei of root cells blue and tag the telomeres with a red fluorescent dye, respectively. The tagged proteins and telomeres along with the stained nucleus will be visualized using confocal microscopy. Ultimately, the goal of this project is to determine if RPA1B and RPA1D localize preferentially on the telomeres. If RPA1B and RPA1D are found to localize on the telomeres, this result would support the genetic data suggesting that the two subunits function in telomere maintenance.

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