2005-07-13

Homologous Strand Exchange and DNA Helicase Activities in Plant Mitochondria

Daqing Song
Brigham Young University - Provo

HOMOLOGOUS STRAND EXCHANGE AND DNA HELICASE ACTIVITIES IN PLANT MITOCHONDRIA

by

Daqing Song

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Microbiology & Molecular Biology

Brigham Young University

August 2005

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Daqing Song

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

_____	_____
Date	Brent L. Nielsen, Chair
_____	_____
Date	Laura Bridgewater
_____	_____
Date	Craig E. Coleman

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Daqing Song in its final form and have found that (1) its format, citation, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures and tables are in place; and (3) the final manuscript is satisfactory to the graduate committee and its ready for submission to the university library.

_____ _____

Date Brent L. Nielsen
Chair, Graduate Committee

Accepted for the Department

_____

Alan R. Harker
Chair, Department of Microbiology and Molecular Biology

Accepted for the College

_____

Rodney J. Brown
Dean, College of Biology and Agriculture

ABSTRACT

HOMOLOGOUS STRAND EXCHANGE AND DNA HELICASE

ACTIVITIES IN PLANT MITOCHONDRIA

Daqing Song

Department of Microbiology & Molecular Biology

Master of Science

Homologous recombination is critical for generating genetic variation in living organisms by exchange and rearrangement of DNA. Most of our knowledge about homologous recombination is limited to processes in bacteria or in eukaryotic nuclei. In *E. coli*, homologous recombination is dependent on the RecA protein. Higher plant chloroplasts have RecA-like strand exchange activity. However, little is known about these mechanisms in higher plant mitochondria. I have detected a RecA-like strand exchange activity in soybean mitochondria. This activity forms joint molecules in the presence of ATP, Mg^{2+}, and homologous DNA substrates. In addition, the *E. coli* single-stranded DNA binding (SSB) protein is a non-sequence-specific DNA binding protein that functions as an accessory factor for RecA protein-promoted strand exchange reactions. Our lab has identified an *Arabidopsis* homologue of *E. coli* SSB that is targeted to mitochondria (mtSSB). The results of my research shows the mtSSB protein

has the same properties as the *E. coli* SSB protein and it can stimulate the *E. coli* RecA protein-promoted strand exchange reactions.

DNA helicases utilize the energy of ATP to separate the two parental DNA strands at the replicating fork or during recombinational strand exchange. Although higher plant chloroplast helicase activity has been reported, no such activity has heretofore been identified in higher plant mitochondria. We report the characterization of a plant mitochondrial DNA helicase isolated from soybean leaves. ATP is required for this enzyme and this enzyme poorly utilizes any other NTPs or dNTPs. The enzyme requires Mg^{2+} for activity. This enzyme only has 3' to 5'unwinding activity. The optimal conditions for mitochondrial DNA helicase are 2 mM ATP, 8 to 10 mM Mg^{2+}, 100 to 200 mM NaCl and 37-42 ^{o}C incubation for one hour or longer time.

ACKNOWLEDGEMENTS

Many people have given me much help for completing this thesis. First of all, I would like to thank Dr. Brent Nielsen for giving me the opportunity to work in his lab and for his patience and helpful advice. Dr. Laura Bridgewater and Dr. Craig Coleman have also provided me important advice and guidance for my thesis. Andrew Edmondson provided valuable information and mitochondria-targeted SSB. Most of all, I would like to thank my wife, Lihua, and my daughter, Yuanjun, who always give me support. This work was supported in part by a grant from the NIH, and by funding from the Department of Microbiology and Molecular Biology.

TABLE OF CONTENTS

LIST OF FIGURES

Background

Mitochondria

The mitochondrion is an organelle found in the cells of most eukaryotes. Mitochondria are sometimes described as "cellular power plants" because their primary purpose is to manufacture adenosine triphosphate (ATP), which is used as a source of energy. "Mitochondrion" literally means 'thread granule', which is what they look like under a light microscope: tiny rod-like structures present in the cytoplasm of most eukaryotic cells. The number of mitochondria found in different types of cells varies widely. At one end of the spectrum, the trypanosome protozoan has one large mitochondrion; by contrast, human liver cells normally have between one thousand and two thousand each (Attardi et al., 1988).

Mitochondria can occupy up to 25% of the cell cytosol. Each mitochondrion has two functionally distinct membrane systems separated by a space: the outer membrane, which surrounds the whole organelle; and the inner membrane, which is thrown into folds or shelves that project inward. These inward folds are called cristae. The number and shape of cristae in mitochondria differ, depending on the tissue and organism in which they are found, and serve to increase the surface area of the membrane. The outer membrane encloses the entire organelle and contains channels made of protein complexes called porins through which molecules and ions can move in and out of the mitochondrion. It is composed of about 50% lipids and 50% proteins. Large molecules are excluded from traversing this membrane except through a specific import process (Alberts et al., 1994). The inner membrane, folded into cristae, encloses the matrix (the internal fluid of the mitochondrion). It contains several protein complexes, and is

about 20% lipid and 80% protein. The intermembrane space between the two membranes contains enzymes that use ATP to phosphorylate other nucleotides and that catalyze other reactions. The matrix contains soluble enzymes that catalyze the oxidation of pyruvate and other small organic molecules. The matrix also contains several copies of the mitochondrial DNA (usually 5-10 circular DNA molecules per mitochondrion), as well as mitochondrial ribosomes, tRNAs, and proteins needed for DNA replication. When the cell divides, mitochondria replicate by fission. They also replicate if the long-term energy demands of a cell increase (Lodish, 2000).

Mitochondrial proteins are found on the outer membrane, the inner membrane, or the intermembrane space. Stop-transfer sequences anchor proteins to the outer membrane. Matrix-targeting sequences target the protein for the mitochondrial matrix. (Weber-Lotfi et al., 2002).

Mitochondria convert the potential energy of food molecules into ATP. The production of ATP is achieved by the Krebs cycle, electron transport and oxidative phosphorylation. Without oxygen, these processes cannot occur. The energy from food molecules (e.g., glucose) is used to produce NADH and $FADH_2$ molecules via glycolysis and the Krebs cycle. The protein complexes in the inner membrane (NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase) that perform the transfer use the released energy to pump protons (H^+) against a gradient (the concentration of protons in the intermembrane space is higher than that in the matrix). An active transport system (energy-requiring) pumps the protons against their physical tendency (in the "wrong" direction) from the matrix into the intermembrane space. As the proton concentration increases in the intermembrane space, a strong diffusion gradient is built

up. The only exit for these protons is through the ATP synthase complex. By transporting protons from the intermembrane space back into the matrix, the ATP synthase complex can make ATP from ADP and inorganic phosphate (P_i). This process is called chemiosmosis and is an example of facilitated diffusion (Kimball, 2005).

Mitochondria have several important functions besides the production of ATP. Disruptions in any of these functions corresponds to a variety of mitochondrial diseases. Some mitochondrial functions are performed only in specific types of cells. For example, mitochondria in liver cells contain enzymes that allow them to detoxify ammonia, a waste product of protein metabolism. These enzymes are not made in the mitochondria of cardiac cells. Mitochondria also play a role in the following: apoptosis; glutamate-mediated excitotoxic neuronal injury; cellular proliferation; regulation of the cellular redox state heme synthesis; steroid synthesis; and heat production (enabling the organism to stay warm) (Esposti et al., 2004).

Mitochondrial DNA

Mitochondrial DNA (mtDNA) is DNA which is located in the matrix of the mitochondria. MtDNA in animals consists of 5-10 rings of DNA per organelle, and consists of 16,500 base pairs with 37 genes (13 proteins, 22 tRNAs and two rRNAs) that are concerned with the production of proteins involved in respiration. However, the majority of proteins involved in respiration and other mitochondrial functions are coded by nuclear DNA (Lodish, 2000). MtDNA is typically passed on only from the mother during sexual reproduction, meaning that the mitochondria are clones. This infers that there is little change in the mtDNA from generation to generation, unlike nuclear DNA which changes by 50% each generation. Since the mutation rate is easily measured,

mtDNA is a powerful tool for tracking family lineage. The existence of mtDNA also supports the endosymbiotic theory (Margulis et al., 1981), which suggests that eukaryotic cells first appeared when a prokaryotic cell was absorbed into another cell without being digested. These two cells then are thought to have entered into a symbiotic relationship, forming the organelle. The existence of separate mtDNA suggests that, at one point, mitochondria were separate entities from their current host cells.

Homologous DNA recombination

Homologous recombination (crossing over and gene conversion) is an inherent part of meiosis. It is essential for DNA repair, and occasionally causes DNA aberrations in nuclei of eukaryotes. Homologous recombination is important in some organisms in the inheritance and stability of mtDNA which is continuously damaged by reactive oxygen species, by-products of respiration. The first direct demonstration of recombination in yeast mtDNA was provided by Shannon and his colleagues (Shannon et al., 1972).

In animals, the first direct demonstration of recombination in mtDNA was provided by Lunt and Hyman in the nematode *Meloidogyne javanica* (Lunt et al., 1997). Heteroplasmy in the control region of mtDNA of several other animals' species has also been attributed to non-homologous recombination (Rokas et al., 2003). The problem of whether recombinant mtDNAs are created in mammalian cells has been controversial for many years. Recently, rare creation of recombinant mtDNA haploptypes were also found by Sato (2005) in human somatic hybrid cells.

Recently, marked progress has been made in our interpretation of unusual variation in plants. One important advance came with the sequencing of the entire

mitochondrial genomes of liverwort (Oda et al. 1992) and Arabidopsis (Unseld et al. 1997). With these genomes elaborated, it is clear that much of the size variation can be accounted for by coding redundancy and changes in genome structure brought about by high levels of recombination and extraneous DNA integration.

Recombinationally active repeated sequences are present within the mitochondrial genomes of nearly all plant species examined, and in direct orientation, they subdivide the genome into a number of different, highly redundant, subgenomic molecules. A second class of repeat, much smaller in size and seldom active, can effect recombinations intragenically, resulting in novel open reading frames (Andre et al., 1992; Vedel et al., 1994; Mackenzie et al., 1999).

Evidence for homologous DNA recombination in plant mitochondria

Plant mitochondria have evolved distinct strategies for genome maintenance, genetic decoding, gene regulation, and organelle segregation. Mitochondrial genomes encode only a fraction of the genetic information required for their biogenesis and function; the vast majority is nuclear derived (Mackenzie et al., 1999). Higher plant mitochondrial genomes are 208 to 2000 kbp, they are much larger than mtDNA in vertebrates (16-17 kbp) and fungi (~25-80 kbp), and genes of higher plant mitochondria are dispersed throughout the genome (Schuster et al., 1994, Mackenzie et al., 1999). Plant mitochondria have some additional expressed genes compared with animal mitochondria, but most of the additional sequences in plants are not expressed and do not appear to be essential (Binder et al., 1996).

The mitochondrial genomes in yeast and higher plants exist as primarily linear and branched DNA molecules of various sizes (Backert et al., 1995, 1997a; Bendich et

al., 1990, 1996; Oldenburg et al., 1996, 2001), and less than 10% of the molecules are circular (Backert et al., 1997b; Bendich, 1996; Jacobs et al., 1996; Oldenburg et al., 1998, 2001; Scissum-Gunn et al., 1998). The branched molecules are very similar to recombination intermediates observed for yeast mtDNA (Sena et al., 1986) or with T4 phage DNA replication (Mosig, 1987, 1998). In addition, the visualization of replication and recombination intermediates in mtDNA from the plant *Chenpodium album* was reported by Backert and Borner (2000), and many of linear DNA molecules observed were found to have one single-stranded end. These ends may be involved in strand invasion.

Restriction mapping of yeast and plant mitochondrial genomes has resulted in circular maps for many species (Andre et al., 1992; Bendich, 1993; Fauron et al., 1992, 1995; Palmer and Shields, 1984). Most higher plant mitochondrial genomes contain at least one pair of large (>1 kbp) direct repeat, which may be involved in homologous recombination to generate subgenomic molecules (Andre et al., 1992; Palmer and Shields, 1984). Many smaller repeats, ranging in size from as little as nine to a few hundred base pairs, may also be involved in homologous recombination (Andre et al., 1992). It has been proposed that in plant mitochondria the large repeats are involved in reversible recombination, while the small repeats result in stable rearrangements (Andre et al., 1992; Mackenzie and McIntosh, 1999).

There are several reports of recombination between mtDNA molecules leading to rearrangement of the genome in a variety of plant species, including *Arabidopsis,* Maize, soybean, petunia (Fauron et al., 1992, 1995; Kanazawa et al., 1998; Kato et al., 1998; Klein et al., 1994; Marienfeld et al., 1997; Rothenberg and Hanson, 1988; Stadler and

Delph, 2002). In *Arabidopsis*, one example of recombination is the *atp6* gene, which is present in two copies, each coding for a preprotein that must be processed to generate the mature protein. Recombination at the protein processing site creates two different presequences, but each is removed precisely to produce active protein (Marienfeld et al., 1996). In *A. thaliana* mtDNA, there are large (6.5 and 4.2 kb) direct repeats and some smaller repeats (30- 560 bp, totaling 144 in number)(Unseld et al., 1997). There is some evidence that recombination across the large repeats occurs often and with no loss of gene function or any resulting rearrangements (Klein et al., 1994), while the small repeats may not be active in frequent recombination (Unseld et al., 1997).

Flower development in plants is mainly controlled by the nuclear genome. The well-known phenomenon of cytoplasmic male sterility (CMS), however, indicates a participation of extra-nuclear genes in flower formation. CMS plants are unable to produce fertile pollen and hence are male sterile. The maternal inheritance of the CMS trait clearly indicates that the genetic information of either the mitochondria or the plastids participates in certain steps of flower formation. Mitochondrial genes have been identified as being causally involved in all well-investigated CMS systems. In many cases of CMS, rearrangements in the mitochondrial genome were found to create unique chimeric genes, and often function of genes encoding subunits of the mitochondrial ATP synthase is impaired, suggesting that reduced mitochondrial respiration and ATP production lead to male sterility (Budar et al., 2001; Mackenzie et al., 1999; Schnable et al., 1998; Linke et al., 2003).

In soybean mitochondria, sequence repeats of 9, 23 and 299 bp have been characterized (Kanazawa et al., 1998; Kato et al., 1998). Genome sequence

rearrangements among various soybean cultivars have been characterized. It has been proposed that homologous recombination across these small repeats or across short elements that are part of a 4.9 kb PstI fragment of soybean mtDNA have led to the rearrangements observed (Chanut et al,. 1993; Grabau et al., 1992; Kanazawa et al., 1994 ; Moeykens et al., 1995; Hanlon and Grabau, 1997). The 299 bp repeat has been found in several copies in the soybean mitochondrial genome and in mtDNA from several other higher plants examined, suggesting that this sequence may represent a hot spot for mtDNA recombination in multiple plant species, similar to *chi* sequence in *E. coli* (Kato et al., 1998). There are three mitochondrial plasmids in various cultivars of broad bean, and additional variant plasmids have been characterized from one cultivar, where the variants appear to have arisen from active double recombination across repeated elements (Flamand et al., 1993). All these suggest that while some rearrangements have occurred in the distant past, active homologous mtDNA recombination also occurs in at least some plant species.

Some reports that individual mitochondria in tobacco and maize may have less than a full genome equivalent of DNA (Satoh et al., 1993; Small et al., 1989; Arimura et al., 2004) suggests the possibility of deletion of non-coding sequences by recombination between direct repeats of portions of some mtDNA molecules. Another possibility is that frequent and transient fusion of mitochondria could lead to exchange of mtDNA copies, leading to coexistence of mitochondria containing differing amounts of mtDNA within a single cell (Arimura et al., 2004). DNA sequence analysis of *Arabidopsis* and rice mtDNA (Unseld et al., 1997; Notsu et al., 2002) and portions of maize (Zheng et al., 1997) and turnip mitochondrial genomes has shown the presence of many remnants of

nuclear and chloroplast genes in plant mitochondria. The mechanism(s) by which these sequences have entered the mitochondria and integrated into mtDNA is not clear, but these remnants show very high homology (more than 90%) with only a small portion of a functional gene. This suggests that rearrangements and deletion of unessential non-coding sequences may have occurred, likely by recombination.

A significant amount of single-stranded DNA has been observed in mtDNA preparations from various plant species (Backert et al., 1997a; Bendich, 1996) and from yeast (Maleska, 1993). Single-stranded regions of DNA molecules may be directly involved in strand-exchange steps of DNA recombination and may be a result of DNA unwinding after introduction of nicks by either a random or sequence-specific process.

RecA in DNA recombination

One method by which genetic variation is generated is homologous recombination. In this process, two double stranded DNA molecules exchange segments of DNA at sites of sequence similarity through breakage and rejoining of strands, leading to recombinant chromosomes with new combinations of alleles at various genetic loci. Homologous recombination occurs in diverse organisms in different contexts. According to one model for homologous recombination, a nick in one of the DNA double helices (catalyzed by the RecBCD oligomeric protein in *E. coli*) allows a region of single stranded DNA to invade a double stranded DNA helix (Taylor et al., 1995). The intruding strand displaces one of the helix strands and binds to the other via Watson-Crick basepairing. The regions of DNA involved in the hetero duplexes enlarges through a process called branch migration. Such "Holliday junctions" are resolved by resolvase (RuvC in *E. coli*) and ligase, leading to recombinant chromosomes in 50% of the resolved

products (Ariyoshi et al., 1994).

The RecA protein is a critical enzyme in this process, as it catalyzes the pairing of ssDNA with complementary regions of dsDNA. The RecA monomers first polymerize to form a helical filament around ssDNA (Story et al., 1992). During this process, RecA extends the ssDNA by 1.6 angstroms per axial base pair. Duplex DNA is then bound to the polymer. Bound dsDNA is partially unwound to facilitate base pairing between ssDNA and duplexed DNA. Once ssDNA has hybridized to a region of dsDNA, the duplexed DNA is further unwound to allow for branch migration. RecA has a binding site for ATP, the hydrolysis of which is required for release of the DNA strands from RecA filaments. ATP binding is also required for RecA-driven branch migration, but non-hydrolyzable analogs of ATP can be substituted for ATP in this process, suggesting that nucleotide binding alone can provide conformational changes in RecA filaments that promote branch migration (Alberts et al., 1994).

Single strand DNA-binding proteins

Single strand DNA-binding (SSB) proteins participate in DNA strand separation during replication though they do not catalyze strand separation. They selectively bind to single-stranded DNA as soon as it forms and coat it so it cannot anneal to reform a double helix (Weaver, 2002). The single-stranded DNA can form by natural "breathing" (transient local separation of strands, especially in A-T rich region) or as a result of helicase action, then SSB protein binds and keeps it in single-stranded form. The best studied SSBs are prokaryotic. In *E. coli*, SSB is the product of the *ssb* gene. SSBs aid helicase action by binding tightly and cooperatively to newly formed single-stranded DNA and keep it from annealing with its partner. By coating the single-stranded DNA,

SSBs also protect it from degradation. They also stimulate DNA polymerases. These activities make SSBs essential for prokayotic DNA replication (Weaver, 2002).

DNA strand exchange

DNA strand exchange is a process whereby a single strand of DNA is exchanged between two homologous double-stranded DNA molecules. The *E. coli* RecA protein was the first recovered RecA enzyme, and is a multifunctional enzyme required for homologous recombination and a variety of cellular responses to DNA damage. RecA protein plays a critical role in promoting pairing and strand exchange between DNA molecules in an ATP-dependent reaction (Shan et al., 1997). The mechanism is complex and proceeds in three steps: presynapsis (Fig. 1a), synapsis (Fig. 1b), and branch migration (post-synapsis) (Fig. 1c) (Weaver, 2002). In the presence of ATP, RecA binds on ssDNA to form a presynaptic nucleoprotein filament. During synapsis, the nucleoprotein filament binds and pairs with homologous dsDNA to form joint molecules. The first contacts are between non-homologous regions, resulting in the formation of a large network of molecules that contributes to acceleration of pairing. This is followed by homologous alignment and formation of joint molecules, with limited unwinding of the dsDNA. Finally, homologously aligned molecules undergo a unidirectional, RecA-mediated extension of the heteroduplex region that results in strand exchange (Friedman-Ohana et al., 1998).

DNA helicase and plant helicase

Helicase is an enzyme that unwinds double-stranded DNA into two single strands. DNA helicases bind to DNA strands and, through conformational changes caused by ATP hydrolysis, break the hydrogen bonds between the bases of double-stranded DNA.

This activity gives helicases an important role in many cellular processes including genome replication, recombination, repair, and transcription (Weaver, 2002). The helicase protein family is universally distributed and numerous recognizable sequence motifs have been discovered in eukaryotic, bacterial and viral genomes. Some of the helicase enzymes share features with ATP synthases.

Helicases can be divided into two classes on the basis of mechanism: those that translocate in a 5'-3' direction along single-stranded DNA and those that operate with the opposite polarity. DNA helicases are molecular motor proteins that use the energy of nucleoside 5'-triphosphate (NTP) hydrolysis to open transiently the energetically stable duplex DNA into single strands and thereby play essential roles in nearly all DNA metabolic transactions. After the discovery of the first prokaryotic DNA helicase from *E. coli* in 1976 and the first eukaryotic one from the lily plant in 1978, many more have been isolated and characterized, including at least eight from plants. All DNA helicases share some common properties, including nucleic acid binding, NTP binding and hydrolysis and unwinding of duplex DNA in the 3' to 5' or 5' to 3' direction. In plants, DNA helicases are present in nuclei, mitochondria and chloroplasts (Tuteja, 2003).

The *in vivo* role of many DNA helicases has not been well investigated in eukaryotic systems, including plants. However, through indirect evidence, the involvement of plant DNA helicases has been suggested at least in the following biological processes: DNA recombination, DNA replication, translation initiation, rDNA transcription and in the early stages of pre-rRNA processing, double-strand break repair, maintenance of telomeric length, nucleotide excision repair, cell division/proliferation during flower development, maintenance of genomic methylation patterns, the plant cell

cycle, and in the maintenance of the basic activities of cells. A recently discovered Helitron insertion in the maize genome has suggested the possible role of plant DNA helicase(s) in a new class of rolling-circle transposons (Eckardt et al., 2003). All these reflect that plant DNA helicases may play an important role in plant growth and development.

Helicases in DNA replication

Replication begins at a specific site in the DNA called the origin of replication. DNA helicases cause the two parental DNA strands to unwind and separate from one another in both directions at this site to form two "Y"-shaped replication forks (http:// student.ccbcmd.edu/courses/bio141/lecguide/unit4/genetics/DNArep/fg13a.html. Dr. Kaiser's website) (Fig. 2). These replication forks are the actual sites of DNA copying. During replication within the fork, helix-destabilizing proteins bind to the single-stranded regions and prevent the strands from rejoining. Helicases unwind duplex DNA and provide single-strand template. Multiple DNA helicases are present in a cell because of the different structural requirements of the substrates at various stages of repair, replication and recombination.

Thesis objective

Genes encoding some higher plant mitochondrial proteins involved in DNA recombination and replication have been characterized by our lab. To demonstrate that higher plant mitochondria contain homologous activities with *E. coli* RecA, SSBs and DNA helicase, I purified soybean mitochondria from young soybean leaves and used partially purified protein fractions to conduct DNA strand invasion and helicase assays. The DNA strand invasion assay was used to test for strand exchange activity in soybean

mitochondria. A helicase assay was used to test the double-strand DNA unwinding activity of soybean mitochondria protein. The details and the results of these assays are described in the following chapters.

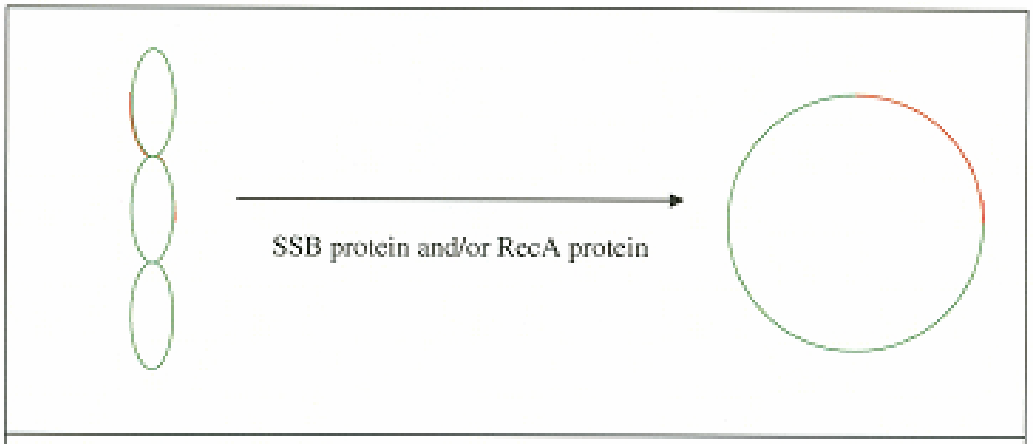

Figure 1a. Presynapsis. SSB protein and/or RecA protein coats single-stranded DNA, removing secondary structure.

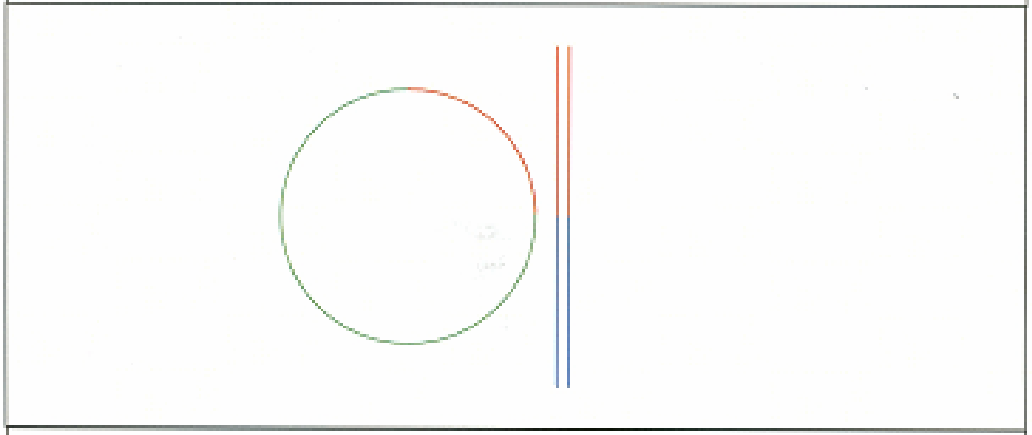

Figure 1b. Alignment of Complimentary Sequences.

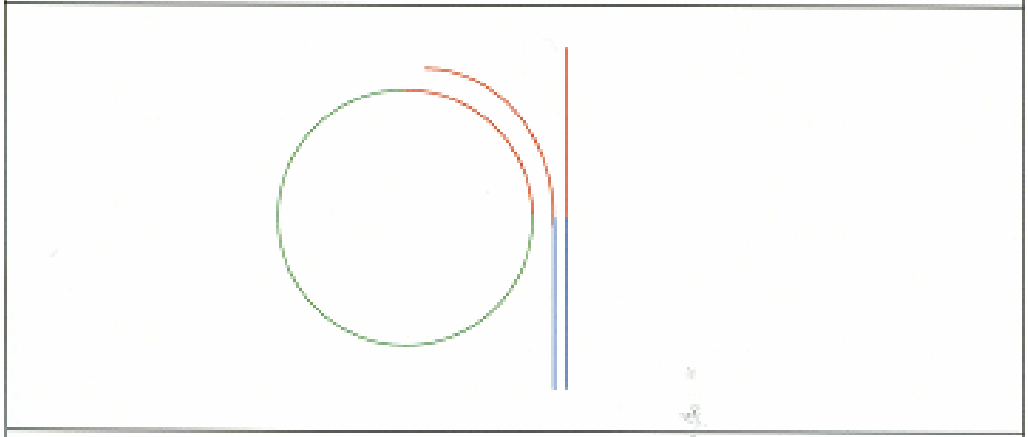

Figure 1c. Strand Exchange. Formation of the joint molecule.

Fig. 1 DNA strand exchange 1a. is presynapsis; 1b. is synapsis; 1c. is postsynapsis, or strand exchange

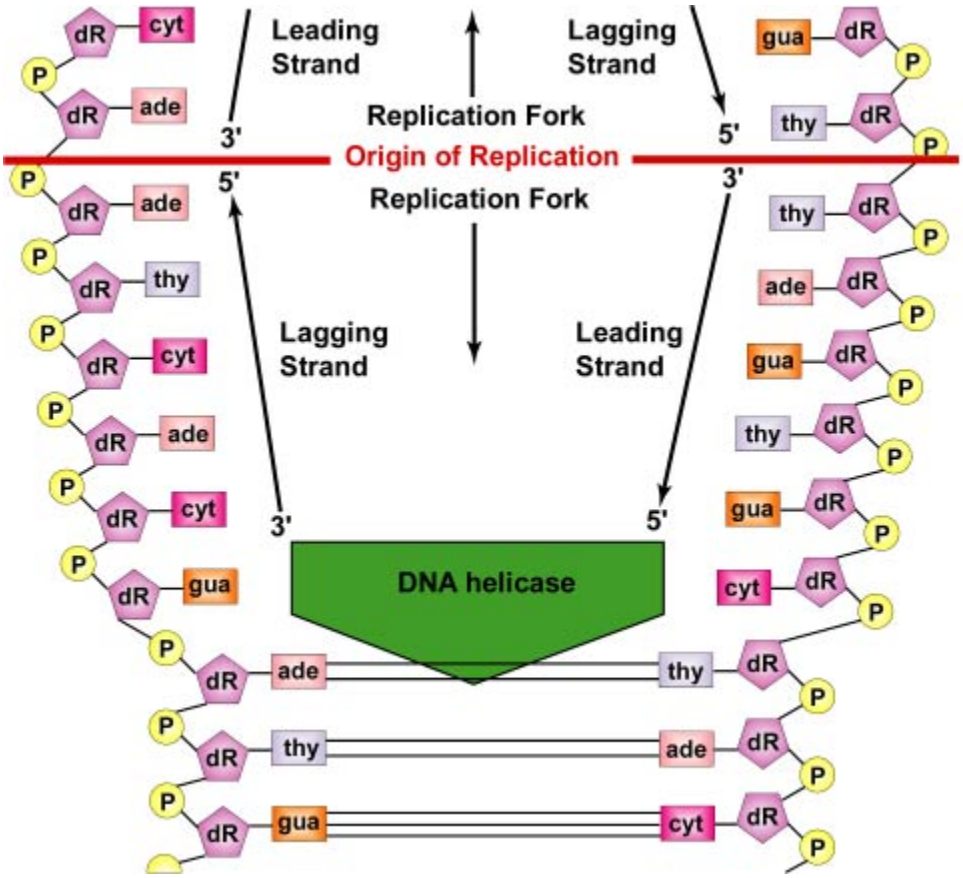

Fig. 2. Unwinding by DNA Helicase. (Permitted to site by Dr. Kaiser from his Microbiology Website http://student.ccbcmd.edu/courses/bio141/lecguide/unit4/genetics/DNA/DNArep/fg13a.html)

Chapter 1

Homologous DNA Strand Exchange Activity in Higher Plant Mitochondria

Introduction

Homologous recombination is critical for rearrangement of DNA and is essential to life. In this process two homologous DNA duplex molecules interact, resulting in exchange of genetic information and rearrangement of DNA fragments. Recombination can provide organisms essential genetic variation for adaptation as environmental conditions change. In bacteria such as *E. coli*, homologous recombination is dependent on the Recombinase A (RecA) protein to catalyze exchange between homologous DNA strands. The RecA protein also plays a major role in response to DNA damage. In eukaryotes, RecA homologues of the Rad51 and Dmcl groups of proteins have been implicated in meiotic recombination and are also associated with DNA repair (Shinohara et al., 1999).

The single-stranded DNA-binding (SSB) protein plays a transient but essential role in DNA replication, homologous recombination, and recombination repair. SSBs participate in DNA strand separation during replication. They bind selectively to single-stranded DNA as soon as it forms and coat it to prevent it from annealing again to form a double helix. The single-stranded DNA can form by natural “breathing” (transient local separation of strands, especially in A-T rich regions) or as a result of helicase action, and then SSB binds and keeps it in single-stranded form.

In the presynapsis step of recombination, RecA protein coats single-stranded DNA that will participate in a recombination reaction. Although the SSB protein is not

absolutely required at this step, it stimulates the recombination process, apparently by melting secondary structure and preventing RecA from trapping secondary structure that would inhibit strand exchange later in the recombination process.

Synapsis occurs when a complementary sequence in single-stranded DNA finds a homologous region in double-stranded DNA and aligns with it. During postsynapsis (strand exchange), RecA and ATP collaborate to promote strand exchange between a single-stranded and double stranded DNA. ATP is necessary to clear RecA off the synapsing DNAs to make way for formation of double-stranded DNA involving the single strand and one of the strands of the DNA duplex (Weaver 2002). The intermediate of this reaction is called a joint molecule. The SSB protein may play an important role in this step. The SSB protein binds to displaced strands to avoid reannealing. It also maintains the stability of the RecA protein/single-stranded DNA complex (Meyer et al., 1990).

The recombination mechanism of RecA and involvement of SSBs in prokaryotes are well characterized. RecA homologues also have been identified in many eukaryotes, including plants. However, in eukaryotes almost all of the information about homologous recombination and its associated proteins are limited to the nucleus. There is very little information about homologous recombination or related activity in plant organelles, such as chloroplasts or mitochondria.

Mitochondria are the “power plants” of both animal and plant cells. They generate the essential energy for metabolic processes in cells. The genomes of mitochondria are separate from the nuclear genome of the cell, but many proteins vital for mitochondrial functions are encoded in the nucleus. These proteins are translated in

the cytoplasm and then the encoded proteins are transported into the mitochondria, where they can assemble with other proteins in order to carry out their intended functions (Binder et al., 1996).

Mitochondrial DNA (mtDNA) molecules undergoing recombination have been identified in yeast (Foury and Lahaye, 1987; MacAlpine et al.,1998; Manna et al., 1991). Studies with a yeast nuclear gene mutation that abolishes mtDNA recombination show its necessity for stable inheritance of yeast mtDNA (Ling et al., 1995). Even though there is some evidence for DNA recombination in plant organelles, the mechanisms are not clear. The evidence for mtDNA recombination activity in yeast (MacAlpine et al., 1998) and mammalian recombinant plasmid (Thyagarajan et al., 1996) along with the observed rearrangements in plant mitochondrial genomes lead to the hypothesis that plant mitochondria should also contain recombination and strand exchange activity. This chapter reports on the characterization of homologous DNA strand exchange in higher plant mitochondria.

Materials & Methods

Isolation of Total Soybean Mitochondria

Approximately 500 grams of 8-9 day old soybean plants were homogenized in 150-200 g batches with 2-3 volumes (ml/g) of cold STM buffer (0.5 M sucrose, 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.2 mM PMSF) in an ice cold blender jar with three 5 sec bursts (Daniell et al., 1995). Subsequent steps were carried out at 4°C. The resulting homogenate was filtered through 4 layers of cheesecloth and 3 layers of Miracloth (Calbiochem). The filtrate was centrifuged at 2500 rpm in a JA-14 (Beckman) rotor for 20 min to remove chloroplasts and nuclei, and the resulting supernatant was centrifuged at 12000 rpm in a JA-14 rotor for 30 min to pellet mitochondria. The pellets were resuspended in a total of 200 ml cold STM buffer, both centrifugation steps were repeated, and the final pellets were resuspended for further purification by a Percoll gradient. The resulting sample was layered on top of a discontinuous Percoll gradient consisting of the following steps: 5 ml each of 15%, 22%, and 27%; and 7 ml 60% (v/v) Percoll solution. 15%, 22%, 27%, and 60% Percoll solutions were made of Percoll (Amersham Biosciences), grinding buffer (0.35 M mannitol, 50 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% BSA (w/v)) and dH_2O. The Percoll gradient was centrifuged at 26,500x g (Beckman JS-13.1 rotor) for 45 minutes. Intact mitochondria appeared as a white or buff band just above the 60% interface. Mitochondria were collected, diluted 3-fold with grinding buffer and washed to remove excess Percoll by centrifuging at 17,000 x g (Beckman JS-13.1 rotor) for 20 minutes. The mitochondrial pellet was then resuspended and washed a second time in grinding buffer by centrifuging at 17,000 x g (Beckman JS-13.1 rotor) for another 20

minutes. The second pellet was resuspended in 10 ml dH_2O with 0.1% TRITON X-100 (Sigma Chemical) and incubated on ice for 20 minutes to lyse mitochondria to get more mitochondria from cells. Lysed mitochondria were centrifuged at 15,000 x g (Beckman JA-20) for 20 minutes. The pellets were discarded and the supernatant was used for chromatography.

DNA Affinity Chromatography

The supernatant was loaded onto equilibrated DEAE cellulose column (~ 70 cm^3 column volume) at 0.3 ml/min using a Biologic LP chromatography system (Bio-Rad). The column was washed with 500 ml buffer A (50 mM Tris-HCl pH 8.0, 50 mM β·mercaptoethanol, 0.2 mM PMSF, 20% glycerol, 50 mM NaCl) at 0.50 ml/min and then eluted with 60 ml buffer A with 0.6 M NaCl at 0.33 ml/min. Selected elution fractions were collected, pooled and dialyzed in buffer A overnight with 2 buffer changes. The dialyzed proteins were then loaded onto an equilibrated heparin sepharose column (~5 cm^3 column volume) at 0.3 ml/min. The column was washed with 50 ml of buffer A at 0.33 ml/min. The bound proteins were eluted with 30 ml buffer A with 0.6 M NaCl at 0.5 ml/min. The fractions were collected and tested for strand invasion activity.

DNA Substrates

M13 replicative form (RF) dsDNA and M13 single-stranded DNA were prepared following the protocol of "Molecular Cloning- A Laboratory Manual" Volume 1 (Sambrook and Russell, third edition). The pUC 19 DNA was isolated from *E. coli* (*dam+*, *dcm+*) by ion exchange chromatography. The prepared M13 RF dsDNA, single-stranded DNA, and pUC 19 DNA were quantified by spectrophotometer (BioRad, SmartSpec 3000). Replicative form DNA was linearized by BamHI (BioLab) and

separated in an agarose gel. The linear dsDNA was extracted by the QIAquick kit (QIAGEN). The pUC 19 single-stranded circular DNA was prepared by digesting double stranded circular pUC 19 with PstI and EcoRI (BioLab), then treating with exonuclease III (TakaRa) to obtain linear single-stranded DNA. The DNA substrates were stored in TE buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA).

Soybean MtDNA Strand Invasion Assay

Protein fractions were assayed for homologous strand invasion activity as follows. Strand-exchange reactions containing 30 mM Tris-acetate (pH 7.5), 2 mM DTT, 50 μg/ml BSA, 20 mM magnesium acetate, 2.5 mM ATP, 15 μl protein fraction (for control, added 2 μg *E. coli* RecA (New England BioLab)), 15 μM ssDNA and 15 μM RF dsDNA, 1.8 μg *E. coli* single-stranded binding protein (SSB) (Promega) and dH_2O to 25 μl final volume. In the first step of the reaction, Tris-acetate pH 7.5, DTT, BSA, $MgCl_2$, ATP, protein fraction (RecA for control), and dH_2O were combined and incubated at 37°C for 5 minutes. In the second step, ssDNA was added and incubation continued at 37°C for 10 minutes. In the third step, SSB was added with incubation at 37°C for another 10 minutes. For the fourth step, RF dsDNA was added and the samples incubated at 37°C for 60 minutes. Stop solution (0.5% SDS, 50 mM EDTA) was added followed by incubation for 5 min at 37°C and 65°C for another 5 min. The samples were then electrophoresed in 1% agarose gels at 12V for 17 hours with 5 μl 10X loading buffer. The gels were checked on the BioRad image analysis system after staining by ethidium bromide (Fisher Biotech).

Southern Blotting

Agarose gels containing separated soybean mitochondrial DNA strand invasion

reaction products were soaked in 0.25 M HCl for 8 min. Then the gels were soaked in 0.5M NaOH for 25 min to denature DNA. A vacuum blot apparatus was used to transfer DNA from the gel to nylon membrane. After DNA was transferred, the membrane was treated with UV light at 150 mJ (GS Gene Linker, BioRad) to crosslink the DNA to the membrane. The blots were hybridized overnight with biotin-labeled pUC 19 ssDNA probe prepared using the BioPrime DNA Labeling System (Invitrogen) following the manufacturer's protocol, using PerfectHyb Plus hybridization buffer (Sigma). After performing low and high stringency washes at 65^{o}C, hybridization was visualized using the North2South Chemiluminescent Nucleic Acid Detection Kit (Pierce) following the manufacturer's protocol. X-ray film was exposed to the blot for different times from 1 second to 30 minutes.

Results

DNA strand exchange assay with mitochondrial protein purified from soybean

Genetic variation is generated by homologous recombination. During *E. coli* recombination, single-stranded DNA invades homologous regions in double strand DNA. RecA protein is a critical enzyme in this process. It helps the invading strand pair with its complementary strand in a homologous dsDNA, forming a D-loop or other joint molecules. In the DNA strand invasion assay, these D-loops or joint molecules can be visualized as bands with altered mobility in agarose gels to demonstrate strand invasion activity. In Fig. 3, M13 single-stranded DNA and M13 replicative form double stranded DNA were used as DNA substrates for a strand invasion assay with *E. coli* RecA and partially purified soybean mitochondria protein fractions. Soybean mitochondria proteins were partially purified by ion exchange and affinity chromatography to remove nucleases and other unwanted protein and to concentrate the strand invasion activity. Elution of the column was monitored by A_{280} to determine peak protein fractions. These fractions were assayed for strand invasion activity. After the reaction, the products were loaded in a 1% agarose gel. Bands representing joint molecules were observed for some fractions (Fig. 3). Reaction products showed similar joint molecule bands with the *E. coli* RecA control reaction. In some lanes the circular ssDNA and linear ssDNA bands disappeared, suggesting there are nucleases in some soybean mitochondria protein fractions. These results indicated that soybean mitochondria contain homologous strand exchange activity analogous to *E. coli* RecA.

Mitochondria strand exchange assay requirement for ATP and Mg^{2+}

In homologous recombination, *E. coli* RecA requires ATP for branch migration

and release from the ssDNA after pairing with complementary dsDNA. RecA also needs Mg^{2+} for proper conformation. For testing whether soybean mitochondria is ATP and Mg^{2+} dependent, different assay conditions were tested for the strand invasion assay by using the peak soybean mitochondria protein fraction. In this assay, M13 single-stranded DNA and M13 double-stranded DNA were used as the substrates. For more sensitivity to detect the strand invasion activity, DNA from the gel was transferred to nylon membrane. Southern blot hybridization result using pUC 19 probe shows in the absence of ATP (Fig. 4 lane 6), the absence of Mg^{2+} (Fig. 4 lane 7), or absence of both (Fig. 4 lane 8), the bands of joint molecules disappeared (lane 7 and lane 8) or were significantly reduced (lane 6). In lane 6, the faint band that remains may be due to the presence of some ATP in the protein fraction.

Soybean mitochondria DNA strand exchange activity is stimulated by mtSSB protein

SSB participates in DNA strand separation during recombination. It selectively binds to single-stranded DNA as soon as it forms and coats it, so it cannot anneal to reform a double helix. It has also been shown to stimulate bacterial RecA activity. The *Arabidopsis* mtSSB protein expressed in *E. coli* and purified by our lab (Edmondson et al., 2005) was tested for its ability to stimulate the strand invasion activity of *E. coli* RecA. MtSSB protein showed similar efficiency with the *E. coli* SSB protein for stimulating RecA activity *in vitro* (Fig. 5, lanes 4, 5, 6), leading to more distinct joint molecules as the amount of mtSSB was increased. Figure 5 also shows that only in lane 3 and lane 6 there are single-stranded DNA bands and strong joint molecule bands, indicating that mtSSB causes stimulation of RecA strand invasion activity. In lane 8, no

mtSSB was added, but there are still some smeared bands, suggesting incomplete strand invasion activity even though no SSB was added.

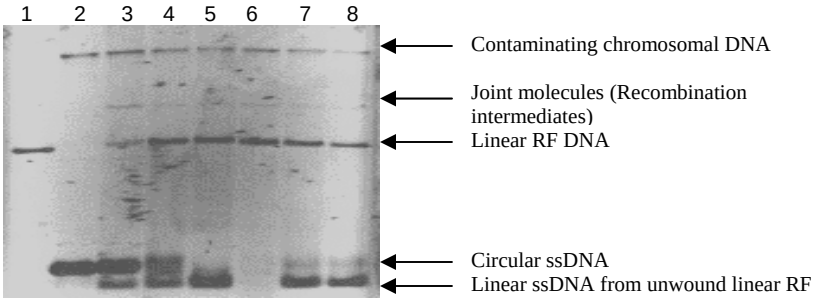

Fig. 3 Results of M13 RF dsDNA and ssDNA strand invasion assay with different protein fractions. Lane 1 contains RF dsDNA alone, lane 2, ssDNA; lane 3, positive control with *E. coli* RecA; and the other lanes are reaction products using soybean proteins from different fractions of the heparin sepharose column. Protein fractions in lanes 4 to 8 show DNA strand exchange activity. Because there may be some nucleases left in fraction lane 5 and 6, the bands of circular ssDNA and the bands of linear ssDNA from unwound linear RF disappeared.

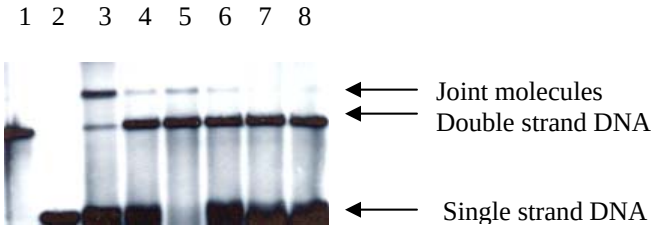

Fig. 4 Soybean mitochondrial strand invasion assay with or without ATP and Mg^{2+}. (Southern blot result) Lane 1: RF double stranded DNA only; lane 2: single stranded DNA only; lane3: RecA control; lane 4: Non-concentrated mitochondrial protein; lane 5: concentrated mitochondrial protein; lane 6: No ATP; lane 7: No Mg^{2+}; lane 8: No ATP or Mg^{2+}. In this experiment, soybean mitochondria protein with peak strand invasion activity was used.

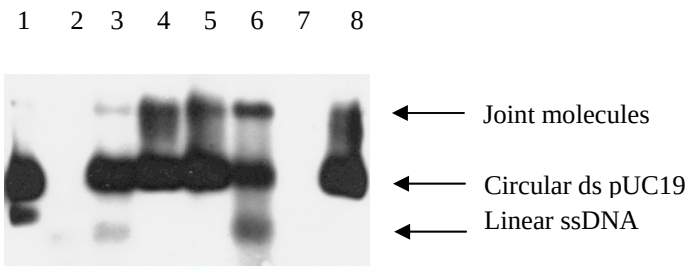

Fig. 5 Stimulation of *E. coli* RecA-catalyzed homologous strand exchange activity by SSB. Reactions were done with double-stranded circular pUC19 DNA and linear single-standed pUC19 and commercially available *E. coli* RecA (5 ug/reaction). For more sensitive detection of strand invasion activity, DNA was transferred to membrane. This figure is the result of Southern blot hybridization using a pUC19 probe. Lane 1, dsDNA only. Lane 2, ssDNA only. Lane 3, reaction with addition of *E. coli* SSB. Lane 4, reaction with addition of 1.2 µg purified mtSSB. Lane 5, reaction with 3.6 µg mtSSB. Lane 6, reaction with 7.2 µg mtSSB. Lane 7, no sample. Lane 8, reaction with no SSB. In this strand invasion assay, soybean mitochondria protein with strongest strand invasion activity was used.

Discussion

The strand exchange activity of RecA can be analyzed by several different assays (Shibata et al., 1979; McEntee et al., 1980; Konforti and Davis. 1987; Griffith and Harris, 1988; McCarthy et al., 1988). In the strand invasion assay, invading ssDNA from one molecule pairs with its complementary recipient in dsDNA, thereby displacing the noncomplementary strand of the acceptor DNA molecule and forming a joint molecule (Cerutti and Jagendorf, 1993). We detected strand exchange activity of soybean mitochondria in vitro based on two assays of this general mechanism. Two pairs of substrates, M13 RF double-stranded linear DNA, M13 circular single-stranded DNA and pUC 19 super coiled double-stranded DNA, pUC 19 linear single-stranded DNA were used for the strand invasion assays. Both the *E. coli* RecA and soybean mitochondrial protein catalyze pairing of ssDNA with complementary regions of dsDNA to form D-loop or joint molecules. When reaction products were analyzed in agarose gels, very similar bands with slower mobility than substrates were observed, which indicated soybean mitochondria have homologous strand invasion activity similar to *E. coli* RecA. The strand invasion activity detected may function in mtDNA recombination involved in DNA replication and/or DNA repair.

As mentioned earlier, *E. coli* RecA is ATP dependent. During DNA recombination, *E. coli* RecA utilizes ATP and Mg^{2+} to facilitate the ssDNA from one molecule to invade its complementary recipient in dsDNA and form a joint molecule. When ATP was absent from the in vitro strand invasion assay with soybean mitochondrial protein, the joint molecule band decreased significantly but not totally. This result indicates that, like *E. coli* RecA, the soybean mitochondria strand invasion

activity is ATP dependent. There appears to be a small amount ATP left in the soybean mitochondria protein fraction. When Mg^{2+} or both ATP and Mg^{2+} were absent from the in vitro reaction, the joint molecule bands totally disappeared. These results provide evidence that the formation of joint molecules by soybean mitochondrial extracts also requires Mg^{2+}, and ATP, analogous to *E. coli* RecA.

Increasing amounts of mtSSB added to in vitro reactions with RecA significantly stimulated strand invasion activity. At high level of mtSSB, a very clear band representing joint molecules is observed. A low level of strand invasion activity is present in the absence of SSB due to the basal level of activity common for RecA proteins. However, there is some unresolved DNA between the circular DNA and joint-molecule band, indicating incomplete strand exchange. These results are similar to the reports by Steffen and Bryant (2000) for the ability of an SSB protein from *Streptococcus pneumoniae* to stimulate the activity of *E. coli* RecA.

The specific pathways that are used by soybean mitochondria for incorporation of ssDNA via recombination are not very clear, but are likely to include an initial invasion of the ssDNA into a homologous region within the duplex chromosome and a subsequent branch migration reaction to bring about an exchange of DNA strands. All these reactions can be catalyzed by soybean mitochondrial proteins, and mtSSB proteins should be important accessory factors for strand exchange. With highly purified soybean mitochondrial proteins and mtSSB proteins, it is possible to analyze the molecular steps involved in plant mitochondrial recombination pathways in detail.

Chapter 2

DNA Helicase Activity in Soybean Mitochondria

Introduction

DNA replication, repair and recombination are critical processes for living organisms and are generally carried out by complex collections of interacting proteins (Lee et al., 1998). The mechanism of DNA replication has been well defined in bacteria, bacteriophages, viruses, plasmids, and yeast. Studies of DNA replication in higher plants have also been conducted in chloroplasts and mitochondria. A number of proteins are involved in this process, including DNA polymerase, origin binding proteins, SSB, and helicase. DNA helicases catalyze the unwinding of double stranded DNA to provide single-stranded templates for DNA replication, repair and recombination utilizing the energy provided by the hydrolysis of the γ–phosphate of ATP (Geider et al., 1981).

DNA helicases have been isolated from a number of organisms, including *E. coli*, its bacteriophages, eukaryotic viruses, yeast, lily, pea, *Xenopus*, mouse, cow, and human. In plants, some DNA helicases have been isolated from pea and soybean chloroplasts (Tuteja et al., 1998). However, mitochondrial DNA helicases which are involved in DNA recombinations have only been found in yeast and mammals (Hehman and Hauswirth, 1992; Foury and Lahaye, 1987). To date, no mtDNA helicase from higher plants has been reported.

In this thesis, characterization of an mtDNA helicase from soybean is reported. Soybean mitochondrial DNA helicase has the activity to unwind duplex DNA in an ATP- and Mg^{2+}-dependent manner. Soybean mitochondrial DNA helicase shows 3’ to

5’polarity for unwinding. The optimal conditions for soybean mtDNA helicase activity are also characterized.

Materials & Methods

Purification of the Soybean Mitochondrial DNA Helicase

All purification steps were carried out at 0-4^{o}C. Mitochondrial protein was isolated from approximately 500 grams of seven day old fresh soybean leaves. The leaves were homogenized in 150-200g batches with 2-3 volumes (ml/g) of cold STM buffer (0.5 M sucrose, 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.2 mM PMSF) in an ice cold blender jar with three 5 second bursts (Daniell et al., 1995). The resulting homogenate was filtered through 4 layers of cheesecloth and 3 layers of Miracloth (Calbiochem). The filtrate was centrifuged at 2500 rpm in a JA-14 (Beckman rotor) for 20 min to remove chloroplasts and nuclei, and the resulting supernatant was centrifuged at 12000 rpm in a JA-14 rotor for 30 min to pellet mitochondria. The pellets were resuspended in a total of 200 ml cold STM buffer, both centrifugation steps were repeated, and the final pellets were resuspended in STM Buffer.

The resulting sample was layered on top of a discontinuous Percoll gradient consisting of the following steps: 5 ml each of 15%, 22%, and 27%; and 7 ml 60% (v/v) Percoll solution. For each step, the volume was adjusted to 22 ml with Percoll (Amersham Biosciences), Grinding buffer (0.35 M mannitol, 50 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0 and 0.2% BSA(w/v)), and dH_2O. The Percoll gradient was centrifuged at 26,500 x g (Beckman JS-13.1 rotor) for 45 minutes. Intact mitochondria appeared as a white band just above the 60% interface. Mitochondria were collected, diluted 3-fold with Grinding buffer and washed to remove excess Percoll by centrifuging at 17,000 x g (Beckman JS-13.1 rotor) for 20 minutes. The mitochondrial pellet was resuspended and washed a second time in Grinding buffer by centrifuging at 17,000 x g

(Beckman JS-13.1 rotor) for another 20 minutes.

The second pellet was resuspended in 10 ml dH_2O containing 0.1% TRITON X-100 (Sigma Chemical) and incubated on ice for 20 minutes to lyse the mitochondria. Lysed mitochondria were centrifuged at 15,000 x g (Beckman JA-20) for 20 minutes. The pellets were discarded and the supernatant was saved on ice for chromatography.

Total mitochondrial proteins were loaded onto equilibrated DEAE cellulose column (~ 70 cm^3 column volume) at 0.3 ml/min using a Biologic LP chromatography system (Bio-Rad). The column was washed with 500 ml buffer A (50 mM Tris-HCl pH 8.0, 50 mM β· mercaptoethanol, 0.2 mM PMSF, 20% glycerol, 50 mM NaCl) at 0.50 ml/min and then eluted with 60 ml buffer A with 0.6 M NaCl at 0.33 ml/min. The eluted fractions were pooled in molecularporous membrane tubing (Spectrum Laboratories, Inc.) and dialyzed in buffer A overnight with 2 buffer changes.

The dialyzed proteins were loaded onto an equilibrated heparin sepharose column (~5 cm^3 column volume) at 0.3 ml/min. The column was washed with 50 ml of buffer A at 0.33 ml/min. The bound proteins were then eluted with 30 ml buffer A with 0.6 M NaCl at 0.5 ml/min. The fractions were collected and tested for DNA helicase activity.

Western analysis to confirm purity of mitochondria

Duplicate gels were prepared, one for staining and one for transfer of proteins to membranes for Western analysis. Prestained molecular weight markers were included for size determinations. Electrophoresis was carried out for 90 min at 125 V. One gel was stained in Commassie Blue for 1 hr, followed by destaining to visualize proteins. Proteins from the other gel were transferred to a PVDF membrane using a semi-dry electroblotter (BioRad).

Membranes were blocked in TBS (50 mM Tris-HCl pH 7.4, 200 mM NaCl) containing 5% nonfat dry milk for 1 hr at room temperature. For one membrane, the antiserum against mtRecA (Khazi et al., 2003) was diluted 1:2000 in 20 ml of blocking buffer, and the membrane incubated in this solution with gentle shaking overnight at 4^{o}C. For another membrane, polyclonal antibody against Chlorophyll a/b binding protein (Rose Biotechnology, 1:2000 dilutions) was used to monitor chloroplast contamination in the purified mitochondria fraction. After incubation with antibody, membranes were washed three times (for 15 min each) with TBS, the second wash containing 0.1% Tween 20. Membranes were blocked again as above, followed by incubation for 1hr with a 1: 2000 dilution of goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP; Promega). The membranes were washed three times as above, followed by incubation for 5 min in SuperSignal West Pico Substrate working solution (Piece). The membranes were wrapped in plastic wrap and exposed to X-ray film for 5 sec-5 min.

DNA oligonucleotides and nucleoside triphosphates

M13 single-stranded DNA was prepared following the protocol of "Molecular Cloning- A Laboratory Manual" Volume 1 (Sambrook and Russell, 3rd Ed.). NTPs were purchased from Sigma Chemical Company. [γ-^{32}P] ATP (111 TBq/mmol) and [α-^{32}P] dCTP (around 29.6 TBq/mmol) were purchased from PerkinElmer Life and Analytical Science. The oligonucleotides used to construct the DNA substrates were synthesized by Invitrogen. The sequences and details of oligonucletides are as follows: oligo 1, 17 nucleotides, 5'-GTAAAACGACGGCCAGT-3'; oligo 2, 32 nucleotides, 5'-TTCGAGCTCGGTACCCGGGGATCCTCTAGAGT-3', both complementary to M13mp19 DNA.

Preparation of DNA helicase substrates

The substrate consists of a partial DNA duplex of ^{32}P-labeled oligonucleotides (Oligo 1, 17 nucleotides, complementary to M13 ssDNA) annealed to M13mp19 ssDNA. To label oligonucleotides, the oligonucleotide was dissolved in dH_2O to 1 μg/μl, then diluted 1: 10, 3 μl added to an eppendorf tube, with addition of 5 μl 10X kinase buffer, 15 μl γ ^{32}P ATP (150 μCi), 25 μl dH_2O, and 2 μl T4 polynucleotide kinase (~ 20 units) (BioLab), and incubated 60 min at 37^{o}C. The product was filtered through a syringe with ~ 0.8 ml Sephadex G-50 (Amersham) by centrifuging at 2000 rpm using an IEC HN SII centrifuge (International Equipment Co.). The labeled oligonucleotide was annealed to M13mp19 ssDNA (2-4 μg) in 40 mM Tris-HCl, pH 7.8, 50 mM NaCl. The mixture was heated at 65^{o}C for 1hr to allow annealing, and then cooled slowly to room temperature (20-30 min). The substrate was purified by filtration through a syringe with ~ 0.8 ml Sephacryl S-300 (Amersham) with centrifugation at 2000 rpm for 5 min.

Preparation of direction specific substrates

The direction-specific substrates to determine polarity of helicase action were prepared in different ways (see Fig.14). For the 3'-to-5'-direction, oligo 2 (32 nucleotides, complementary to M13) was labeled at the 5'end, filtered by centrifugation through a Sephadex G-50 syringe and then annealed to M13mp19 ssDNA. The annealed substrate was filtered through a Sephacryl S-300 syringe and then digested with SmaI (BioLabs) following the manufacturer's protocol. For the 5'-to-3'-direction-unwinding substrate, oligo 2 (32 nucleotides, complementary to M13 ssDNA) was annealed to M13mp19 ssDNA first, filtered through Sephacryl S-300, labeled at the 3'end with [α ^{32}P] dCTP by DNA pol I (Klenow fragment USB) (0.1 to 4 μg DNA with a 5'

overhanging end, 2 μl 10X Klenow buffer, 20 μCi [α ^{32}P] dCTP, 1 unit of Klenow fragment), filtered through Sephadex G-50 and digested with SmaI (BioLabs).

Soybean MtDNA Helicase Assay

The standard reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 1 mM dithtothreitol, 5 mM ATP, 1 ng ^{32}P-labeled helicase substrate (1000 cpm), and the soybean mitochondrial fraction, with incubation at 37^{o}C for 60 min, except as otherwise stated. The reaction was terminated by addition of 40 mM EDTA, 30% glycerol, and 0.001mg/ml bromophenol blue, and the products were separated by 7% native polyacrylamide gel (8 cm X 10 cm) electrophoresis in 0.5 X TBE. The gel was exposed to Kodak film overnight and the film was analyzed using the BioRad imaging system.

Results

Western blot analysis confirms the purity of the mitochondrial protein fraction

Chloroplast DNA helicase activity in pea and in other higher plants was reported by Tuteja in 1996 (Tuteja et al., 1996). However, there has been no report for mitochondria DNA helicase activity until today. To make sure there is no chloroplast contamination in our purified soybean mitochondria protein, Western blot analysis was carried out to confirm the purity of the mitochondrial protein fractions. Polyclonal antibody (Rose Biotechnology) prepared against a synthetic 16-amino acid peptide representing a unique region near the N-terminal end of the mature mtRecA protein (Khazi et al., 2003) recognized a single specific polypeptide of the expected size (~43 kDa) in protein extracts from purified soybean mitochondria (Fig. 6a). Similar size bands were also found in the soybean total protein and *Arabidopsis* total protein. An antibody against chlorophyll a/b binding protein (Rose Biotechnology), a chloroplast-specific protein, (~ 25.2 kDa) was also tested. Approximately 25.2 kDa bands were found from soybean total protein and *Arabidopsis* total protein on Western blot but not with the purified mitochondria lysate (Fig. 6b). These results confirm there is undetectable chloroplast contamination in the mitochondrial protein fraction.

Soybean mitochondria have DNA helicase activity

The function of helicase is to utilize the chemical energy of ATP to separate the two parental DNA strands at the replicating fork or during recombination. For testing whether soybean mitochondria have this activity, a substrate was prepared by annealing a ^{32}P-labeled oligonucleotide (17 bp) with its complementary M13 ssDNA. If soybean mitochondria do not have helicase activity, a large size band only (same as M13 ssDNA)

will be obtained on the polyacrylamide gel because the substrate was not separated. If soybean mitochondria have helicase activity (or heated to denature the substrate as a control), the substrate will be separated. A 17 bp band (^{32}P-labeled oligonucleotide) at lower region of gel which is the unwound labeled oligonucleotide will be found on the polyacrylamide gel. The result (Fig. 7) indicates soybean mitochondria have helicase activity.

Optimal conditions for soybean mitochondrial DNA helicase activity

As mentioned before, DNA helicases are molecular motor proteins that use the energy of nucleoside 5'-triphosphate (NTP) hydrolysis to open transiently the energetically stable duplex DNA into single strands. For testing which NTPs and dNTPs can be utilized for soybean mitochondria DNA helicase activity, a helicase assay with different NTPs and dNTPs was carried out. The result (Fig. 8) indicates that among the NTPs and dNTPs be tested, ATP was by far the most active nucleotide. Other nucleotides were poorly utilized. Another helicase assay (Fig. 9) showed that the optimum concentration requirement of ATP is 2 mM. At higher ATP concentrations (such as 8 or 16 mM) the enzyme was inhibited.

Helicase needs divalent cations for activity because divalent cations can support ATP hydrolysis and support helicase binding to DNA. The result of a helicase assay with different concentration of Mg^{2+} indicated soybean mitochondrial DNA helicase had a requirement for divalent cations in the helicase assay. It showed very little activity at low concentrations of Mg^{2+} (such as 1, 2, or 4 mM Mg^{2+}). The optimum concentration of $MgCl_2$ was 8 to 10 mM (Fig. 10).

DNA helicases have been shown to have a range of requirement for monovalent

salt ions. A helicase assay was also carried out with different concentrations of NaCl. The result shows soybean mitochondrial DNA helicase was active at a range of concentrations of monovalent cations, between 100-200 mM NaCl (Fig. 11). At higher NaCl concentrations (400 mM) the activity was inhibited.

Different incubation temperatures affected the soybean mitochondrial DNA helicase activity (Fig. 12). At higher temperature, such as 50 ^{o}C, the soybean mitochondrial DNA helicase activity was inhibited (Fig. 12a). Soybean mitochondrial helicase activity was also tested at room temperature, 30^{o}C, 37^{o}C, and 42^{o}C (Fig. 12b). The result shows the optimum temperature was 37-42^{o}C. However, at 42^{o}C some unwinding occurs in the absence of the mitochondria protein due to the short length of the oligonucleotide used to prepare the substrate (Fig. 12c). All subsequent reactions to determine optimal conditions were done at 37^{o}C.

The incubation time also affected soybean mitochondrial DNA helicase activity (Fig. 13). The result of the helicase assay with different incubation times shows with one hour or longer incubation the soybean mitochondria has optimal DNA helicase activity.

Unwinding direction of soybean mitochondrial DNA helicase

Another important property of DNA helicase is the unwinding direction. In order to determine the direction of unwinding, two substrates with long linear ssDNA molecules bearing short stretches of duplex DNA at both ends were constructed. A helicase assay with 3'-to-5'-direction and 5'-to-3'-direction specific substrates was carried out. The results show soybean mitochondrial DNA helicase only moves from 3' to 5' along the DNA strand to which it binds (Fig. 15). It lacks 5' to 3' unwinding activity.

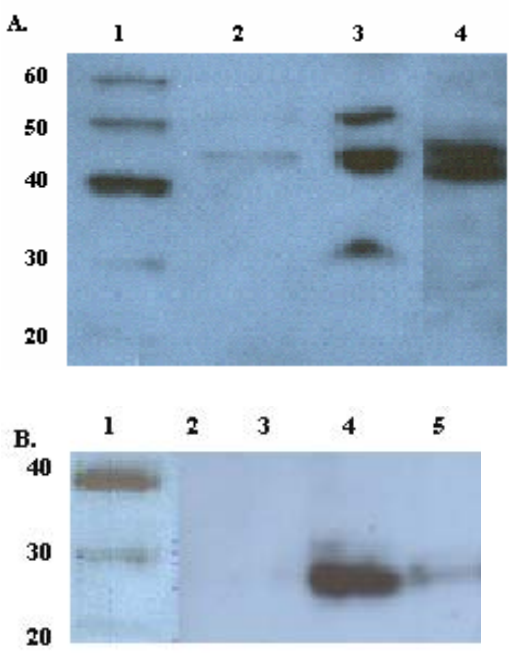

Fig. 6 Results of western blot analysis. Fig. A is the result with antibody against *Arabidopsis* mtRecA protein. Lane 1 is the marker (with sizes indicated at left (kDa)); lane 2 is purified mitochondria protein. There is a band ~ 43 kDa; lane 3 is the soybean total protein; lane 4 is the *Arabidopsis* total protein. Both lane 3 and lane 4 have a band ~ 43 kDa. Fig. B is the result with antibody against chlorophyll a/b protein (~25.2 kDa). Lane 1 is the marker; lane 2, 3 are different amounts (10 and 15 μl) of the purified soybean mitochondrial proteins, no band observed at 25.2 kDa; lane 4 is *Arabidopsis* total protein; lane 5 is the soybean total protein. Both of them have a band ~25.2 kDa. These figures show there is no detectable chloroplast protein in our purified mitochondrial protein fraction.

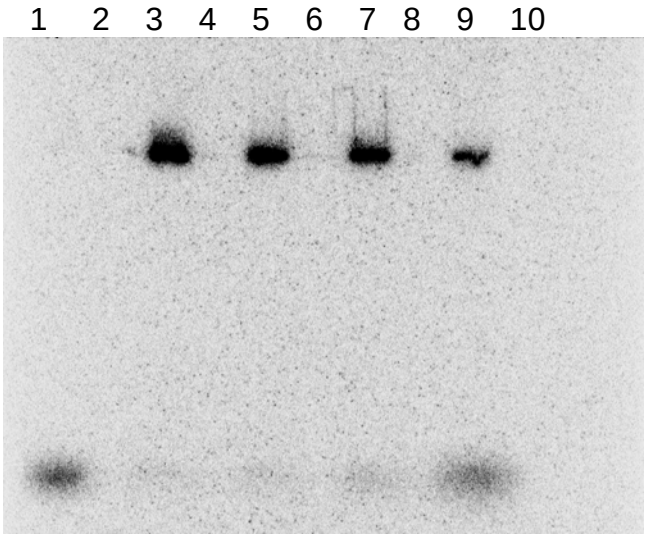

Fig. 7 Soybean mitochondria contain DNA helicase activity. Lane 1 is heat-denatured substrate; lanes 2, 4, 6, 8, and 10 are empty. Lanes 3,5,7,9 are helicase assays with different soybean mitochondria fractions from the Herparin Sepharose column. The bands at the bottom show soybean mitochondria contain DNA helicase activity (Peak in fraction 9).

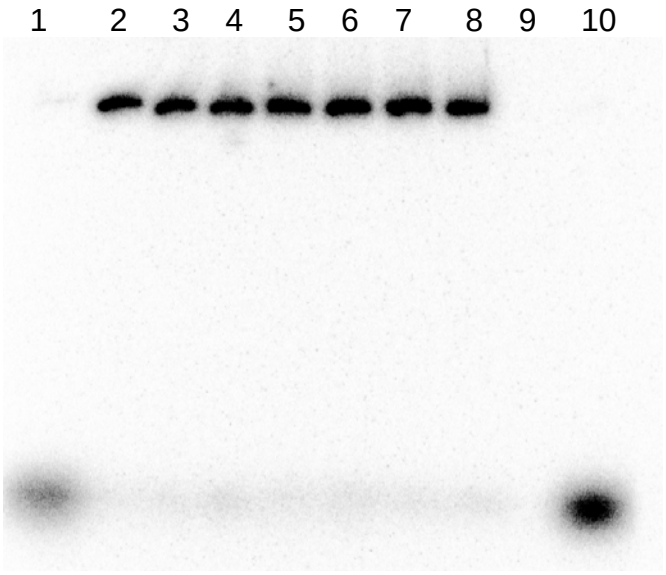

Fig. 8 Nucleotide preference of soybean mitochondrial DNA helicase. Lanes 1 to 8 show assay results using ATP, dATP, CTP, dCTP, GTP, dGTP, UTP, and dTTP (5 mM) respectively; lane 9 is empty; lane 10 is heat denatured substrate. Only in lane 1 (ATP), there is a strong band. This figure shows ATP is the most favored cofactor for soybean mitochondria helicase activity, the other NTPs and dNTPs are poorly utilized.

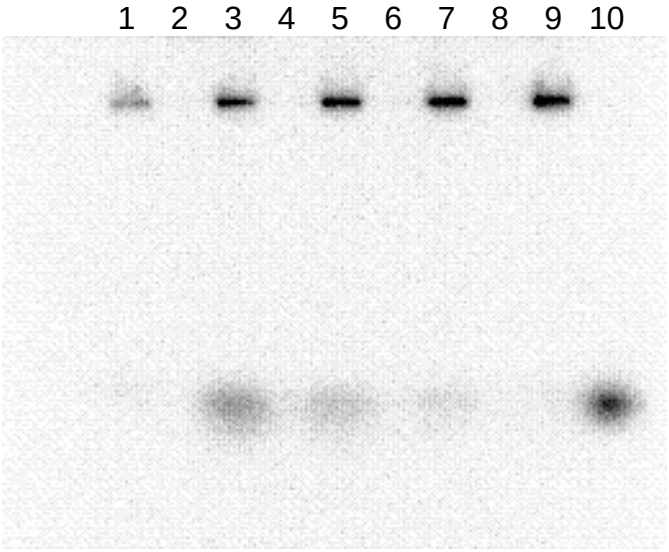

Fig. 9 Different concentrations of ATP on soybean mitochondrial DNA helicase activity. Lane 1 is with 1 mM ATP; lane 3, 2 mM ATP; lane 5, 4 mM ATP; lane 7, 8 mM ATP; lane 9, no enzyme; lane10, heat-denatured substrate. Lanes 2, 4, 6, and 8 are empty. The strongest band in lane 3 (2 mM ATP) indicates 2 mM ATP is optimal for soybean mitochondria helicase activity.

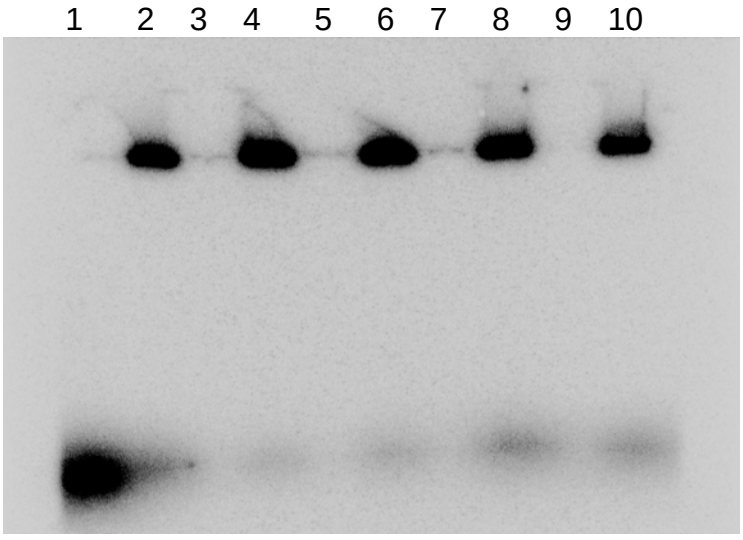

Fig. 10 Soybean mitochondrial DNA helicase assay with different concentrations of Mg^{2+}. Lane 1 is heat denatured substrate; lane 2 is with 1 mM Mg^{2+}; lane 4, 2 mM Mg^{2+}; lane 6, 4 mM Mg^{2+}; lane 8, 8 mM Mg^{2+}; lane 10, 10 mM Mg^{2+}; lanes 3, 5, 7, and 9 are empty. This figure shows soybean mitochondria have the optimal helicase activity at 8 to 10 mM Mg^{2+}.

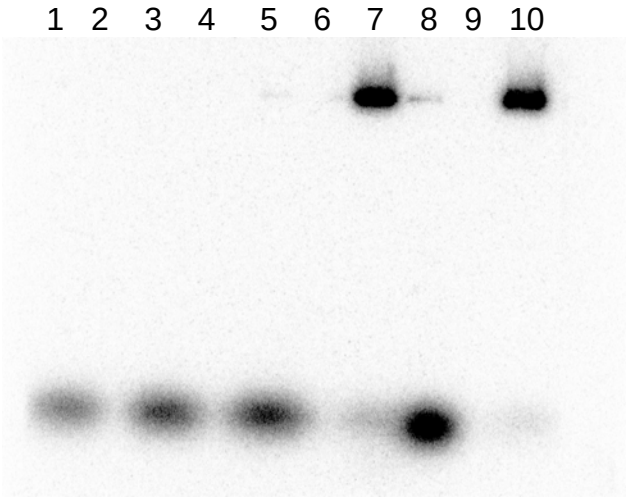

Fig. 11 Soybean mitochondrial DNA helicase assay with different concentrations of NaCl. Lane 1 is with 50 mM NaCl; lane 3, 100 mM NaCl; lane 5, 200 mM NaCl; lane 7, 300 mM NaCl; lane 8, denatured substrate; lane 10, 400 mM NaCl. Lanes 2, 4, 6, and 9 are empty. This figure shows the strongest helicase activity at 100 to 200 mM NaCl.

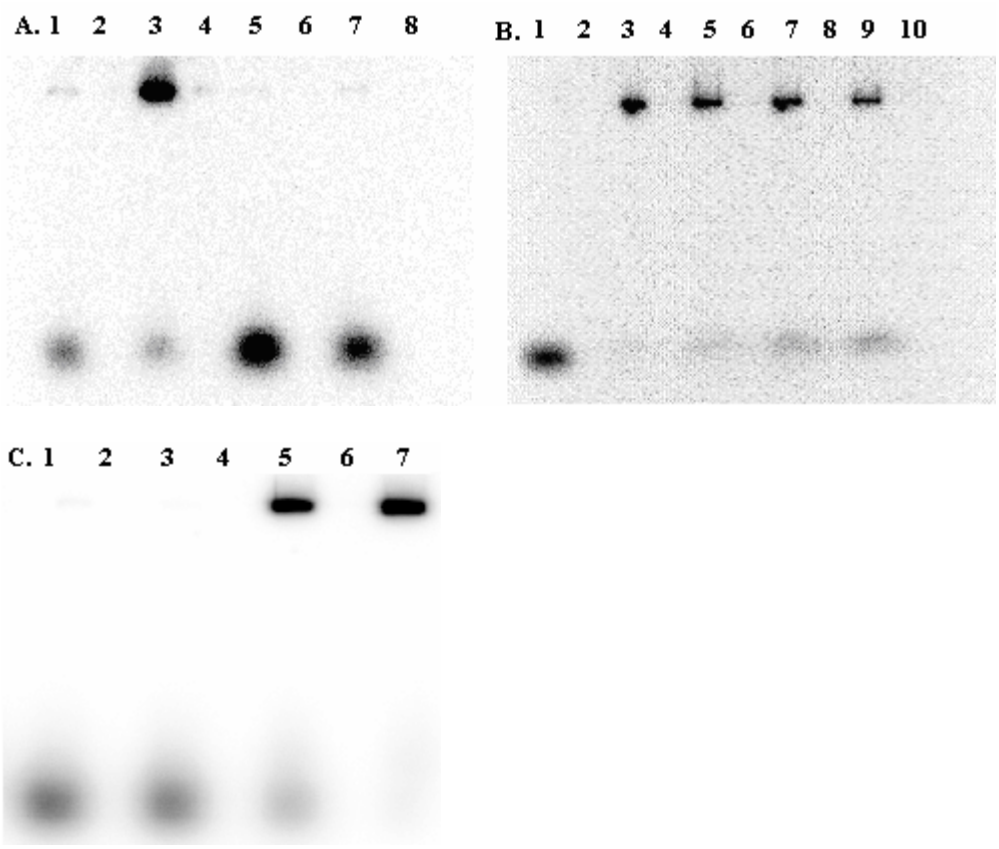

Fig. 12 Soybean mitochondrial DNA helicase assay at different incubation temperatures. A. Lane 1 is heat-denatured substrate; lane 3 was incubated at 25^{o}C; lane 5, at 37^{o}C; lane 7, at 50^{o}C. Lanes 2, 4, 6 and 8 are empty. This figure shows that of the temperatures tested, 37^{o}C is the best for the helicase assay. When the temperature was raised to 50^{o}C, the helicase activity of soybean mitochondria was inhibited. B. Base on the on the results from Fig. 12a, the helicase assay was conducted to test the accurate optimal temperature for the soybean mitochondria helicase activity. Lane 1 is heat-denatured substrate; lane 3 was incubated at 25^{o}C; lane 5, at 30^{o}C; lane 7, at 37^{o}C; lane 9, at 42^{o}C. Lanes 2, 4, 6, 8, and 10 are empty. This figure shows that 42^{o}C is the best temperature for the helicase assay. C. The control helicase assay was also conducted to test the unwinding of substrate at different temperatures in the absent of soybean mitochondria protein. Lane 1 is at 95 ^{o}C, lane 3 is at 50 ^{o}C, lane 5 is at 42 ^{o}C, and lane 7 is at 37 ^{o}C. Lane 2, 4, 6 are empty. This figure shows high temperatures (above 37 ^{o}C) can cause unwinding of substrates.

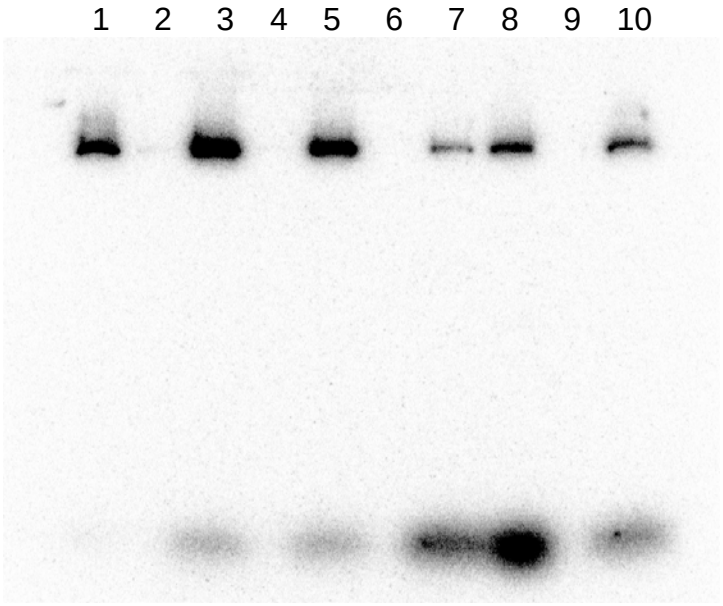

Fig. 13 Soybean mitochondrial DNA helicase assay at different incubation times. Lane 1 was incubated for 0 min; lane 3, 10 min; lane 5, 30 min; lane 7, 60 min; lane 8, denatured substrate; lane 10, 120 min. Lanes 2, 4, 6, and 9 are empty. This figure shows that 1 hr or longer time incubation of the mixture for the helicase assay is optimal.

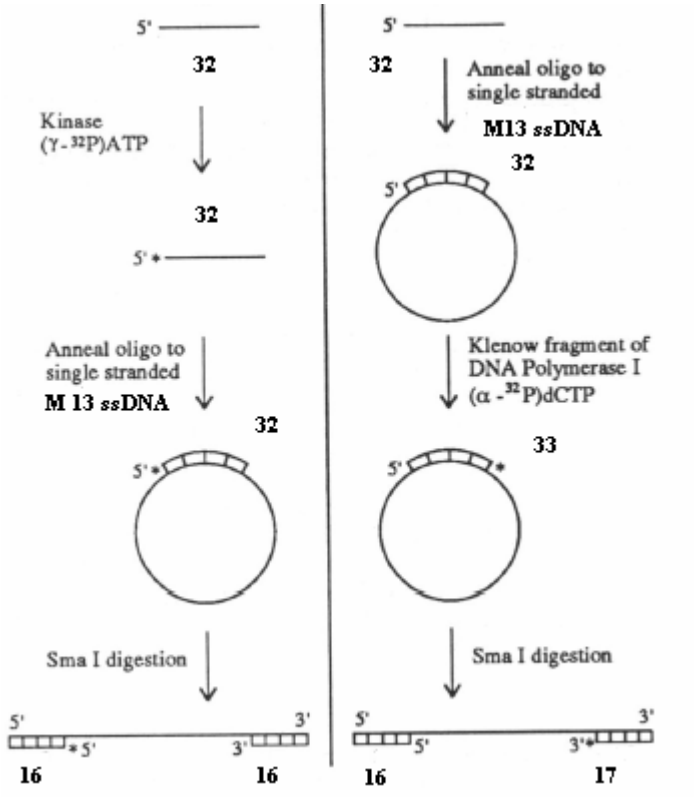

Fig. 14 Schematic diagram of the assay DNA substrates to test 3' to 5' (left) and 5' to 3' (right) unwinding activities of DNA helicase (Based on Hehman et al., 1992)

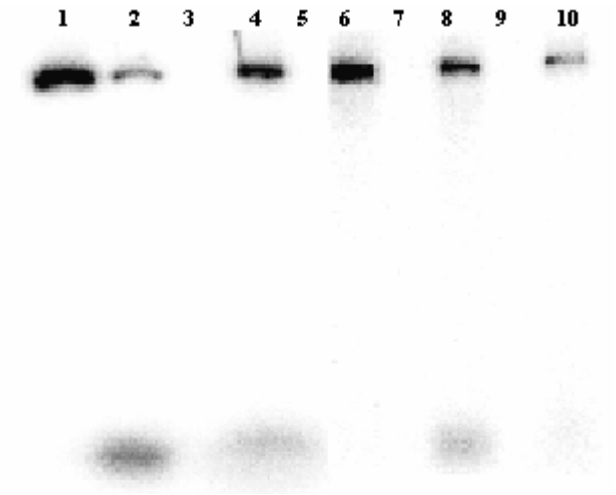

Fig. 15 Soybean mitochondrial DNA helicase has 3' to 5' DNA helicase unwinding activity and lacks 5' to 3' unwinding activity. Lane 1 is the 3' to 5' unwinding substrate; lane 2 is the heat denatured 3' to 5' substrate; lane 4 is the helicase assay with soybean mitochondria fraction and 3' to 5' substrate; lane 6 is the 5' to 3' substrate; lane 8 is the denatured 5' to 3' substrate; lane 10 is the reaction with the soybean mitochondria fraction with 5' to 3' substrate. Lanes 3, 5, 7, 9 are empty. This figure shows that soybean mitochondria has 3' to 5'unwinding activity but appears to lack 5' to 3' activity.

Discussion

DNA helicases have been isolated and characterized from a number of prokaryotes and eukaryotes (Nielson et al., 1991; Thommes et al., 1990; Matson et al., 1992). Very little information about DNA helicase has been reported in higher plants. The first reported helicase from plants was the unwinding protein from *lilium* (Hotta and Stern,1978). More recently DNA helicases from pea chloroplasts were reported (Tuteja et al.,1996). DNA helicase has been isolated and characterized from mammalian mitochondria (Hehman and Hauswirth, 1992). Until now there has not been any report of DNA helicase in higher plant mitochondria. We have partially purified and characterized mitochondrial DNA helicase from soybean.

Soybean mitochondria has DNA helicase activity

Mitochondrial DNA helicase has been partially purified from soybean leaves. Because chloroplast and mitochondria are difficult to separate, two methods were used to purify soybean mitochondria. In one method, differential centrifugation was used and a Percoll gradient procedure was not included. The other method utilized a Percoll gradient for soybean mitochondria purification. The results from the different purification fractions were similar and indicated that the helicase activity subsequently detected was from mitochondria. The results of western blotting of these partially purified proteins also indicated the absence of chloroplast protein contamination, so the identified helicase activity is a mitochondrial intrinsic activity.

Soybean mitochondria DNA helcase and other DNA helicases

DNA helicases can transiently abolish the stable helical structure of DNA by harnessing energy derived from the hydrolysis of NTPs. This energy is required to

overcome the thermodynamic energy barrier imposed by molecular interactions between the bases of DNA and for the helicase to translocate along the DNA strands to which it is bound. The single-stranded DNA generated in the helicase reaction is utilized by other enzymes that participate in DNA replication, repair and recombination (Lee et al., 1998).

The NTP/dNTP requirements for DNA helicase activities are varied from helicase to helicase. Some mammalian DNA helicases can utilize different NTPs and dNTPs for breaking the hydrogen bonds between the bases of double-stranded DNA, but most of them prefer ATP over dATP. Soybean mitochondrial DNA helicase was found to be ATP-dependent. ATP is the most important cofactor for soybean mitochondrial DNA helicase to have optimal activity. Other NTP or dNTPs could also be utilized but to a much lesser extent.

The soybean mitochondrial DNA helicase appears to be very similar to pea chloroplast DNA helicases (Tuteja et al., 1996), but with minor differences. Mg^{2+} is essential for the activity of soybean mitochondrial DNA helicase because Mg^{2+} can promote some structural transition in duplex DNA, but the optimal concentrations of $MgCl_2$ are different from the chloroplast activity. The optimal concentration of $MgCl_2$ for soybean mitochondrial DNA helicase activity was found to be 8 to 10 mM. The optimal concentration of $MgCl_2$ for pea chloroplast DNA helicase was reported to be 1 mM (Tuteja et al., 1996), and for soybean chloroplast DNA helicase was reported also to be 10 mM (Cannon et al., 1990).

The helicase unwinding process is very complex. Helicases have optimal concentration requirements for monovalent cations (NaCl) for activity. At low concentrations of NaCl, DNA helicase remains associated with other protein like

ATPase; at high concentrations these associations are broken. This may explain why at high concentrations of NaCl the helicase activity was inhibited.

The unwinding activity catalyzed by a helicase exhibits a specific directionality (5' to 3' or 3' to 5') with few exceptions. Most prokaryotic DNA helicases that are involved in origin activation and growing-fork movement move in the 5'-to-3' direction with respect to the strand to which they are bound. The soybean mitochondrial DNA helicase moves in a 3'-to-5' direction along the bound strand similar to the pea chloroplast and many other DNA helicases (Tuteja et al., 1998). The reason why soybean mitochondria has 3' to 5' direction unwinding activity is not very clear. It seems related to the unique primary linear and branched circular structure of mitochondria DNA and recombination dependent replication forms of mtDNA. Soybean mitochondria DNA helicase may play an important role in DNA replication, repair and recombination.

Potential role of soybean mitochondrial DNA helicase in mtDNA replication

The *in vivo* roles of most DNA helicases isolated from *E. coli* have been determined and many are involved in DNA replication (Matson et al., 1990). The yeast PIFI gene product has been shown to be a DNA helicase used in repair and recombination of mtDNA (Bessler et al., 2001). The reported soybean mitochondrial DNA helicase activity in this thesis, to our knowledge, is the first documented higher plant mitochondrial DNA helicase. A comparison of its characteristics with pea chloroplast DNA helicase suggests its possible role in mtDNA replication. The data presented shows that soybean mitochondria has 3'-to-5' unwinding ability, which may be involved in leading strand synthesis for mtDNA replication. MtDNA replication in plants may be initiated by recombination events that create branched structures of

multigenomic concatemers (Oldenburg and Bendich, 1996). Further purification and characterization of the activity from soybean mitochondria and identification of the gene encoding this protein may help us understand the possible role of soybean mitochondrial DNA helicase in replication and DNA repair.

References

Alberts B, Bray D, Lewis J, Raff M, Roberts K, and Watson JD (1994) Molecular Biology of the Cell 3rd Ed. Garland Publishing, Inc: New York

Andre CP, Levy A, Walbot V (1992) Small repeated sequences and structure of plant mitochondrial genomes. Trends Genet. 8: 128-132

Arimura G, Ozawa R, Kugimiya S, Takabayashi J, Bohlmann J (2004) Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (E)-b-ocimene and transcript accumulation of (E)-b-ocimene synthase in *Lotus japonicus*. Plant Physiol. 135:1-8

Ariyoshi M, Vassylyev DG, Iwasaki H, Nakamura H, Shinagawa H, and Morikawa K (1994) Atomic structure of the RuvC resolvase: A Holliday junction-specific endonuclease from *E. coli*. Cell 78: 1063-1072

Attardi G, Schatz G (1988**)** Biogenesis of Mitochondria. Annual Review of Cell Biology 4: 289-331

Backert S, Borner T (2000) Phage T4-like intermediates of DNA replication and recombination in mitochondria of higher plant *Chenopodium album* (L.) Curr. Genet. 37:304-314

Backert S, Dorfel P, Borner T (1995) Investigation of plant organeller DNAs by pulsed-field gel electrophoresis. Curr. Genet. 28: 390-399

Backert S, Lurz R, Oyarzabal OA, Borner T (1997a) High content, size and distribution of single-stranded DNA in the mitochondria of *Chenopodium album* (L.) Plant Mol. Biol. 33: 1037-1050

Backert S, Nielsen BL, Borner T (1997b) The mystery of the rings: structure and replication of mitochondrial genomes from higher plants. Trends Plant Sci. 2: 477-483

Bendich AJ (1993) Reaching for the ring: the study of mitochondrial genome structure. Curr. Genet. 24:279-290

Bendich AJ (1996) Structure analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. J. Mol. Biol. 255:546-588

Bendich AJ, Smith SB (1990) Moving pictures and pulsed-field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria. Curr. Genet. 17: 421-425

Bessler JB, Torres JZ, Zakian VA (2001) The Pif1p subfamily of helicases: region-specific DNA helicases? Trends Cell Biol. 11: 60-65

Binder S, Marchfelder A, Brennicke A (1996) Regulation of gene expression in plant mitochondria. Plant Mol. Biol. 32: 303-314

Blanco PR, Kowalczykowski SC (2002) Translocation step size and mechanism of the RecBC DNA helicase. Nature 405: 368-372

Cannon GC, Heinhorst S (1990) Partial purification and characterization of a DNA helicase from chloroplasts of *Glycine max*. Plant Mol Biol. 15: 457-464

Cerutti H, Jagendori AT (1993) DNA strand-transfer activity in pea (*Pisum sativum L.*) chloroplasts. Plant Physiol 102: 145-153

Chanut FA, Grabau EA, Gesteland RF (1993) Complex organization of the soybean mitochondrial genome: recombination repeats and multiple transcripts at the *atpA* loci. Curr. Genet. 23:234-247

Daniell H, Zheng D, Nielsen BL (1995) Isolation and characterization of an in vitro DNA replication system from maize mitochondria. Biochem. Biophys. Res. Comm. 208: 287-294

Eckardt NA (2003) A new twist on transposons: The maize genome harbors helitron insertion. Plant Cell 15: 293-295

Edmondson AC, Song D, Alvarez LA, Wall MK, Almond D, McClellan DA, Maxwell A, Nielsen BL. (2005) Characterization of a mitochondrially targeted single-stranded DNA-binding protein in *Arabidopsis thaliana.* Mol. Gen. Genomics 273: 115 - 122

Esposti MD (2004) Mitochondria in apoptosis: past, present and future. Biochem. Soc. Trans. 32: 493–495

Fauron C, Casper Y, Gesteland R, Albertson M (1992) A multi-recombination model for mtDNA rearrangements seen in maize cmsT regenerated plants. Plant J. 2:949-958

Fauron C, Casper Y, Gao Y, Moore B (1995) The maize mitochondrial genome: dynamic, yet functional. Trends Genet. 11:228-235

Flamand MC, Duc G, Goblet JP, Hong L, Louis O, Briquet M, Boutry M (1993) Variant mitochondrial plasmids of broad bean arose by recombination and are controlled by the nuclear genome. Nucl. Acids Res. 21:5468-5473

Foury F, Lahaye A (1987) Cloning and sequencing of the PIF gene involved in repair and recombination of yeast mitochondrial DNA. EMBO. J. 6: 1441-1449

Friedman-Ohana R, Cohen A (1998) Heteroduplex joint formation in *Escherichia coli* recombination is initiated by pairing of a 3'-ending strand. Genetics 95: 6909-6914

Geider K, Hoffmann-Berling H (1981) Proteins controlling the helical structure of DNA Ann. Rev. Biochem. 50: 233-260

Grabau EA, Davis WH, Phelps ND, Gengenbach BG (1992) Classification of soybean cultivars based on mitochondrial DNA restriction fragment length polymorphisms. Crop. Sci. 32:271-274

Griffith JD, Harris LD. (1988) DNA strand exchanges. Crit Rev Biochem. Suppl 1: S43-86

Hanlon RW, Grabau EA (1997) Comparison of mitochondrial organization of four soybean cytoplasmic types by restriction mapping. Soybean Genet. Newsletter 24:208-210

Hehman GL, Hauswirth WW (1992) DNA helicase from mammalian mitochondria. Proc. Natl. Acad. Sci. USA 89:8562-8566

Hotta Y, Stern H (1978) DNA unwinding protein from meiotic cells of *lilium*. Biochemistry 17: 1872-1880

Hrubec TC, Robinson JM, and Donaldson RP (1985) Isolation of mitochondria from soybean leaves on discontinuous percoll gradients. Plant Physiol. 77: 1010-1012

Jacobs MA, Payne SR, Bendich AJ (1996) Moving pictures and pulsed-field gel electrophoresis show only linear mitochondria DNA molecules from yeast with linear-mapping and circular-mapping mitochondria genomes. Curr. Genet.30: 3-11

Kaiser GE Web page of Dr. Kaiser**:** http://student.ccbcmd.edu/courses/bio141/lecguide/unit4/genetics/DNA/DNArep/fg13a.html

Kanazawa A, Tozuka A, Kato S, Mikami T, Abe J, Shimamoto Y (1998) Small interspersed sequences that serve as recombination sites at *cox2* and *atp6* loci in the mitochondrial genome of soybean are widely distributed in higher plans. Curr. Genet. 33:188-198

Kato S, Kanazawa A, Mikami T, Shimamoto Y (1998) Evolutionary changes in the structures of *cox*2 and *atp6* loci in the mitochondria genome of soybean involving recombination across small interspersed sequences. Curr. Genet. 34:303-312

Khazi FR, Edmondson AC, Nielsen BL (2003) An *Arabidopsis* homologue of bacterial RecA that complements an *E. coli* recA deletion is targeted to plant mitochondria. Mol Gen. Genomics 269: 454-463

Kimball JW (2005) Biology Online Page: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/

Kline M, Eckert-Ossenkopp U, Schmiedberg I, Brandt P, Unseld M, Brennicke A, Schuster W(1994) Physical mapping of the mitochondrial genome of *Arabidopsis thaliana* by cosmid and YAC clones. Plant J. 6:447-455

Konforti BB, Davis RW (1987) 3' Homologous Free Ends are Required for Stable Joint Molecule Formation by the RecA and Single-Stranded Binding Proteins of *Escherichia coli* Proc. Natl. Acad. Sci. USA 84: 690-694

Lahaye A, Stahl H, Thines-Sempoux D, Foury F (1991) PIF1: a DNA helicase in yeast mitochondria. EMBO J. 10: 997-1007

Lee C, Seo YS (1998) Isolation and characterization of a processive DNA helicase from the fission yeast *Schizosaccharomyces pombe* that translocates in a 5'-to-3' direction. Biochem. J. 334: 377-386

Ling F, Makishima F, Morishima N, Shibata T (1995) A nuclear mutation defective in mitochondrial recombination in yeast. EMBO J. 14: 4090-4101

Linke B, Nothnagel T, Borner T (2003) Flower development in carrot CMS plants: mitochondria affect the expression of MADS box genes homologous to *GLOBOSA* and *DEFICIENS* Plant J. 34: 27-37

Lodish, Harvey, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J (2000) Molecular Cell Biology. W. H. Freeman and Company

Lockshon D, Zweifel SG, Freeman-cook LL, Lorimer HE, Brewer BJ (1995) A role for recombination junctions in the segregation of mitochondrial DNA in yeast. Cell 81: 947-955

Lunt, DH, Hyman BC (1997) Animal mitochondrial DNA recombination. Nature 387: 247

MacAlpine DM, Perlman PS, Butow RA (1998) The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates in vivo. Proc. Natl. Acad. Sci. USA 95: 6739–6743

Mackenzie S, Mclntosh L (1999) Higher Plant Mitochondria. Plant Cell 11: 571-585

Maleska R(1993) Single-stranded regions in yeast mitochondria DNA revealed by pulsed-field gel electrophoresis. Appl. Theor. Electrophor. 3:259-263

Manna F, Massardo DR, Del Giudice L, Buonocore A, Nappo AG, Alifano P, Schafer B, Wolf K (1991) The mitochondrial genome of Schizosaccharomyces pombe. Stimulation of intra-chromosomal recombination in *Escherichia coli* by the gene product of the first cox1 intron. Curr. Genet. 19: 295-299

Margulis L (1981) Symbiosis in Cell Evolution New York: W.H. Freeman

Marienfeld JR, Unseld M, Brandt P, Brennicke A (1997) Mosaic open reading frames in *Arabidopsis thaliana* mitochondrial genome. Biol. Chem. 378:859-862

Matson SW (1991) The DNA helicases of *Escherichia coli*. Prog. in Mol. Biol. and Nuc. Acids Res. 40: 289-326.

McCarthy JG, Sander M, Lowenhaupt K, Rich A (1988) Sensitive homologous recombination strand-transfer assay: partial purification of a drosophila melanogaster enzyme and detection of sequence effects on the strand-transfer activity of RecA protein. Proc. Natl. Acad. Sci. USA 85: 5854-5858

McEntee K, Weinstock GM, Lehman IR (1980) RecA protein-catalyzed strand assimilation: stimulation by *Escherichia coli* single-stranded DNA-binding protein. Proc. Natl. Acad. Sci. USA 77: 857-861

Meyer RR, Laine PS (1990) The single-stranded DNA-binding protein of *Escherichia coli.* Microbio. Rev. 54:342-380

Moeykens CA, Mackenzie SA, Shoemaker RC (1995) Mitochondrial genome diversity in soybean: repeats and rearrangements. Plant Mol. Biol. 29:245-254

Mosig G (1987) The essential role of recombination in phage T4 growth. Ann. Rev. Genet. 21-347-371

Mosig G (1998) Recombination and recombination-dependent DNA replication in bacteriophage T4. Ann. Rev. Genet. 28:113-121

Naqvi A, Tinsley E, Khan SA (2003) Purification and characterization of the PcrA helicase of *Bacilllus anthracis.* J. Bacteriol. 185:6633-6639

Nielson PE, Egholm RH, Berg RH, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254: 1497-1500.

Notsu Y, Masood S, Nishikawa T, Kubo N, Akiduki G, Nakazono M, Hirai A, Kadowaki K (2002) The complete sequence of rice (*Oryza sativa L.*) mitochondrial genome: frequent DNA sequence acquisition and loss during the evolution of flowering plants. Mol. Genet. Genomics 268: 434-445

Oh EY, Grossman L (1989) Characterization of the helicase activity of the *Escherichia coli* UvrAB protein complex. J. Biol. Chem.264:1336-1343

Oldenburg DJ, Bendich AJ (1996) Size and structure of replicating mitochondrial DNA in cultured tobacco cells. Plant Cell 8: 447-461

Oldenburg DJ, Bendich AJ (1998) The structure of mitochondrial DNA from the liverwort, *Marchantia polymorpha.* J Mol. Biol. 276: 745-758

Oldenburg DJ, Bendich AJ (2001) Mitochondrial DNA from the liverwort Marchantia polymorpha; circularly permuted linear molecules, head-to-tail concatemers, and a 5'protein. J. Mol. Biol. 310: 549-562

Palmer JD, Shield CR (1984) Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307:437-440

Pham XH, Reddy MK, Ehtesham NZ, Matta Bharati, Tuteja N (2000) A DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase I activity. Plant J. 24: 219-229

Phan TN, Ehtesham NZ, Tuteja R, Tuteja N (2003) A novel nuclear DNA helicase with specific activity from *Pisum sativum* catalytically translocates in the 3'->5' direction. Eur. J. Biochem. 270: 1735-1745

Rokas A, Ladoukakis E, Zouros E (2003) Animal mitochondrial DNA recombination revisited. Trends Ecol. Evol. 18: 411-417

Rothenberg M, Hanson MR (1988) A functional mitochondrial ATP synthase proteolipid gene produced by recombination of parental genes in a petunia somatic hybrid. Genetics 118:155-161

Sambrook J, Russell D (2001) Molecular Cloning- A Laboratory Manual, Cold Spring Harbor Laboratory Press.

Sato A, Nakada K, Akimoto M, Ishikawa K, Ono T, Shitara H, Yonekawa H, Hayashi JI. (2005) Rare creation of recombinant mtDNA haplotypes in mammalian tissues. Proc. Natl. Acad. Sci. USA 102: 6057–6062

Satoh M, Nemoto Y, Kawano S, Nagata T, Hirokawa H, Kuroiwa T (1993) Organization of heterogeneous mitochondria DNA molecules in mitochondrial nuclei of cultured tobacco cells. Protoplasma 175:112-120

Schuster W, Brennicke A (1994) The plant mitochondrial genome: physical structure, information content, RNA editing, and gene migration to the nucleus. Ann. Rev. Plant Physiol. Plant Mol. Biol. 45:61-78

Scissum-Gunn KD, Gandhi M, Backert S, Nielsen BL (1998) Separation of different conformations of plant mitochondrial DNA molecules by field inversion gel electrophoresis. Plant Mol. Rep. 16: 219-229

Sedman T, Kuusk S, Kivi S, Sedman J (2000) A DNA helicase required for maintenance of the functional mitochondrial genome in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 20:1816-1824

Sehorn MG, Sigurdsson S, Bussen W, Unger VM, Sung P (2004) Human meiotic recombinase Dmc1 promotes ATP-dependent homologous DNA strand exchange. Nature 429: 433-437

Sena EP, Revet B, Moustacchi E (1986) In vivo homologous recombination intermediates of yeast mitochondrial DNA analyzed by electron microscopy. Mol. Gen. Genet. 202: 421-428

Shan Q, Cox MM (1997) RecA filament dynamics during the DNA strand exchange reaction. J. Biol. Chem.272: 11063-11073

Shannon MP, Kaufman TC, Shen MW, Judd BH (1972) Lethality patterns and morphology of selected lethal and semi-lethal mutations in the *zeste-white* region of *Drosophila melanogaster*. Genetics 72:615-638

Shen JC, Gray MD, Oshima J, Loeb LA (1998) Characterization of Werner syndrome protein DNA helicase activity: directionality, substrate dependence and stimulation by replication protein A. Nucl. Acids Res. 26:2879-2885

Shibata T, Cunningham RP, DasGupta C, Radding CM (1979) Homologous Pairing in Genetic Recombination: Complexes of recA Protein and DNA. Proc. Natl. Acad. Sci. USA 76: 5100-5104

Shin JH, Jiang Y, Grabowski B, Hurwitz J, kelman Z (2003) Substrate requirements for duplex DNA translocation by eukaryal and archaeal minichromosome maintenance helicases. J. Biol. Chem. 278: 49053-49062

Shinohara A, Ogawa T (1999) Rad51/RecA protein families and the associated proteins in eukaryotes. Mutation Research/DNA repair. 435: 13-21

Small ID, Isaac PG, Leaver CJ (1989) Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondria genome diversity in maize. EMBO J. 6:865-869

Stadler T, Delph LF (2002) Ancient mitochondrial haplotypes and evidence for intragenic recombination in a gynodioecious plant. Proc. Natl. Acad. Sci. USA 99: 11730-11735

Story RM, Weber IT, and Steitz TA (1992). The structure of the *E. Coli* RecA protein monomer and polymer. Nature 355: 318-325.

Taylor AF, Smith GR (1995) Monomeric RecBCD enzyme binds and unwinds DNA. J Biol. Chem. 270(41):24451-24458.

Thommes P, Ferrari E, Jessberger R, Hubscher U (1990) Four different DNA helicases from calf thymus. J. Biol. Chem. 267: 6063-6073

Thyagarajan B, Padua RA, Campbell C (1996) Mammalian mitochondria possess homologous DNA recombination activity. J. Biol. Chem. 271: 27536-27543

Tuteja N (2003) Plant DNA helicases: the long unwinding road. J. Exp. Bot. 54:2201-2214

Tuteja N, Beven AF, Shaw PJ, Tuteja R (2001) A pea homologue of human DNA helicase I is localized within the dense fibrillar component of the nucleous and stimulated by phosphorylation with CK2 and cdc2 protein kinases. Plant J. 25: 9-17

Tuteja N, Phan T-N, Tewari KK (1996) Purification and characterization of a DNA helicase from pea chloroplasts that translocates in the 3'-to-5'direction. Eur. J. Biochem 238: 54-63

Tuteja N, Phan T-N (1998) A chloroplast DNA helicase II from pea that prefers fork-like replication structures. Plant Physiol. 118:1029-1039

Tuteja N, Rahman K, Tuteja R, Falaschi A (1991) DNA helicase IV from hela cells. Nucl. Acids Res. 19: 3613-3618

Tuteja N, Tuteja R (2004a) Prokaryotic and eukaryotic DNA helicases-essential molecular motor proteins for cellular machinery. Eur. J. Biochem. 271: 1835-1848

Tuteja N, Tuteja R (2004b) Unraveling DNA helicases-motif, structure, mechanism and function. Eur. J. Biochem. 271: 1849-1863

Tuteja N, Tuteja R, Rahman K, Kang LY, Falaschi A (1990) A DNA helicase from human cells. Nucl. Acids Res. 18:6785-6792

Zheng D, Nielsen BL, Daniell H (1997) A 7.5 kbp region of the maize (T cytoplasm) mitochondrial genome contains a chloroplast-like *trnI* (CAT) pseudo gene and many

short segments homologous to chloroplast and other known genes. Curr. Genet. 32:125-137

Zhou JQ, Monson EK, Teng SC, Schulz VP, Zakian VA (2000) Pif1p helicase, a catalytic inhibitor of telomerase in yeast. Science 289: 771-774

Zhou JQ, Qi HY, Schulz VP, Mateyak MK, Monson EK, Zakian VA (2002) *Schizosaccharomyces pombe* pfh1^{+} encodes an essential 5' to 3' DNA helicase that is a member of the PIF1 subfamily of DNA helicases. Mol. Biol. Cell 13: 2180-2191

Unseld M, Marienfeld J, Brandt P, Brennicke A (1997) The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nature Genetics 15:57-61

Vindigni A, Ochem A, Triolo G, Falaschi A (2001) Identification of human DNA helicase V with the far upstream element-binding protein. Nucl. Acids Res. 29:1061-1067

Weaver, RF (2002) Molecular Biology, McGraw-Hill

Weber-Lotfi F, Dietrich A, Russo M, Rubino L (2002) Mitochondrial targeting and membrane anchoring of a viral replicase in plant and yeast cells. Virology J. 76: 10485-10496

Wu L, Hickson ID (2001) RecQ helicases and topoisomerases: components of a conserved complex for the regulation of genetic recombination. Cell Mol Life Sci. 58: 849-901