Molecular Characterization of OsMre11 and OsRad52 from *Oryza sativa* (rice)

By

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ANURADHA NAIR

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

ANURADHA NAIR

List of Publications arising from the thesis

Journal

1. "Biochemical characterization of plant Rad52 protein from rice (Oryza sativa)", Nair A, Agarwal R, Chittela RK. Plant Physiology Biochemistry. 2016 Sep; 106:108-17.
2. "Characterization of the functional N- terminal domain of Mre11 protein from rice, *Oryza sativa*". (Manuscript under preparation) Anuradha Nair, Rajani Kant Chittela

Conferences

1. Presented the poster "Over- expression, purification and biochemical characterization of OsRad52 protein from rice (*Oryza sativa*)" at the 14th FAOBMB and 84th SBC (I) meeting, held at the BITS PILANI campus, Hyderabad from 27th - 30th November 2015.
2. Presented the poster "Characterization of N-terminal end of Mre11 protein from rice *Oryza sativa*" at the 2017 FASEB Science Research Conference on "Genetic Recombination and Genome Rearrangements" held during July 16 - July 21, 2017 at Colorado (USA).

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SYNOPSIS OF Ph. D. THESIS

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2. **Name of the Constituent Institution:** Bhabha Atomic Research Centre, Mumbai
3. **Enrolment No.:** LIFE01201304002; January 01, 2013
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SYNOPSIS

(Limited to 10 pages in double spacing)

The thesis "Molecular characterization of OsMre11 and OsRad52 from *Oryza sativa* (rice)" comprises of 5 chapters: Chapter 1 comprises of detailed introduction to DNA damage repair and its significance in plant system. Chapter 2 deals with the materials and method which were used in the study. Chapter 3 describes the cloning and biochemical characterization of OsMRe11 and chapter 4 describes the cloning and biochemical characterization of OsRad52. Discussion and future directions of the present work will be presented in chapter 5.

INTRODUCTION:

DNA, the blueprint of an organism, is often exposed to several endogenous and exogenous challenges resulting in damage to genetic material, causing harmful effects

at cellular and organism level. The cellular response to DNA damage includes both cytotoxic response (cell death) and cytostatic response (suppression of cell growth). However, all living organism have developed several DNA damage check points and DNA damage repair pathways to restore the integrity of the genetic material**.** DNA damage comprises of simple mutations, deamination, missing bases, chemical modification of bases, formation of dimers and strand breaks. Strand breaks include both single strand breaks (SSBs) and double strand breaks (DSBs), caused by exposure to xenobiotics or during physiological processes like mitotic and meiotic recombination, DNA replication, transposition, transformation, and transduction or during VDJ recombination in mammals (1)**.** Among all, double stranded breaks (DSBs) are most lethal and the if not repaired leads to chromosomal rearrangements. DSBs are mainly repaired by three major pathways: Homologous Recombination (HR), Non Homologous End Joining (NHEJ) or Single Strand Annealing (SSA) processes. HR pathway is mainly prevalent in prokaryotes where as the SSA and NHEJ pathways operate in eukaryotes. In HR, DSB sites are processed to generate single strand DNA (ssDNA) which initiates homology search in homologous chromosomes. This is followed by different biochemical and biophysical steps including strand exchange and branch migration. The resulting Holliday junctions are resolved leading to the formation of either crossovers or non- crossovers. Several proteins are involved in the HR process and many of these have been extensively studied using mutant's sensitive to DNA damaging agents like UV and IR (which showed deficiency in recombination). HR in *E. coli* occurs mainly by the RecBCD pathway. However, in yeast genetic and biochemical studies have pointed to several genes in the RAD52 epistasis group to be

involved in HR (2,3). Using available literature from *E. coli* and yeast recombinases, several orthologues have been identified and widely studied in higher eukaryotes.

The MRN/X complex:

MRN/X is a multifunctional complex involved in many aspects of DNA metabolism and is involved in HR pathway. It comprises of Mre11, Rad50 and Nbs1 or Xrs2and is one of the early responder to DSBs. The MRN complex tethers, processes and signals DSBs, thereby promoting HR and thus genomic stability. Mre11is proposed to have 3'–5' exonuclease, divalent cation Mn^{++}dependent endonuclease, DNA unwinding activities and is involved in the 5'–3'resection of DSB ends to produce 3' single- strand overhangs. This complex has been extensively studied in humans, yeast and bacteria.

Rad52:

The Rad52 (Radiation sensitive 52) epistasis group of genes includes RAD50, RAD51, RAD54, RAD55, RAD57, RAD59, RDH57, MRE11 and NBS1, were identified by complementation assay (2) in yeast. Data on human, yeast Rad52 suggests that Rad52 is involved in both HR and SSA pathways. Rad52 mediates Rad51 loading onto ssDNA ends, thereby initiating HR and catalyzing DNA annealing.Rad52 protein also promotes DSB repair independent of Rad51 by binding to ends of breaks and promoting annealing of 3' overhangs between two direct repeats a process termed as SSA.

DNA repair in plants:

Plants are continuously exposed to several exogenous agents and endogenous processes causing damage to its genetic material, which ultimately reduces plant genome stability, growth and productivity. HR in plants has been reported during

somatic development and during meiosis. Several genes involved in HR in plants have been identified by mutational studies (4), but the biochemistry of these proteins are yet to be elucidated. Studies on plant counterparts of MRN/X complex has been reported for *Arabidopsis* and the functions of AtMre11 and AtRad50 have been studied (5). These studies showed that mutations in *Arabidopsis* MRE11 and RAD50 genes lead to severe developmental defects, sensitivity to DNA damaging agents and shortened telomeres in the mutants. In plants, RAD51 and RAD54 homologs have been identified, leaving the question of whether plants contain RAD52 homologs or whether their absence may be compensated by plant BRCA2 homologs. However, computational and experimental analyses provide clear evidence for the presence of functional RAD52 homologs in plants. In crop plants, knowledge about Mre11 and Rad52 is limited to only expression studies and biochemical studies have not yet been reported. The objective of the thesis is to study the biochemical properties of OsMre11 and OsRad52 from rice.

Objectives of the thesis:

1. To identify MRE11 and RAD52 homologues from *Oryza sativa* (Japonica) based on the sequence information available from *Arabidopsis thaliana*.
2. Cloning of cDNA's corresponding to *Os*MRE11 and *Os*RAD52 into *E. coli* over-expression strains.
3. Over-expression and purification of *Os*Mre11 and *Os*Rad52 proteins.
4. Biochemical analysis (DNA binding, annealing, exonuclease properties) of purified proteins

Chapter 2 of the thesis includes the materials and methods used in the thesis to characterize the proteins. Cloning of the corresponding genes to suitable expression

vector and host was carried out by standard procedures. Affinity and anion exchange chromatographic techniques were employed for the purification of the over- expressed protein. JASCO J-815 CD Spectrometer was used to record the far-UV CD of purified protein and the identity of the purified protein was established by peptide mass fingerprint (PMF) spectra obtained on a Brucker MALDI-TOF/TOF system in reflectron mode. To determine the *in vitro* properties of the purified protein, nucleic acid binding assays were performed by EMSA. A 31mer oligonucleotide for ssDNA binding assay and a 33mer oligonucleotide for dsDNA binding assay were used for this purpose. Fluorescence based assays were carried out to test the binding of OsMre11 protein to different forms of DNA, where a change in tryptophan fluorescence of the protein OsMre11 upon binding to DNA was monitored. The endonuclease property of OsMre11 was determined by its ability to convert RF I DNA (CCC form) to RF II DNA (Nicked Circular), upon incubation with increasing amount of protein in presence and absence of $MnCl_2$ using agarose gels. To demonstrate the exonuclease property of OsMre11 protein, the nucleolytic degradation of linearized pSK plasmid in presence and absence of $MnCl_2$was monitored. To determine the renaturation ability of OsRad52, a 2.0 kb fragment was heat denatured in water bath at 100 °C for 10 min and immediately frozen in liquid nitrogen and used. For renaturation assay by FRET, Phi-C oligonucleotide labelled with rhodamine at 3′ end and its complimentary oligonucleotide Phi- W labelled at the 5′ end with fluorescein was used. The decrease in fluorescence emission intensity of fluorescein at 520nm after excitation at 480nm as a result of renaturation was measured. Result from all these experiments were presented in chapter 3 (OsMre11) and chapter 4 (OsRad52).

Chapter 3 of the thesis discusses the characterization of OsMre11 in detail. Mre11 has been genetically characterized in *Oryza sativa* and in *Arabidopsis.* Functional and full-length AtMRE11has been shown to be essential for activation of the cell cycle arrest, transcriptional regulation and DNA repair upon induction of DSB (6). Genetic studies have shown that OsMRE11 deficient rice plants exhibited normal vegetative growth but could not develop seeds, homologous pairing of chromosomes was abolished and extensive chromosomal fragmentation occurred during anaphase I (7). However, OsMre11 has not yet been biochemically characterized.

Using *Arabidopsis* MRE11 sequence, analysis of rice genome showed the presence of MRE11 gene. The full length cDNA corresponding to OsMRE11 was procured from Rice Genome Resource Center, (RGRC, Japan). The OsMRE11cDNA (~2115 bp) was cloned into pET28a vector and transformed into *E. coli* BL21 (DE3) pLysS cells. Upon over- expression, an induced band (~80kDa) corresponding to OsMre11 (705 amino acids) was observed. OsMre11 protein was observed to form inclusion bodies and several methods were followed to purify the full length protein in the native state. These methods included use of N-lauroylsarcosine and arginine for protein refolding, solubilization and purification, cloning OsMRE11 into pCold-TF vector, chaperon (Takara Chaperon plasmid set) assisted co-expression and purification of OsMre11, use of *Kluyveromyces lactis* expression system (yeast) to clone OsMRE11, use of Shuffle T7 Express lysY competent *E. coli* system to express OsMre11 protein in native form. In all these methods, the full length protein was expressed in either very low levels or not induced at all. Further, purification of the full length protein from the inclusion bodies was carried out, however extensive degradation was observed.

Upon analysis of the sequence of full length OsMre11 and it showed presence of 42% random coils, which probably might be a reason for the degradation of protein upon purification in vitro. Hence, it was proposed to study the functional domains of OsMre11 protein. Upon conserved domain analysis, it was observed the protein has 2 domains namely

1. Mre11 nuclease, N-terminal metallophosphatase domain (9-277)
2. Mre11 DNA-binding domain (300-452).

We have initiated the study of N- terminal domain of OsMRE11 gene, which harbors both DNA binding and nuclease domains (N-terminal 452 amino acids). cDNA corresponding to this region of OsMRE11(~1380 bp) was cloned into pET16b vector and transformed into *E. coli* shuffle T7 Express lysY system. Upon over- expression, a ~55kDa band corresponding to N- terminal OsMre11 (460amino acids) was observed. Purified OsMre11 N-terminal domain was subjected to following biochemical characterization.

a) The CD spectrum of OsMre11 showed a negative peak at 200nm, typical of helical proteins, confirming the theoretical prediction that purified rice Mre11 protein has helical structures in its native state.
b) The ability of OsMre11 protein to bind to different forms of DNA was probed and a protein concentration dependent ssDNA, dsDNA and G4 DNA binding is observed.
c) Fluorimetric assays showed an increase in tryptophan fluorescence of the protein, upon binding of the protein to all 3 different forms of DNA (dsDNA/ssDNA and G4 DNA) mentioned above.

d) Endonuclease experiments showed that OsMre11 protein facilitated the conversion of RFI DNA to RFII DNA, only in presence of $MnCl_2$.

e) OsMre11 showed divalent cation dependent endonuclease activity (Ca^{2+}, $Mg2^{+}$and Mn^{2+}), but not in presence of monovalent cation like K^{+}.

f) Exonuclease experiments showed that OsMre11 protein facilitated the degradation of dsDNA only in presence of $MnCl_2$.

g) Upon testing different forms of metal ions (monovalent and divalent) required for exonuclease activity, it showed that only Mn^{2+} is able to exhibit the exonuclease property but not any other divalent or monovalent cations.

Chapter 4 of the thesis discusses the characterization of OsRad52 in detail. The RAD51 and RAD54 homologues had already been identified in plants and it was reported in a recent study that RAD52 homologues are present in all those plants whose genome has been thoroughly sequenced (8). In 2012, Janicka et al. proposed that Organellar DNA Binding protein 1 (ODB1) found in plant mitochondria may assume functions similar to that described for nuclear RAD52 (9). However, no biochemical data is available for crop plant Rad52 protein. For the first time, in this study Rad52 protein of plant origin i.e. from *Oryza sativa* rice (OsRad52-2a) has been purified and biochemically characterized.

Using the Rad52 sequence from *Arabidopsis*, bioinformatic analysis showed the presence of its homolog in rice. The full length cDNA clone for RAD52 available with the RGRC cDNA library was procured. The OsRAD52 cDNA (~700bp) was cloned into pET28a vector and transformed into *E. coli* BL21 (DE3). Upon over- expression, a ~30kDa band corresponding to OsRad52 (192amino acids) was observed.

Purified OsRad52 protein was subjected to following biochemical characterization.

a) The CD spectrum of OsRad52 showed two negative peaks at 209 and 225nm, typical of helical proteins, confirming the theoretical prediction that purified rice rad52protein has helical structures in its native state.

b) Native PAGE analysis showed that OsRad52 protein appeared as a single band with an approximate molecular weight of 226 kDa (undecameric structure). The molecular sieve column chromatography confirmed an undecameric structure of the protein with the protein eluting at a size of 26kDa.

c) MALDI-TOF analysis was carried out to determine the over-expressed and purified protein's identity. A sequence coverage of 66% confirmed that the over-expressed protein was indeed OsRad52.

d) Nucleic acid binding assays exhibited that as protein concentration increased, ssDNAand dsDNA binding also increased.

e) OsRad52 protein mediated duplex DNA formation of complementary single stranded DNA at a higher protein concentration as compared with control having no protein. Renaturation activity was also studied by FRET assay, where in FRET efficiency was expressed as a function of time at different concentration of the protein. The extent and rate of renaturation was high, i.e. within 100sec the reaction did reach a steady state level at the highest protein concentration tested.

Chapter 5 deals with the summary and conclusion of the work and future directions of the project. It has already been established that HR is an important process in all living organisms, responsible for maintaining genomic stability and also for establishing genetic diversity. Although the proteins involved in the HR pathway is

well characterized in yeast, *E. coli* and humans, the same is yet to happen with plants. Several studies have been led to study of genes involved in HR in plants such as *A. thaliana, O. sativa, L. longiflorum, Zea mays, P. patens.* In 2013, Jianhuiji et al. showed that Mre11 gene in rice if silenced, the OsMre11 deficient plants did not seed or showed severe chromosomal fragmentation and inhibition of HR. These plants displayed defective pollen, reduced fertility and defective meiosis (almost all events of DSB processing and cross over formation is inhibited). AtMre11 has been shown to be essential for activation of cell cycle arrest and DNA repair initiation upon encountering a DSB (6). In consistent with these findings, our results show biochemically how the OsMre11 protein binds to different forms of DNA. OsMre11 protein is also able to exhibit the endonuclease and exonuclease property, a highly conserved function of Mre11 protein upon encountering a DSB. It is concluded that the Mre11 nuclease activity is highly conserved which is dependent on divalent cation like Mn^{++}.

Till recent time it was thought that RAD52 is absent in plant systems and its function was compensated by BRCA2. Samach et al. (2011) suggested that Rad52 protein plays an important role in both somatic and meiotic recombination where as Janicka et al. (2012) reported the presence of mitochondrial Rad52 called ODB1 and its role in mitochondrial DNA repair. In both these reports, the authors have proposed the presence of RAD52 homologues in plants and their role in DNA repair. Based on the binding properties of OsRad52 protein to different forms of DNA, and the annealing of complementary strands to from duplex DNA, which is an important function of Rad52 group of proteins supports our conclusion that the rice Rad52 protein indeed belongs to Rad52 family proteins. In the present we have characterized two important

proteins OsMre11 and OsRad52 from rice and conclude that both the proteins mediated the important functions in homologous recombination and DNA repair.

Significance and future directions: The thesis details the biochemical characterization of OsMre11 and OsRad52 which is an important milestone in determining the biochemistry of HR in plants. These proteins being important in HR, it would be worth to investigate the structural properties of both these proteins and study the interacting partners which might give an insight into the functioning of the protein in vivo and detail the immediate requirements of the protein to carry out its function in vivo.

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Signature of Student:

Date: 20/11/2017

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1.	Dr. T.R. Ganapathi	Chairman		20-11-17
2.	Dr. Rajani Kant Chittela	Guide/ Convener		20/11/17
3.	Dr. P. Mukherjee	Member		
4.	Dr. H.S.Misra	Member		23/11/17
6.	Dr. H. Rajaram	Member	Hema Rajaram	21/11/17

List of abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BME	ß-mercaptoethanol
cDNA	Complementary DNA
DS	Double stranded
DSBs	Double strand breaks
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetate
FRET	Fluorescence Resonance Energy Transfer
HR	Homologous Recombination
kDa	Kilo dalton
MALDI	Matrix-assisted laser desorption/ionization
M.Wt	Molecular weight
NHEJ	Non-homologous End Joining
Ni-NTA	Nickel chelating affinity matrix
OsMre11	Oryza sativa Meiotic Recombination 11
PAGE	Polyacrylamide gel electrophoresis
RF	Replicative form
SDS	Sodium dodecyl sulphate
SS	Single stranded
SSA	Single strand annealing
TEMED	Tetramethyl ethylene diamine
Tris	Tris-(hydroxymethyl) aminomethane

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CHAPTER I

INTRODUCTION

DNA, the genetic material of living organisms is often exposed to several damaging agents, even in the best of situations, which in most cases may prove lethal to the survival of the cell and ultimately the organism itself. DNA, being the template for basic processes such as replication and transcription, essential for maintaining life, makes genomic stability an important process for viability of the cell. Genomic instability can be defined as higher than normal rates of mutation, which if unrepaired can lead to catastrophic consequences (1). Even before DNA double helix structure was proposed in 1953, it was known that exogenous agents such as X-rays, ultraviolet (UV) light, and various chemicals, can cause genetic changes (2). Therefore, it was evident that DNA can be damaged by both endogenous agents such as oxidative stress, replication errors etc. as well as exogenous agents, such as exposure to xenobiotic agents, radiation etc.

DNA damage can be broadly classified into three types: mismatched bases, double-strand breaks, and chemically modified bases which can lead to mutations, cell cycle arrest etc. Each of these classes of lesions, is in turn, corrected via distinct repair pathways, to conserve the genomic integrity. Whenever the cell encounters a DNA damage, the lesion triggers specific repair mechanisms to repair the lesion and hence retains genomic integrity (3). Damaged DNA is mainly repaired by Base Excision Repair (BER), Nucleotide Excision Repair (NER), Photo-reactivation, Mis-Match Repair (MMR) and Double Strand Break Repair (DSBR) pathways.

Of all types of DNA damage studied till date, DNA double-strand breaks (DSBs) are considered to be more lethal to living systems. DSBs can arise during natural cellular processes, such as immunoglobulin gene rearrangement, replication fork collapse, and meiotic recombination (4, 5). Likewise, exogenous agents, including ionizing radiation (IR), radiomimetic, and topoisomerase II inhibitors, such as etoposide, also cause DSBs. If left unrepaired, DSBs pose a severe threat to genome stability, leading to chromosomal rearrangements and fragmentation (5).

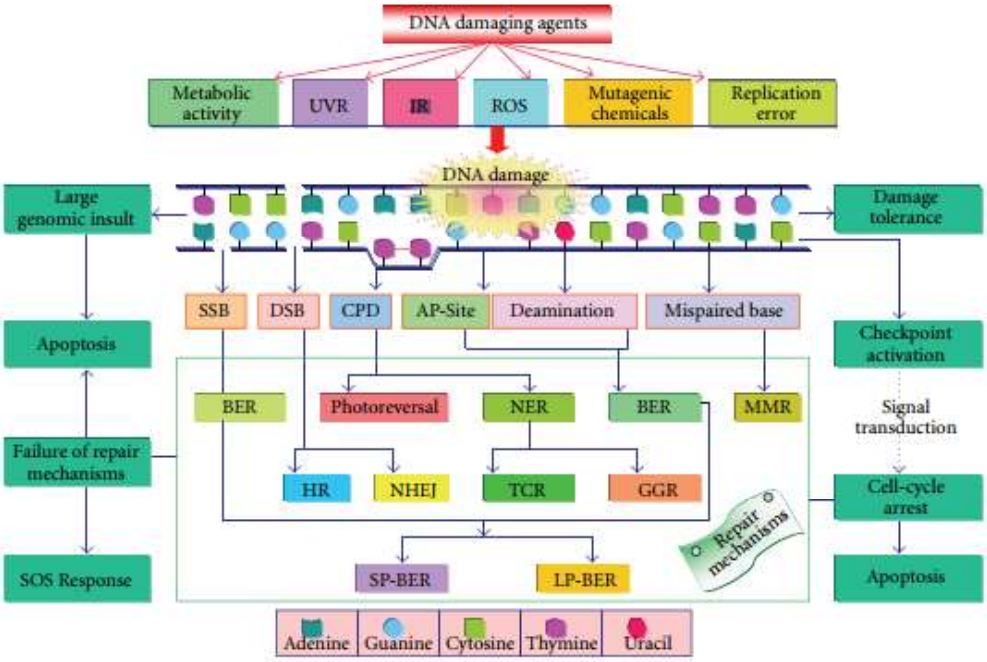

Figure 1.1: Types of DNA damage and its repair mechanism (adopted from Sinha, 2010) *Damage to the genomic DNA can be caused by several DNA damaging agents, which in turn triggers several specific repair machineries to repair the damage and help conserve the genomic integrity. If at all the repair mechanism fails due to severe damage, the cell goes into apoptosis or induces the SOS response. The cellular response to DNA damage activates the cell-cycle checkpoint mechanism with the help of a signalling network pathway that gives the cell time to repair genomic lesions or may induce programmed cell death (PCD).*

[UVR: Ultraviolet Radiation, ROS: Reactive Oxygen Species, SSB: Single Strand Breaks, DSB: Double Strand Breaks, CPD: Cyclobutane Pyrimidine Dimers, AP site: Apurinic/ Apyrimidinic site, BER: Base Excision Repair, NER: Nucleotide Excision Repair, MMR: MisMatch Repair, HR: Homologous Recombination, NHEJ: Non- Homologous End Joining, TCR: Transcription Coupled DNA Repair/ Transcription Coupled NER, GGR: Global Genomic NER, SP- BER: Short Patch BER, LP- BER: Long Patch BER].

Three major pathways operate to repair DSBs: Homologous Recombination (HR), Non-Homologous End Joining (NHEJ) or Single Strand Annealing (SSA) processes. Homologous recombination pathway involves DNA sequence homology between interacting pairs where as non-homologous end joining is independent of significant homology. The former is an accurate pathway and the latter is an inaccurate or error prone pathway as the genetic information between the two DNA ends is lost during processing. HR is important, as it is involved in repairing DSBs formed during both meiosis and mitosis in eukaryotes. An alternate pathway, SSA operates when a DSB is formed between two closely spaced direct repeats on DNA. This pathway shares proteins involved in the above two processes, HR and NHEJ.

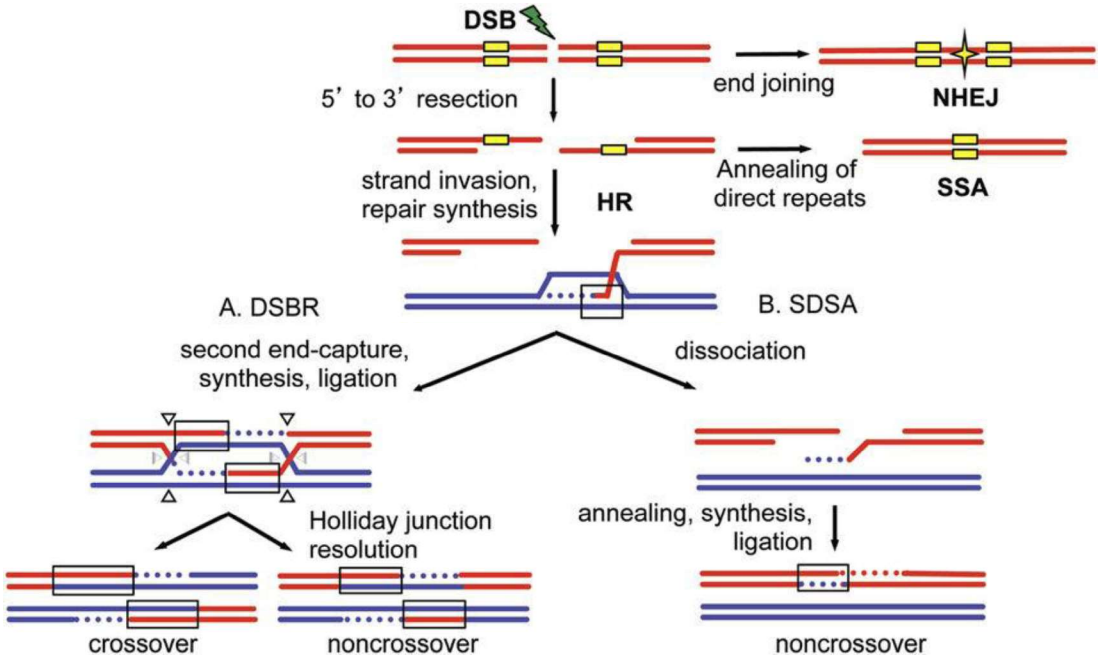

Figure 1.2: Mechanism of DSB repair (adopted from LaRocque, 2015)

Upon encountering damage to DNA, a cell with the help of proteins involved in the signalling pathway initiates the repair pathway to overcome damage to its DNA. The major mechanisms found to be conserved across species includes the homologous recombination (HR) pathway, the non-homologous end joining pathway (NHEJ), the double strand break repair pathway (DSBR) and the synthesis dependent annealing pathway (SDSA).

In Homologous Recombination (HR), whenever a DSB occurs, the ends are resected to generate 3' overhangs, which are then bound by HR proteins to form a nucleoprotein filament, capable of strand invasion and homology search. This 3' overhangs invade the sister chromatid/ homologous chromosome and extends, while the displaced strand forms a D- loop. At this point, the free 3' overhang finds homology with the D- loop and extends. Branch migration facilitates the formation of Holliday junctions. Resolution of the Holliday junction followed by ligation leads to the formation of cross over and non-cross over products.

In Non-Homologous End Joining (NHEJ) pathway, the ends of DSBs are bound by the Ku70-Ku80 heterodimer, which repairs DSB with the help of DNA-Pkc, MRN complex, DNA ligase 4 and Atremis nuclease. These proteins cause end modifications, gap filling followed by ligation.

The Single Strand Annealing pathway, operates when a DSB occurs between two adjacent repeat sequences. In this process also, ends are resected to create 3' overhangs. The overhangs find homology with each other and anneals. As a result, two flaps are formed, which are subsequently removed, gaps are filled and ligated. The major difference between DSBR and Synthesis Dependent Strand Annealing (SDSA) being, after extensive DNA synthesis is primed from the invading strand, the elongated strand is displaced and pairs with the other side of the break in SDSA. Hence, non-crossovers are generated by SDSA and crossovers by DSBR on resolving the Holliday junctions. Thus, differences in the mode of repair mechanism, result in the loss or conservation of the information after DSB repair. Major enzymes participating in the HR pathway are evolutionarily conserved in eukaryotes and prokaryotes.

Since the thesis deals with biochemical characterization of proteins involved in the HR pathway in plants, a brief outline of homologous recombination in *E. coli* and eukaryotes and an introduction to homologous recombination in planta is included below.

1.1: Homologous Recombination:

Homologous recombination (HR) is defined as a DNA metabolic process, present in all known forms of life which ensures high-fidelity, template-dependent repair or tolerance of complex DNA damages including DNA gaps, DNA double-stranded breaks (DSBs), and DNA interstrand crosslinks (ICLs) (6). This process is essential for the generation of genetic diversity, maintenance of genome integrity and for proper segregation of homologous chromosomes during meiosis (7) and for telomere maintenance (8).

Homologous recombination reaction is classified into two types namely generalized and site specific depending upon the sequence homology required for the process. Generalized homologous recombination occurs between the two DNA molecules, that share identical sequence. In eukaryotes, meiotic recombination is the best example of the generalized recombination. The site specific recombination as the name suggests, occurs at a specific, pre-determined sites in the genome. Lambda phage integration at *cos* site in *E. coli* genome and mating type switching in the yeast at *mat* locus are the examples for this type of recombination.

In HR, use of one DNA molecule as a template for repairing a damage encountered in the second DNA molecule requires close association between these two DNA molecules. Robin Holliday in 1964, suggested that recombination can also be attained through the physical exchange of DNA strands from different DNA duplexes, by forming a crossover intermediate that later was termed as the Holliday junction. The Holliday model has since been polished to accommodate increasing knowledge of HR and several models have also been developed to account for recombination in particular circumstances, such as those that occur between repeated homologous sequences.

Though the importance of double strand breaks and its repair by homologous recombination in prokaryotes and eukaryotes was realized long time ago, the molecular mechanisms of this

process were revealed with the advances in genetic and molecular approaches. The genes and proteins involved in HR was determined by studying the mutant's sensitive to DNA damaging agents like UV and ionizing radiation, which showed deficiency in recombination (9, 10, 11).

1.2: Homologous recombination in *E. coli*:

Most of our knowledge concerning the biochemistry of HR pathway has been gained through extensive studies on simple, well studied bacteria *E. coli*. About two dozen proteins are known to be involved in homologous recombination in *E. coli* and several of these have already been purified, to study their biochemical roles. Of these, only a handful of key proteins are involved in the fundamental steps of strand exchange, branch migration and resolution.

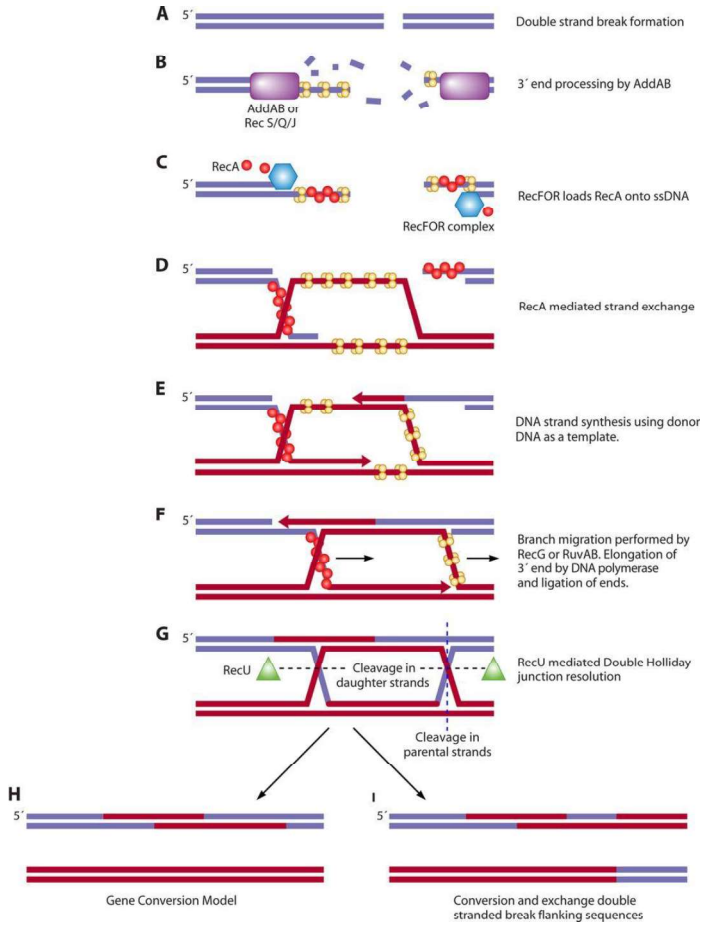

Figure 1.3: Homologous recombination in E. coli (adopted from Simmons, 2012)

Figure 1.3 outlines the mechanism of HR and double Holliday junction formation in E. coli. Once the cell encounters a DSB, the ends are processed by the AddAB helicase-nuclease complex, or by the RecQ or RecS in combination with RecJ. The 3'-ssDNA strand formed on both sides of the DSB, is then bound by SSB. This is followed closely by recruitment of RecFOR which helps to load RecA, generating a 3'-ssDNA–RecA nucleoprotein filament, capable of homology search. This leads to displacement loop (D loop) formation, wherein one strand of the template DNA is displaced by the RecA filament during pairing. DNA polymerase then extends the 3' ends of both invading strands, using the homologous strand as a template for DNA synthesis. The RecG protein or the RuvAB complex facilitates migration of the D loop. Resolution of the double Holliday junctions is facilitated by RecU or RecV. Depending on the location where the endonuclease cut occurs, different exchanges between the two strands is generated, leading to either gene conversion or exchange of homologous sequence.

The protein machinery capable of carrying out the three important stages of HR in *E. coli* is the RecA recombinase. It alone can mediate homology recognition and DNA strand exchange. DNA unwinding, nucleolytic processing and it's loading, the first step of the pre- synaptic stage of HR is co- ordinated by the action of a single protein complex the RecBCD. Alternatively, a recombination competent DNA end can also be produced through unwinding by RecQ, nucleolytic processing by RecJ, following which RecF, RecO, RecR helps load RecA. The RecA coated nucleoprotein filament is capable of mediating homology recognition, strand invasion followed by exchange. The RuvAB protein complex is the molecular motor which helps to migrate the Holliday junction generated by the extension of heteroduplex DNA molecules. The RuvC protein interacts with RuvAB protein and functions as the Holliday junction resolvase, thereby helping in resolving or nicking the junction liberating the recombining molecules for repairing the broken ends (12). The resolution of these Holliday

junctions, is the major factor in determining whether or not the flanking sequences are exchanged after recombination (13).

1.3: Homologous recombination in higher organisms:

Most of the available reports on recombination in eukaryotic organisms has been obtained from yeast genetics. Several of these genes and thereby proteins identified in yeast have direct homologues in humans. Therefore, it can be stated that, eukaryotes and higher organisms have molecular machinery similar to those described for HR mediated repair in *E. coli*. The one and only protein conserved at the amino acid sequence level is RecA in *E. coli* and Rad51 in eukaryotes. Similar to the function of RecA, the Rad51 protein binds DNA to form a nucleoprotein filament that catalyses homologous pairing and strand exchange. But strand exchange by Rad51 in eukaryotes is stimulated by the presence of additional factors such as the Rad52 protein or a complex containing the Rad55 and Rad57 proteins. Another protein complex biochemically similar to the RecBCD complex in prokaryotes is the MRN complex in eukaryotes, which consists of the Mre11, Rad50 and Nbs1 proteins. The MRN complex has nuclease properties and can apparently migrate along the DNA. The MRN complex also aids in keeping the DNA broken ends in close proximity so that they can be efficiently processed (12).

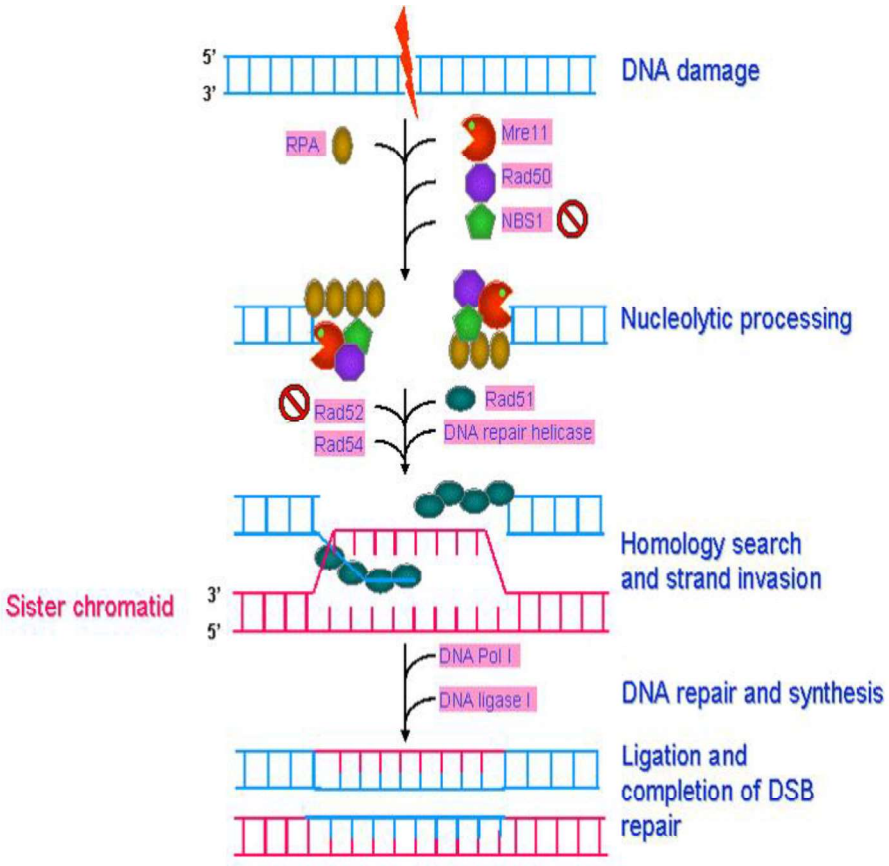

Figure 1.4: Mechanism of HR in eukaryotes (adapted from malarial parasite metabolic pathways tutorial, DSB repair and HR, http://mpmp.huji.ac.il/maps/dsbRecomb.html)

In the double strand break repair model shown in Figure 1.4, the double strand break introduced in one of the two homologous chromosome is resected by nuclease MRX/MRN to expose the 3'ends. With the help of RPA, Rad52, Rad55, Rad57 and Rad51 the ssDNA locates the complementary region on homologous chromosome, resulting in strand invasion. The 3' ends are then used as primers for new DNA synthesis, at the gap sites. Resulting Holliday junction is resolved and gaps sealed by ligase to generate repaired molecules.

Once a DSB occurs, the MRN complex, which is one of the earliest responders to DSBs, binds to the broken ends and trims the 5' ends on either side of the break to generate 3' overhangs of ssDNA. To these 3' overhangs, RPA protein binds which has high affinity to ssDNA. Rad52

protein then helps to load the Rad51 protein onto the RPA coated ssDNA, thereby forming a nucleoprotein filament capable of homology search and strand invasion, leading to formation of D- loops. DNA polymerase then extends the 3' overhangs of the invading strand thereby forming Holliday junctions, which are then resolved by endonucleolytic cleavage to generate cross overs or non- cross overs.

1.4: Homologous recombination in plants:

Plants, because of their sessile, phototrophic nature of lifestyle and intrinsic immobility, are bound to be exposed to different environmental agents (UV, IR, cross linking agents like mitomycin C and cisplatin, fungal and bacterial toxins) and endogenous processes(reactive oxygen species, hydroxyl radicals, superoxide and nitric oxides, and damages occurring during normal processes of replication and recombination) that pose a threat to its genome(14).As a result, plants are highly prone to DNA damage which leads to immense genotoxic stress, which in turn lessens the plant genome stability. Oxygen and sunshine (UV) are the two major genotoxic agents for most organisms. Plants because of their need for both oxygen and sunshine to carry out the process of photosynthesis, are obliged to be exposed infinitely to both of these mutagens (15). Hence, plants, like all other living entities, have evolved mechanisms that enable them to tolerate or repair the DNA damage they inevitably experience. Damage to a plants genome, results in various physiological effects, such as reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins, which in turn affects the growth and development of the whole organism. Though most of the studies on genome repair and genome stability maintenance have been carried out in *E. coli* and higher organisms as already explained, in recent years, there has been increased interest in plant DRR (DNA Repair and Recombination) mechanism and in using plants as model for understanding these processes. However, it is necessary to point out that plant DRR genes are still not explored as much as human, rodents, yeast or microorganism counterparts (16).

The *Arabidopsis* genome sequence project (genome size ~127 Mbp) and the rice genome sequence project (genome size ~389 Mbp), revealed many repair proteins which are similar to those of humans. Ingenious use of several different techniques has led to the identification of genes and regulatory elements in these plants. Several functional studies carried out in these plants, have shed light on how plants cope with DNA damage caused by exo- and/or endogenous factors. It has been widely reported that the genome of the plant encodes several orthologues of most of the proteins involved in eukaryotic genomic integrity (16).

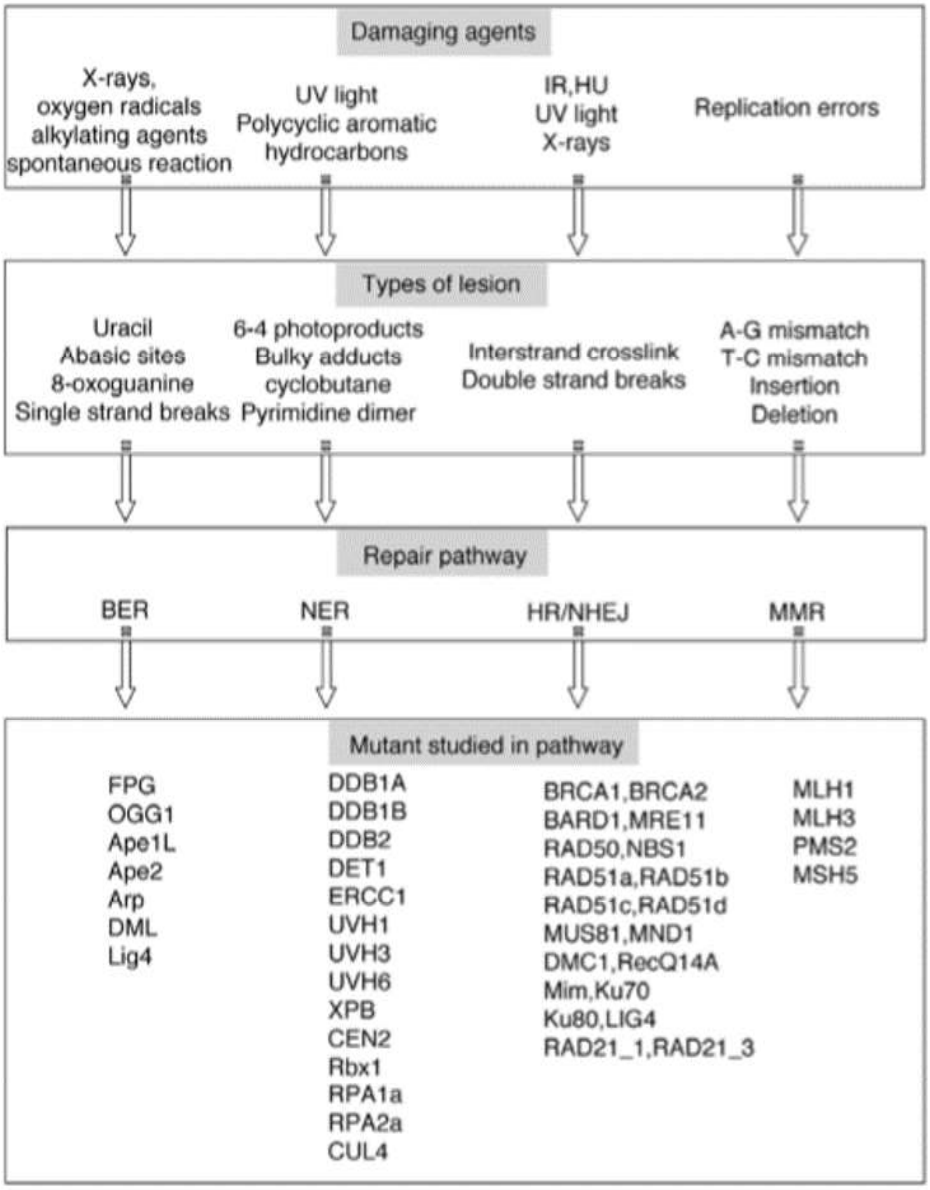

Figure 1.5: List of Arabidopsis mutants of DNA repair and recombination genes (Sengupta, 2010)

The genome of the plant has been found to encode orthologs of most proteins used by eukaryotes to maintain genomic integrity. Several DRR genes mutant line which leads to embryonic lethality or infertility in mammals are tolerated by plants. Plants are therefore, projected as excellent models to study the DRR because of their high tolerance to DRR gene mutations and substantial homology to mammalian DRR proteins.

The plant DNA repair and recombination machinery is more closely related to human as compared to yeast because both plant genomes (rice and *Arabidopsis*) were found to retain more mammalian homologs than their yeast counterparts. Many of the plant DRR proteins identified were anticipated to function in a number of diverse activities unrelated to DRR process, hence suggesting a probable role in normal plant growth and development (14, 16).

In plants, homologous recombination occurs in meiosis and during somatic development. In meiosis, a single round of DNA replication followed by two rounds of cell division take place to generate haploids (17). During this process, HR allows information on both the parental chromosomes to recombine and to generate new combinations. This leads to evolution in subsequent generations. Hence the frequency of recombination is supposed to govern the speed of the evolution process in the living organisms (18). However, at chromosomal level, the frequency of HR events is not constant (19; 20; 21). It was proposed that meiotic HR process is confined to structural genes as number of genes and genetic length across the organisms is almost constant in spite of complexity and genome sizes increased (22). The meiotic HR is confined to coding regions, however, its frequency is not equal for all genes i.e. different gene loci showed different recombination frequency (23; 24). The differences in the frequency of HR may be due to the sensitive nature of the upstream sites of the genes for nucleases to generate DSBs. During the transcription process, chromatin structure changes to access the DNA by transcription factors. At the same time, these active areas may be substrates for meiotic DSBs formation (25).

Studies on DSB repair in plants concentrate mostly on HR. This is because of immense interest in HR for its application to genome manipulation (‘gene targeting’), and also on the consequences, DSBs as intermediates in meiotic recombination (26). As already stated, most of the information on mechanisms of DSB repair in plants is mainly available from the studies in *Arabidopsis* (26, 17, 27). AtRad51 (28), AtRad50 (29; 30) and AtMre11 (31) which are all

involved in HR, have been isolated and analysed. Differing requirements for the activities of *Arabidopsis* AtRad51 paralogues in DNA repair and meiosis have been observed (32). Xrs2 mutant in *Arabidopsis thaliana* plants showed hypersensitivity to DNA damaging agents like X-rays, MMC and MMS with decreased somatic HR and increased meiotic HR (33, 34). Using reverse genetics approach, Rad9, Rad50 and Rad17 mutants have been characterized in *Arabidopsis*. These mutants show increased somatic HR, sensitivity to DNA damaging agents like MMS, MMC, UV radiation, bleomycine and defects in DSB repair mechanism (26, 29, 30, 35)). Though several genes involved in HR pathway in plants have been identified, biochemistry of most of these proteins are yet to be elucidated except Dmc1, Rad51, RadA which have already been biochemically characterized in plants. OsDmc1 and OsRad51 has been shown to bind to both ssDNA and dsDNA and mediate renaturation of complementary single strand into duplex DNA molecules leading to D-loop formation (36, 37, 38). Two isoforms of these two proteins have also been characterized in plants. OsDmc1A and OsDmc1B bound to both ssDNA and dsDNA and also promoted strand exchange irrespective of the presence or absence of RPA (39). On the other hand, OsRad51A1 and OsRad51A2 bound to both ssDNA and dsDNA and their DNA binding activity strictly required ATP (40). Another RecA like protein however differing from Dmc1 and Rad51 was characterized biochemically (41) and the protein was designated OsRadA. This novel protein was found to be present in plants but not in animals or yeast. The protein showed D-loop and ssDNA dependent ATPase activities.

This thesis deals with the biochemical characterization of two important proteins participating in HR, the Mre11 protein which is one of the earliest responder to a DSB, and a part of the MRN complex. The other is the Rad52 protein which is responsible for efficient loading of Rad51 protein on to resected 3' overhang of a DSB, to form a nucleoprotein filament capable of homology recognition and strand invasion. Although the role of both these proteins have

been discussed as a part of mechanism of HR in eukaryotes, a literature survey of these proteins is detailed below.

1.5: MRN Complex:

The MRX/N complex consists of Mre11, Rad50 and Xrs2 in case of yeast and Nbs1in case of vertebrates, are the first responders to double strand breaks. The role of MRN complex includes scanning DNA double strand for a break, and consequently binding to DNA as soon as it encounters a DNA break (42). It was shown that MRN complex has a globular DNA binding head, elongated mobile Rad50 heads and a distal Rad50 hook domain (43, 44, 45). Upon encountering a DSB, MRN recruits the ATM kinase by binding to Nbs1 domains (46, 47). The Mre11 protein of the MRN complex is highly conserved in eukaryotes, with the N- terminal end sharing several sequence motifs with *E. coli* SbcD protein, protein phosphatases and bacteriophage T4ngp46 nucleases (48).

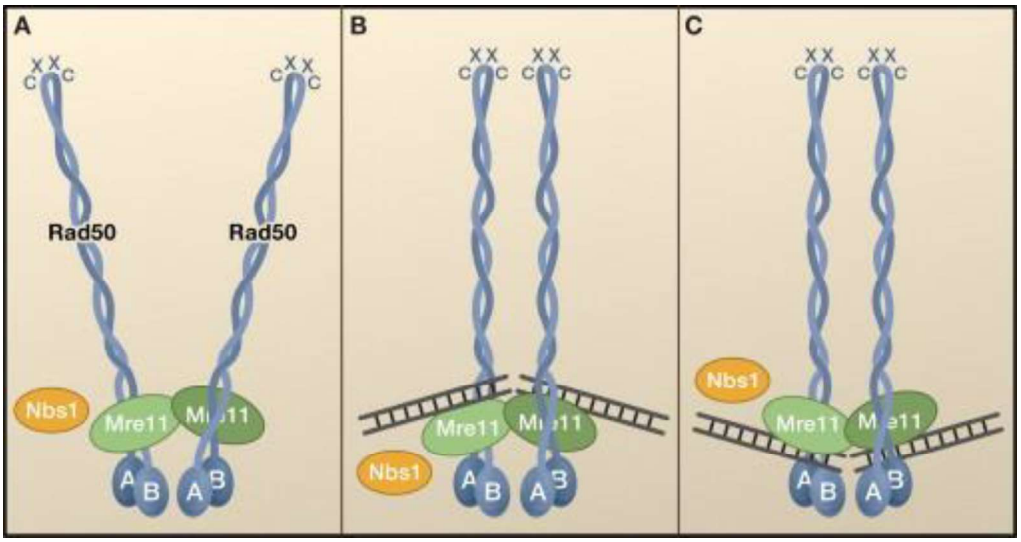

Figure 1.6: MRN complex and its interaction with DNA upon encountering a DSB (adopted from Wyman, 2008)

The MRN complex consists of a globular domain that contains the Walker A and B ATPase domains of both Rad50 molecules and the Mre11 dimer. The Nbs1 protein is part of the

globular domain. Emanating from the globular domain are the 50 nm flexible coiled coils ofRad50, which carry the intercomplex interacting zinc hook domain (CxxC) at their apex. (B and C).

Although, null mutants of MRX complex in yeasts are viable, they show similar phenotypes of reduced growth, high sensitivity to IR and defects in meiosis (49). But the null mutants of this complex in vertebrates and mouse embryonic cell lines are lethal. Along with the genetic studies, yeast and human Mre11 has been widely characterized biochemically. These studies have revealed that Mre11 protein alone has several enzymatic activities such as DNA binding, exo- and endonuclease in a Mn^{2+} dependent manner (50). Functional elucidation revealed that yeast Mre11 has an important role to play in telomere length maintenance, meiotic recombination and in cell cycle checkpoint (51). It was also shown that the ScMre11 protein can bind with high affinity to G4 DNA, over ss or dsDNA and binding of OsMre11 protein to G4DNA was much stronger than binding to any forms of G rich DNA and it showed endonuclease activity which is dependent on Mn^{2+}. Human Mre11 have been shown to possess 3'- 5' exonuclease and endonuclease activity upon binding to hairpin substrates (50).

Apart from these studies, the significance of Mre11 proteins have also been studied in plants, though most of the studies are limited to genetic and mutant analysis in rice, *Oryza sativa* and *A. thaliana*. Samanic et al. (51), performed comparative analysis of several Mre11 alleles to study the genome stability and meiosis in *A. thaliana.* According to the study, Mre11 mutants showed different phenotypes in regards to growth morphology, genome stability and meiosis. T-DNA insertion mutagenesis in the MRE11 gene shown that the region between 499-529 amino acids in the N- terminal region of the protein (which is widely conserved) is critical for the meiotic function in *A. thaliana*.

Samanic et al. (52), studied the impact of MRE11 mutations on DNA damage response and repair in *A. thaliana*. A functional full- length AtMRE11 gene is essential for several in-vivo activities such as the cell cycle arrest, transcriptional regulation and DNA repair upon induction of DNA damage.

Hong et al. (53), studied the rice ortholog of Mre11 protein, the pOsMre11. This protein was constitutively expressed in several tissues and 10-day old rice seedlings that were exposed to several genotoxic agents. Level of OsMRE11mRNA increased after 3 days of irradiation. Treatment with defence-related hormones also activated the OsMRE11 gene. It was concluded that the possible function of OsMre11 protein, is to maintain the stability of rice chromosome.

JianhuiJi et al. (54), reported that MRE11 is required for DSB processing in rice meiosis. MRE11 gene was silenced and its function studied by cytological and molecular methods. Although the OsMRE11- deficient plants showed normal vegetative growth, seed formation was hampered, homologous pairing of chromosomes was severely repressed and the chromosomes were entangled causing fragmentation during anaphase. Taken together, they concluded that OsMRE11 performed a function essential for preserving normal HR process.

1.6: Rad52 protein:

The RAD52 epistasis group of genes RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH57, MRE11 and NBS1 were identified using complementation assays (49). Mutations in these genes have been shown to cause defects in meiotic or mitotic recombination or both. Two pathways for homologous recombination have been defined, based on the involvement of Rad51 recombinase, namely the Rad51 dependent and the Rad51 independent pathways. In the Rad51 dependent pathway (error free pathway), Rad52 mediates the loading of Rad51 onto ssDNA ends, thereby forming a nucleoprotein filament which invades the homologous duplex partner and acts as a template for DSB repair. In the alternate Rad51

independent pathway, also known as the single strand annealing pathway, Rad52 directly promotes annealing of two direct repeats by binding to the broken ends. This pathway is error prone and results in loss of genetic material between two direct repeats.

RAD52 is reported to be highly conserved across the kingdoms and homologues of RAD52 have been identified in several eukaryotes, in some prokaryotes and in archaea, further signifying its role in HR (49). Though RAD52 is well-conserved across different species, majority of the biochemical studies on the protein is available either from yeast or human systems. Based on these reports, Rad52 is thought to imitate the DNA annealing functions of RecT (*E. coli*) and β- protein of bacteriophage (55). Human and yeast Rad52 has been proposed to preferentially bind to ssDNA and promote annealing of complementary ssDNA into duplex dsDNA (56, 57). Both yeast and human Rad52 proteins promote Rad51 mediated strand exchanges (55, 57). Rad52 protein also participates in the maintenance of telomeres in cells that are devoid of essential components of yeast telomerase (58). The protein is reported to have greater binding affinity for ssDNA than for dsDNA (55, 59). Rad52 protein is also found to be proficient in capping DNA termini (55) and hence protect DSBs from nuclease attack once the ends are resected to form ssDNA tails and promote ligation (60).

The electron microscope studies of human and yeast Rad52 proteins have shown the formation of oligomeric ring (9-13 nm in diameter) like structures as well as higher order aggregates (61, 62). Ring formation depends on the self-association domain present in the N-terminal half of Rad52 and the higher order complexes of the ring are formed by elements found in the C-terminal half of the molecule (63, 64). X-ray crystallographic studies of human Rad52 protein have revealed the formation of an undecameric ring structure with a groove on the surface layered by a large quantity of basic and aromatic residues (65). Mutational analysis of these residues on both human and yeast Rad52 proteins has shown that these residues are important for ssDNA binding. Two DNA binding domains have been suggested to be present in the

Rad52 protein. ssDNA binding domain located at the N-terminal portion of the protein and a second DNA binding site at the C- terminal half of the protein which can bind to both ssDNA and dsDNA (66, 67).

Till recent, it was thought that Rad52 homologues are absent in plant systems. The DMC1, RAD51, RAD54 homologues have already been identified in plants leaving the question of whether RAD52 homologues are present in plants or its absence is compensated by the presence of two BRCA2 homologues (68, 69), which are believed to participate in a common pathway to facilitate orderly HR and hence maintain genetic stability (70). However, in a recent study, it was reported that RAD52 homologues are present in plants also (71). A computational analysis has shown that in vascular land plants, RAD52 genes are conserved throughout but are yet to be characterized. The plant RAD52 homologues were identified in *Arabidopsis thaliana* by using yeast RAD52 sequence as a probe. The RAD52 homologues of rest of the plants were identified by multiple sequence analysis of conserved domains from *A. thaliana* RAD52. Based on this analysis, the plant RAD52 family was sub- divided into two groups, which is attributed to the genes early duplication and alternative splicing, leading to its localization within all DNA containing organelles, thereby signifying its role in maintaining nuclear and organellar genomes. Samach et al. (71) further carried out several expression studies such as sub cellular localization, sensitivity to mitomycin C (MMC) treatment and RNAi lines which depicted a somatic role for *A. thaliana* Rad52 homologues. Reduced fertility was also observed in RAD52 knockout lines which might be the result of defective somatic or meiotic DNA recombination and repair. Based on the results reported on *A. thaliana* RAD52, it is evident that the protein plays an important role in both meiosis and mitosis in plants.

In 2012, Janicka et al. proposed that Organellar DNA Binding protein 1 (ODB1) found in plant mitochondria may assume functions similar to that described for nuclear RAD52. It was reported that ODB1 promoted base pairing of complementary sequences, a function performed

by RAD52 in homologous recombination. Similarity in the DNA binding domain of RAD52 and ODB1, high affinity of ODB1 towards ssDNA and its ability for DNA annealing suggested that ODB1 promotes DNA repair by recombination. Moreover, the report showed that the ODB1 DNA binding domain resembled the structural domain of yeast and human Rad52. Janicka et al. (72) also showed that ODB1 has affinity for both dsDNA and ssDNA, with preference for ssDNA, forms homo-oligomers and is required for efficient HR mediated mitochondrial DNA repair.

Taken together, although some work has been done in plants to show the importance of Mre11 and Rad52 proteins, no biochemical characterization has yet been carried out, where binding of this protein to different forms of DNA or their nuclease activities or renaturation properties have been documented. Hence, the current study focuses on the biochemical characterization of OsMre11 and OsRad52 protein from *Oryza sativa*, including the purification of the protein, followed by biophysical and biochemical analysis.

1.7: Objectives and scope of the study:

The current study involves the cloning, over- expression and purification of OsMre11 and OsRad52 proteins from rice. Following purification of the proteins, we studied the ss and ds DNA binding properties, nuclease properties and renaturation properties of OsMre11 and OsRad52. To the best of our knowledge, this is the first report on in vitro biochemical properties of OsMre11 and OsRad52 proteins from crop plants which would be interesting to the researchers working in the field of homologous recombination in plants.

CHAPTER II

MATERIALS AND METHODS

2.1: Materials

γ^{32P}-ATP and Taq DNA polymerase were procured from the Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India**.** Plasmid DNA isolation kit, gel purification kit was purchased from Qiagen, Germany**.** Lowry's protein assay kit was obtained from Sigma Aldrich, USA. Restriction enzymes (*BamH1, Nde1, Sac1, Xho1, EcoR1*), DNA molecular weight markers 100 bp ladder, 500 bp ladder and 1 kb ladder was purchased from New England Biolabs (NEB, UK). Nitrocellulose membrane, and NBT-BCIP solutions were obtained from Roche life sciences, UK. T4 poly nucleotide kinase was procured from Amersham Biotech, United Kingdom. Lysozyme, BSA, Anti-poly histidine antibody, Ni-NTA resin from Sigma Chem. Co, U.S.A. Aseptic conditions were maintained during the molecular and microbiological work. All media components, reagents, solutions, glassware and plastic ware were autoclaved at 121°C/15psi for 15 min and glass wares were sterilized by dry heat (180°C/ 1.0 hr) whenever required. Antibiotic solutions and heat labile solutions were subjected to filter sterilization using 0.2 µM filter.

2.1.1: Bacterial strains, plasmids and primers used in this study:

Escherichia coli strains DH5α and Jm109 were used as cloning host and *E. coli* BL21 (DE3) pLysS, *E. coli* BL21 (DE3) and *E. coli* Shuffle T7 Express lysY cells from NEB were used for over-expression of the recombinant proteins. All the bacterial strains used in this study are shown in Table 2.1. The pET28a, pET16b and pSK^{+} vector (Novagen) was used for over-expression of recombinant proteins in *E. coli*. All the plasmids used in this study are listed in Table2.2.

Table 2.1 Bacterial strains used in this study:

Strain	Description	Source
E. coli DH5α	F- recA41 endA1 gyrA96 thi-1 hsdR17 (rk-mk-) supE44 relAλ lacU169	Lab collection
E. coli BL21(DE3) pLysS	F- ompThsdSB(rB- mB-) gal dcm (DE3) pLysS (CamR)	Novagen
E. coli BL21(DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ s*Bam*HIo Δ*Eco*RI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5	Novagen
E. coli Shuffle T7 Express lysY	MiniF lysY (CamR) / fhuA2 lacZ::T7 gene1 [lon] ompTahpC gal λatt::pNEB3-r1-cDsbC (SpecR, lacIq) ΔtrxB sulA11 R(mcr- 3::miniTn10--TetS)2 [dcm] R(zgb 210::Tn10 --TetS) endA1 Δgor Δ(mcrC-mrr)114::IS10	NEB

Table number 2.2: Plasmids used in this study

Plasmid	Description	Source
pET16b	*E. coli* over-expression vector	Novagen
pET28a	*E. coli* over-expression vector	Novagen
pBluescript SK^+	*E. coli* over-expression vector	Addgene

Table 2.3: Primers used in this study

Primer	Sequence
OsMRE11 fwd	5'ATAATACATATGGGAGACGAAAGCAACACACTC 3'
OsMRE11 rev	5' TTTTTTGGATCCTCATCTCCTCCTAACAGCT 3'
OsMRE11-A fwd	5'ATAATACATATGGGAGACGAAAGCAACACACTC 3'
OsMRE11- A rev	5' GATCGGATCCTTAATCAATCAGGGAGGTTGC 3'
OsMRE11- B fwd	5' AGTCCAT ATGGGTTTTCACATCACTCAACCA 3'
OsMRE11- B rev	5' GCATGGATCCTTACGCCATCTTGTCATCCTTGCTCAC 3'
OsMRE11- C fwd	5'ATAATACATATGGGAGACGAAAGCAACACACTC 3'
OsMRE11- C rev	5' GCATGGATCCTTACGCCATCTTGTCATCCTTGCTCAC 3'

Table 2.4: Oligonucleotides used in this study

Oligo	Sequence
4G3	5'-AATTCTGGGTGTGTGGGTGTGTGGGTGTGTGGGTGTGG-3'
4G3C	5'-CCACACCCACACACCCACACACCCACACACCCAGAATT-3'
NBS1	5'-TAAATTGTGT CGAAATCCGCGA CCTGCTCCATG-3'
NBS1C	5'-CATGGAGCAGGTCGCGGATTTC GA CACAAT TTA-3'
Oligo 1	5'- TTTTTTGG ATCCTCATCTCCTCCTAACAGCT- 3'
Phi W	5'- CGTTCTTATTACCCTTCTGAATGTCACGCTGATTATTTT GACTTTGAGCGTATCG -3'
Phi C	5'- CGATACGCTCAAAGTCAAAATAATCAGCGTGACA TTCAGAAGGGTAAT AAGAACG -3'

Table 2.5: List of reagents used for nucleic acid electrophoresis:

Reagent	Composition
10X loading dye	0.4% bromophenol blue, 0.4% Xylene cyanol, 50% Glycerol
1X Tris borate EDTA buffer (TBE)	8.9 mM Trizma base, 8.9 mM Boric acid, 0.2 mM EDTA
TE_{100}	100 mM Tris-HCl, 100 mM EDTA, pH 8.0

Table 2.6: List of reagents used for protein electrophoresis and western blotting:

Reagent	Composition
Acrylamide Solution	29.2 g Acrylamide and 0.2 g Bis Acrylamide / 100 ml water
5X gel loading buffer for SDS-PAGE	0.25% Bromophenol blue, 0.5 M DTT, 50% Glycerol, 10% SDS
Running Buffer	0.3% Trizma base, 1.44% Glycine, 0.1% SDS
Destaining Solution	I: 10% Glacial acetic acid, 50% Methanol II: 10% Glacial acetic acid, 10% Methanol, 2% Glycerol
Coomassie Brilliant Blue R- 250	0.2% Coomassie Brilliant Blue R- 250, 20% Glacial acetic acid, 40% Methanol
Transfer Buffer	0.125 M Trizma Base, 0.192 M Glycine, 20 % methanol
Tris Saline Buffer	0.05 M Trizma Base, 0.9% NaCl
Maleic Acid Buffer	0.1 M Maleic acid, 0.15 M NaCl
Reaction Buffer	0.1 M Trizma Base, 0.1 M NaCl

Table 2.7: List of composition of media used:

Reagent	Composition
Luria Bertani broth (LB broth)	5.0 gm Sodium Chloride, 5.0 gm Bacto Yeast Extract, 10 gm Bacto Tryptone were dissolved in 1.0 L of water and adjusted the pH to 7.4 with 10 N NaOH.
LB agar plates	LB agar broth with 1.8 % agar agar
Transformation buffer- Solution A	MOPS 10 mM, RuCl (Rubidium Chloride) 10 mM, pH 7.0
Transformation buffer- Solution B	MOPS 100 mM, RuCl (Rubidium Chloride) 10 mM, $CaCl_2$ (Calcium Chloride) - 50 mM, pH 6.5

Table 2.8: List of stock solutions:

Name	Stock concentration	Working concentration	Storage	Sterilization Method
Kanamycin	25 mg/ml	25 µg/ml	-20˚C	Filtered
Carbenecillin	100 mg/ml	50 µg/ml	-20˚C	Filtered
Chloramphenicol	20 mg/ml	10 µg/ml	-20˚C	Filtered
Tris HCl	1.0M	As required	Room temp	Autoclaved
Et Br	10 mg/ml	50 µg/ml	Room temp	Filtered
SDS	10 %	As required	Room temp	Filtered
IPTG	50 mg/ml	1.0 mM	-20˚C	Filtered

All the stock solutions were prepared in milli-Q water and sterilized by autoclave or filter sterilization accordingly. Antibiotic containing LB plates or LB liquid medium were prepared

freshly by adding required amount of antibiotic to the pre-prepared media. LB plates containing antibiotic were poured by adding the required antibiotic at ~50˚C. All the stock solutions and respective working concentrations are given in the Table2.8

2.1.2: CULTURE MEDIUM AND GROWTH CONDITIONS:

E. coli strains DH5α, Jm109, BL21 (DE3), BL21 (DE3) pLysS or shuffle cells were grown in Luria Bertani (LB) medium at 37°C (20°C for shuffle cells) with shaking at 120-140 rpm with appropriate antibiotics as and when required. Antibiotics were used at 100 µg/ ml carbenecillin (Cb), 33 µg/ml chloramphenicol (Cm), 20 µg/ml Cm for shuffle cells or 25 µg/ ml kanamycin (Km).

2.2: METHODS

2.2.1: Polymerase Chain Reaction:

A 50 µl of reaction mixture (100 pg of template DNA, 0.2 µM of upstream and downstream primers, 200 µM of dNTP mixture, 1 unit of Taq DNA polymerase in 1X reaction buffer) was incubated at 94˚C for 5 minutes. Amplification cycles were performed at 72˚C for extension, 94˚C for 30 seconds and 50-60˚C (depending up on the template and primer combination) for annealing. Extension time for was set up depending on the primers and template combination considering the amplification speed is 1 kb/min. All the reactions were performed for 35 cycles and finally 5 minutes' extension at 72˚C was given to complete the aborted extensions. PCR products were analysed on agarose gel in 1X TBE buffer. For colony PCR reaction, single colony was touched with platinum wire and cells were suspended in sterile distilled water and lysis was done by boiling the samples at 95˚C for 5 min before PCR reaction.

2.2.2: Restriction Digestion:

Restriction digestion was carried out using kit from New England Biolabs Ltd following manufacturer's protocol. By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes. A 30 µl of reaction mixture containing 0.1 µg of DNA (plasmid DNA), 1 µl of concerned NEB restriction enzyme each (in case of double digestion), 1 µl of 10X NEB buffer and made up the remaining volume with nuclease free water. The mixture was incubated at 37˚C for 3 hours. The digested DNA was mixed with the appropriate amount of loading dye and resolved on 0.8% agarose gel. DNA fragments were visualized under UV trans-illuminator. The DNA marker (100 bp and/or 1kb, NEB) were used to estimate sizes of the products.

2.2.3: Ligation Reaction:

The ligation reaction was carried out using quick DNA ligase (T4 DNA ligase, Roche Biochemicals) as per the manufacturer's protocol. The insert to vector ratio was maintained in molar ratio 3:1- 5:1 for sticky end ligation. Total DNA concentration was kept at 200 ng/20µl. Ligation reaction was set up with Rapid DNA Ligation kit (Roche Diagnostics). Procedure briefly involves 10 µl of DNA (150 ng of vector + 50 ng of insert) in 1.0X DNA dilution buffer. To this, 10 µl of T4 DNA ligation buffer (2X) was added and mixed. 1.0 µl of T4 DNA ligase was added and reaction mixture was incubated at 25˚C for 5.0 min. This ligation mixture was used directly for transforming the competent cells.

2.2.4: Cloning of full length OsMRE11 cDNA:

Based on the *Arabidopsis* MRE11 sequence, analysis of rice genome showed the presence of MRE11 homolog. The cDNA corresponding to OsMRE11 was available with Rice Genome Resource Centre, (RGRC), Japan. Hence a clone corresponding to full length OsMRE11 cDNA was procured from RGRC (Accession No: AK070546, Clone name: J023054N24). The OsMRE11 cDNA fragment was amplified by PCR using a set of specific primers, the **OsMRE11 fwd** and **OsMRE11 rev** primers containing restriction sites for *Nde*I and *Bam*HI respectively. Followed by PCR amplification, 2115 bps of OsMre11 amplicon and pET28a expression vector were double digested with *Nde*1 and *Bam*H1. The OsMRE11 cDNA fragment was gel eluted and ligated into the similarly digested pET28a expression vector. This ligation mixture was then used directly for transforming chemically competent *E. coli* strain JM109 cells and plated on LB plate containing antibiotic (25µg/ml of kanamycin) for the selection. Random colonies were picked and tested for the presence of insert by colony PCR. The recombinant plasmid was isolated from these positive colonies and subjected to restriction digestion with *Nde*1and*Bam*H1. As expected, a ~2.1 kb OsMRE11 fragment was released.

Randomly selected recombinant plasmid was transformed into *E. coli* expression host BL21 (DE3) pLysS and plated on LB plate containing 25µg/ml of kanamycin and 34µg/ml of chloramphenicol. Colonies were checked for the presence of the recombinant plasmid by colony PCR and positive clones were subjected to over-expression of OsMre11 protein with IPTG.

2.2.5: Cloning of N- terminal end of OsMRE11 cDNA (OsMRE11- N):

cDNA corresponding to N- terminal end of OsMRE11 (1380 bp) was amplified with the gene specific primers (**OsMRE11 fwd and OsMRE11- B rev primers**) using the full length cDNA clone procured from Rice Genome Resource Centre, Japan as template DNA. Upstream primer with *Nde*1 and downstream primer with *Bam*H1 restriction sites were synthesized and used to amplify the above cDNA fragment corresponding to N- terminal end of OsMRE11. Amplicon was purified using PCR clean up kit (Qiagen). This fragment and pET16b expression vectors were double digested with *Bam*H1 and *Nde*1 restriction enzymes. Digested vector backbone and OsMRE11-N cDNA fragment bands were excised from LMP agarose gel and purified using gel elution kit(Qiagen). Ligation reaction was set up with Rapid DNA Ligation kit (Roche Diagnostics). Procedure briefly involves 10 µl of DNA (150 ng of vector + 50 ng of insert) in 1.0x DNA dilution buffer. To this, 10 µl of T4 DNA ligation buffer (2x) was added and mixed. 1.0 µl of T4 DNA ligase was added and reaction mixture was incubated at 25˚C for 5.0 min. This ligation mixture was used directly for transforming the competent cells. After transformation, random colonies were picked and tested for the presence of insert by colony PCR. The recombinant plasmids were isolated from these positive colonies and subjected to restriction digestion with *Nde*1 and*Bam*H1. Randomly selected recombinant plasmid was transformed into *E. coli* expression host Shuffle T7 Express Lys competent cells and plated on LB plate containing 50µg/ml carbenecillin and 20 µg/ml of chloramphenicol. Colonies were

checked for the presence of the recombinant plasmid by colony PCR and positive clones were subjected to over-expression of OsMre11-N protein with 0.4mM IPTG.

2.2.6: Cloning of OsRAD52 cDNA:

The full length cDNA obtained from RGRC was sub-cloned into pBluescript SK^+ between *Sac*1 and *Eco*R1 restriction sites. Subsequently, full length insert was cloned in to pET28a vector between *Sac*1 and *Xho*1restriction sites. Digested vector and OsRAD52 cDNA bands were excised from LMP agarose and purified using gel elution kit (Sigma Aldrich). Ligation reaction was set up with rapid ligation kit (Roche Diagnostics) and transformed into *E. coli* DH5α cells. Random colonies were picked and tested for the presence of insert by digestion of isolated recombinant plasmids with *Sac*1 and *Xho*1. Randomly selected recombinant plasmid was transformed into *E. coli* BL21 (DE3) chemically competent cells and plated on LB plate containing 25µg/ml kanamycin. Colonies were checked for the presence of the recombinant plasmid by restriction digestion of isolated plasmids with *Sac*1 and *Xho*1 and positive clones were subjected to over-expression of OsRad52 protein with 1.0 mM IPTG.

2.2.7: Competent cells preparation and transformation:

E. coli cultures stored at -70˚C were streaked on to LB agar plates and incubated at 37˚C overnight. Isolated colony was inoculated into 2.0 ml of LB medium and grown over night with shaking at 200 rpm, 37˚C. Next day morning, 10 ml of LB medium was inoculated with 0.1 ml of overnight grown culture and shaking was continued till the OD_{600nm} of the culture reached to 0.5 units. Cells were harvested by centrifuging the culture at 6000 rpm, 4.0˚C for 10 min and resuspended in 2.5 ml of Solution A (MOPS 10 mM, RuCl (Rubidium Chloride) 10 mM, pH 7.0), followed by centrifugation at 5000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 2.5 ml of Solution B (MOPS 100 mM, RuCl (Rubidium Chloride) 10 mM, $CaCl_2$ (Calcium Chloride) 50 mM, pH 6.5) and incubated on ice for 30 min.

The cell suspension was centrifuged at 3000 rpm for 8 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 1.0 ml Solution B. 200 µl aliquots of the competent cells were used for further transformation reactions. 20 µl of ligation mixture containing 50-100 ng of DNA was added and the cells incubated on ice for 30 min. Cells were subjected to heat shock at 42˚C for 90 secs in a water bath. Tubes were immediately transferred on to ice and kept for 5.0 min. 1.0 ml of LB medium was added to the tube and cells were grown by shaking at 200 rpm, 37˚C for 1.0 h. 100 µl of above culture was plated on LB plate containing appropriate antibiotic (20 µg / ml of carbenecillin, 10 µg / ml of chloramphenicol for *E. coli* Shuffle T7 Exp Lys cells with pET16b and 25µg / ml of kanamycin for *E. coli* BL21 (DE3) cells with pET28a) for selection. Plates were kept at 37˚C, over-night. To check the recombinants, colonies were touched with a sterile platinum needle and dipped into the PCR reaction mixture. Reaction was carried out as described earlier and products were analysed on agarose gels.

2.2.8: Plasmid DNA purification by 'NucleoSpin Plasmid' kit(MACHEREY-NAGEL):

1.5 ml of overnight grown culture was centrifuged at 10000 rpm for 5 min. To the pelleted cells 250 µl of Buffer A1 (100 mM Tris HCl pH: 7.5, 10 mM EDTA, 10 µg/ml RNase) was added and the pellet re-suspended by vortexing. To this, 250 µl of Buffer A2 (NaOH: 1.0 M, SDS: 0.5 %) was added and thoroughly mixed by inverting the tube 6-8 times. This mixture was incubated at RT for 5 min. Then, 300 µl of Buffer A3 (Potassium: 3.0 M and Acetate: 5.0 M) was added and mixed by inverting the tube 6-8 times. The mixture was centrifuged at 11,000 g for 10 min. A NucleoSpin® Plasmid column was placed onto a 2ml collection tube and the supernatant decanted into the column. The column was centrifuged at 11,000 g for 1 min and flow through was discarded. The column was washed by adding 600 µl Buffer A4 (Tris-HCl (pH: 7.5):20 mM; EDTA: 2.0 mM and NaCl: 200 mM) and centrifuged at 11,000 g for 1 min.

To elute Plasmid DNA, the NucleoSpin® Plasmid column was placed onto a new 1.5 ml micro centrifuge tube. Plasmid was eluted with 50 µl of sterile distilled water and used immediately.

2.2.9: DNA elution from Agarose gel by QIAquick Gel Extraction Kit (Qiagen):

QIAquick Gel Extraction Kit was used for gel elution, following manufacturer instructions. The electrophoretically resolved DNA fragment was excised from the agarose gel with a clean and sterile scalpel and weighed. 3 volumes (V/W) Buffer QG was added to 1 volume gel i.e. for every 100 mg of agarose gel, 300 µl of Buffer QG (Gel solubilisation solution) was added. The gel mixture was incubated at 50-60 ºC for 10 min and mixed properly until the gel is completely dissolved. To this, one gel volume of isopropanol was added. A QIAquick spin column was placed in a 2 ml collection tube, provided with the kit. To bind DNA, the sample was applied to the QIAquick column and centrifuged at6,000 x g for 1 min. The flow through was discarded and the column placed back in the same collection tube. 500 µl of Buffer QG was added to the QIAquick column and centrifuged at 6,000 x g for 1 min. The flow through was discarded and column placed back in the same collection tube. For washing, 750 µl of Buffer PE was added to QIAquick column and centrifuged at6,000 x g for 1 min. The flow through was discarded and column placed back in the same collection tube. The QIAquick column was centrifuged for an additional 2 min at 10,000 x g to remove the residual wash buffer, and then placed onto a clean 1.5 ml micro centrifuge tube.45 µl of sterile stage II water was added to the centre of the QIAquick column and allowed to stand for 5 min and then centrifuged at 10,000 x g for 1 min. 2 µl of the eluted sample was analysed on 0.8% agarose gel to check the plasmid preparation. The rest of the eluted sample was stored at -20ºC until further use.

2.2.10: Over-expression of OsMre11, OsMre11-N and OsRad52:

Recombinant plasmid DNA was transformed into expression host cells (*E. coli* BL21 (DE3) pLysS cells for OsMre11, *E. coli* Shuffle T7 Express LysY cells for OsMre11-N and *E. coli* BL21 (DE3) cells for OsRad52) for protein expression. Bacterial cells harbouring the recombinant plasmid construct pET28a with OsMRE11 cDNA, pET28a with OsRAD52 cDNA and pET16b with OsMRE11-N cDNA insert were selected on LB-kanamycin plates and LB-carbenecillin/ chloramphenicol plates respectively. Single colony for each protein were inoculated in LB media containing respective antibiotic and were grown over night at 37˚C by shaking at 200 rpm. 1.0 L of LB media containing the required antibiotics was inoculated with 1.0% of above overnight grown culture and grown at 37˚C by shaking at 200 rpm until the O. D_{600nm} of the culture reached 0.6. Expression of proteins was induced by adding 1.0 mM isopropyl-ß-D thiogalactoside (IPTG) for BL21 (DE3) cells or 0.4mM IPTG for shuffle cells, followed by shaking for another 3.0 hours on orbital shaker at 37˚C or overnight at 20˚C for shuffle cells. Cells were harvested by centrifugation at 6000 rpm in Sorvall RC-5C centrifuge using a SS-34 rotor. Cell pellets expressing OsMre11, OsMre11-N and OsRad52 were collected and stored at -20˚C until further use.

2.2.11: Purification of OsMre11:

OsMre11 protein was purified under denaturing conditions of 8.0 M urea. For this purpose, inclusion bodies were first isolated from the cell extract to high purity by repeated sonication in lysis buffer containing 1% Triton X-100, 0.01% sodium azide, 0.01% PMSF, protease inhibitor cocktail and 1mM DTT followed by centrifugation at 8000 rpm for 30 minutes at 4˚C. Relatively pure inclusion bodies preparation was obtained. The histidine tag at the N-terminal end of the over-expressed protein was utilized to purify the protein by affinity chromatography using Ni-NTA affinity matrix under denaturing conditions (8.0M urea) at pH 8.0. The matrix

was equilibrated with 10 ml phosphate buffer containing 8.0 M urea pH 8.0. Inclusion bodies were dissolved in5 ml of phosphate buffer containing 8.0 M urea pH 8.0 and incubated with the equilibrated matrix. The resin- inclusion body suspension was loaded onto the column and washed with phosphate buffer (10mM Tris-Cl, 100mM sodium dihydrogen phosphate) pH 6.3 and eluted to near homogeneity using the same buffer containing 8.0M urea at decreasing pH of 5.5 and 4.5. Eluted fractions were analysed on 12% SDS-PAGE. The Mre11 containing fractions were pooled and refolded to its native state by dialyzing against dialysis buffer (25mM Tris-Cl, 100mM NaCl, 5mM DTT) containing decreasing concentrations of urea (8.0M to without urea) over a period of 16 hrs at 4˚C.The refolded protein was then concentrated and stored in storage buffer at 4˚C.

2.2.12: Purification of OsMre11-N:

Frozen cells were resuspended in 10 volumes (w/v) of lysis buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, and 1mg/ml lysozyme, protease inhibitor cocktail) and incubated at 37˚C for 30 min followed by freeze thawing in liquid nitrogen for, 4 times. Thick, turbid cell suspension was sonicated with Branson W -220F sonicator (Heat systems Ultrasonic Inc) with the output of 30, for 10 min total till the suspension turned semi-transparent. The lysate was centrifuged in SS-34 tubes at 12,000 rpm for 30 min at 4˚C. A clear supernatant representing crude extract was collected. The crude extract was loaded onto Ni-NTA agarose resin pre-equilibrated with equilibration buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl). The flow through was collected and the column washed with 3 column volumes of wash buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl and 20 mM of imidazole). Proteins were eluted with 2 column volumes of elution buffer with increasing concentration of imidazole (100 mM-500 mM imidazole).

2.2.13: Purification of OsRad52:

For the purification of OsRad52 protein, cell pellet from 2.0 L of induced culture was re-suspended in 15 ml of lysis buffer containing 25 mM Tris- Cl, 100 mM NaCl and 8.0 M urea, pH 8.0. Cell suspension was subjected to repeated freeze- thaw cycles in liquid nitrogen followed by sonication with Branson W-220 F sonicator (Heat systems ultasonics Inc.) at 10 s on/off cycles for 10 minutes to ensure proper lysis on ice. The lysate was centrifuged at 8000rpm for 20 minutes and the clear supernatant loaded onto Ni-NTA agarose column pre-equilibrated with equilibration buffer (25 mM Tris- HCl, 100mM NaCl, 8.0 M urea and 10 mM imidazole, pH 8.0). Column was washed with five column volumes of the wash buffer (25 mM Tris- HCl, 100 mM NaCl, 8.0 M urea and 20 mM imidazole, pH 8.0). Bound proteins were eluted with two column volumes of elution buffer containing increasing amounts of imidazole from 100- 500 mM. Denatured OsRad52 protein was refolded by stepwise dialysis for 8 hours each against 1 litre of dialysis buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 1.0 mM ß-ME, 0.1 mM DTT) containing 6.0 M, 3.0 M, 1.5 M, and 0 M urea respectively at 4 ˚C. Finally, two changes were given with 1.0 L of storage buffer (20 mM Tris-HCl pH 8.0, 10% glycerol, 500 mM NaCl, 1.0 mM ß-ME, 0.1 mM DTT). The protein solution was stored at -20 ˚C in the same buffer.

2.2.14: SDS-PAGE and Western blotting:

Gel apparatus was washed with water and wiped with ethanol. The apparatus was assembled and sealed with clamps provided with the apparatus. 30% acrylamide solution (containing 0.8% bis-acrylamide), 1.5 M Tris-HCl pH 8.8, and 10% SDS were used to prepare (12-14%) resolving gels. APS (0.1% final concentration) and TEMED (10 µl per 20 ml of gel mix) were used as free radical generator and catalyst respectively and poured between the plates leaving 3.0 cm space from the top. A small volume of isobutanol was layered carefully to prevent

contact of gel with air. After 20 min, the gel was polymerized and excess solution was removed and wiped with filter papers. 1.0 mm comb was inserted and stacking gel solution (4% acrylamide/bisacrylamide, 125 mM Tris-HCl, pH 6.5, and 0.1% SDS) was poured on top of the resolving gel by appropriately diluting stock solutions of 30% acrylamide, 0.5 M Tris-HCl pH 6.8, 10% SDS and was allowed to polymerize for at least 20 minutes. Combs were removed and wells were washed with electrophoresis buffer, which was used for running the SDS PAGE gels. Composition of the solutions is given in Table 2.6. Appropriate volume of SDS PAGE sample loading buffer (5x) was added to the protein sample and mixed well. Samples were boiled in water bath for 5 min and centrifuged at 12,000 rpm for 10 min. Required amount of supernatant (20 µl) was loaded in the wells and electrophoresis was performed at constant voltage (90 V). After the dye front reached the bottom of the gel, gel apparatus was dismantled and the gel fixed in the de-staining solution before staining. Proteins on the gels were detected by staining the gels with coomassie brilliant blue R-250 (Table 2.6). For Western blotting, proteins were electro transferred onto nitrocellulose membranes. For this, the gel was carefully removed and the proteins transferred by electro blotting at 300 mA current for 3 hrs. The blot was then blocked in Maleic acid-NaCl (MaNa) buffer containing 1% blocking reagent for 2 hours. This was followed by addition of Anti-His antibody (primary antibody), at appropriate dilution (1: 10,000) in 1X Blocking buffer. The blot was incubated overnight at 4ºC. The blot was then washed thrice with 1X TBS (Tris Buffered Saline) buffer for 15 min each, followed by incubation with alkaline phosphatase (AP) tagged secondary antibody in 1X TBS buffer (1: 3000 dilution) for 1 hour. The blot was then washed again, thrice with 1X TBS. The substrate NBT-BCIP (Nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate) solution (diluted 1:100 in reaction buffer) was added and blot incubated in dark. As the bands appeared on the blot, the reaction was stopped, by rinsing the blot with water. Blot was dried and scanned for records. Composition of the buffers is given in the Table 2.6.

2.2.15: Native PAGE analysis purified OsMre11- N and OsRad52 protein:

Purified OsMre11- N and OsRad52 protein samples were prepared for native PAGE analysis using 5X sample buffer (containing 62.5mM Tris-HCl, pH 6.8, 25% glycerol, 0.01% Bromophenol Blue) without DTT. The protein sample was separated on 10% native polyacrylamide gel run at a constant voltage of 100V at 4°C. The gel was stained with coomassie brilliant blue R-250, after the completion of electrophoretic run.

2.2.16: Circular dichroism of OsMre11- N and OsRad52 protein:

JASCOJ-815CD spectrometer was used to record the far-UV circular dichroism of purified OsMre11-N and OsRad52proteins. The purified OsMre11-N and OsRad52 proteins were diluted in buffer containing 25mM Tris-HCl, 100mM NaCl, 5mM DTT to a final concentration of 0.4mg/ml. The spectrum was recorded at 20°C in the wavelength range of the 200–260 nm using 1.0 mm path-length quartz cuvette. An average of three scans were taken for constructing the final spectra. A reference spectrum was also recorded with the same buffer which was later subtracted from the protein spectra. The observed ellipticity measured in milli-degree units was converted to molar ellipticity i.e. Delta epsilon (M^{-1} cm^{-1}) and plotted against the wave length.

2.2.17: Gel-filtration analysis of OsRad52 protein:

To determine the molecular weight of OsRad52 protein in its native state, molecular sieve column chromatography was performed using Superdex™200GL column (GE Healthcare) on AKTA purifier (GE Healthcare). The purified OsRad52 protein was loaded onto the column in storage buffer containing 25mM Tris-HCl, 250 mM NaCl, pH 8.0 and eluted at a flow rate of 0.5ml/min. The column was formerly calibrated with gel filtration molecular weight markers (Amersham-Pharmacia: carbonic anhydrous-29kDa, carbonic dehydrogenase-150kDa, β-amylase-200kDa, ferritin-440kDa). Standard calibration curve was plotted with elution volume of marker against the logarithm of molecular weight of markers. The molecular weight

ofthepurifiedOsRad52 protein in the native condition was determined by fitting the elution volume into the calibration curve.

2.2.18: MALDI-TOF analysis of purified OsMre11- N and OsRad52 protein:

The purified OsMre11-N and OsRad52 protein was resolved on 12% SDS-PAGE and the protein band excised and subjected to in-gel tryptic digestion. Briefly, the gel pieces were washed, twice with sterile double distilled water followed by 50 mM NH_4HCO_3: acetonitrile (1:1, v/v) (15 min. each at room temperature), dehydrated with acetonitrile and dried. The protein was reduced with 10 mM DTT (in 50 mM NH_4HCO_3), alkylated with 55 mM iodoacetamide (in 50mM NH_4HCO_3) and subjected to overnight trypsin digestion at 37°C. The peptides were extracted with 0.1% tri fluoro acetic acid in 50% acetonitrile, dried and re-suspended in 5.0 µl of the same solution. Protein mass fingerprint (PMF) spectra was obtained on a Bruker MALDI-TOF/TOF system in reflectron mode. As the sequence of the OsRad52 is not available in SwissProt and NCBI, the data was analysed using the Masssorter v3.1 program (73) available at http://services.cbu.uib.no/software/massSorter using the known protein sequence as the target. The search parameters for both the proteins were carbamidomethylation of Cysteine, variable oxidation of methionine, one missed cleavage and a fragment mass-tolerance of 200 ppm.

2.2.19: Radioactive labelling of Oligonucleotides:

To study the DNA binding activity of OsMre11-N protein, 4G3 oligonucleotide was labelled with γ^{32}P-ATP. A reaction mixture containing 10 pmol of 4G3 oligonucleotide, PNK buffer (polynucleotide kinase buffer), PNK enzyme (20 units), 50 pmol of γ^{32}P-ATP and nuclease free water was prepared. This reaction was then incubated for 30 minutes at 37˚C followed by heat inactivation at 65˚C for 10 minutes. Radioactive labelled oligonucleotide was purified by

Quickspin columns, which contain gel filtration matrices (Sephadex G-25 in STE buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 100 mM NaCl).

2.2.20: DNA binding assays by Electrophoretic Mobility Shift Assay (EMSA):

DNA binding activity was monitored by EMSA, also referred to as gel shift assay or gel mobility assay or band shift assay. It determines whether a protein is capable of binding to a DNA sequence followed by separation of the protein- DNA complex on a polyacrylamide gel or agarose gel.

Several DNA substrates were used in this study, to determine the binding of OsMre11-N and OsRad52 protein to DNA.

Table 2.9: List of DNA substrates used in this study:

Form of DNA	Oligo Name	Procedure	Concentration (pmol/ µl)
DNA substrates used for OsMre11 binding			
ssDNA	4G3*	Radioactive labelled 4G3 and used for the assay	3
dsDNA	4G3* + 4G3C	Radioactive labelled 4G3 and 4G3C oligos were mixed in equimolar concentrations, heat denatured at 100°C for 10 min and renatured over a period of 2.0 hrs.	3
G4 DNA	4G3*	Radioactive labelled 4G3 incubated with 100 mM Tris- Cl and 100 mM KCl at 100°C for 10 min and allowed to form G4 over a period of 2.0 hrs.	3
DNA substrates used for OsRad52 binding			
ssDNA	Oligo1	Oligo1 was used for the assay	100

dsDNA	NBS1 + NBS1C	NBS1 and NBS1C oligos were mixed in equimolar concentrations, heat denatured at 100°C for 10 min and renatured over a period of 2.0 hrs.	100

2.2.21: OsMre11- N DNA binding assay:

DNA binding activity of OsMre11-N protein was carried out according to procedure mentioned by Muniyappa et.al (8). A 15.0 µl of reaction mixture containing increasing amounts of OsMre11 protein, 20mM Tris-HCl, 0.1mM DTT was incubated at 37°C for 5.0 min and reaction was started by adding 3 pmol/µl of radioactively labelled single-stranded DNA or3 pmol/µl double-stranded DNA or 3 pmol/µl G4 DNA. Samples were incubated at 37°C for 10.0 min. The samples were then mixed with DNA loading sample buffer containing 0.1% each of bromophenol blue and xylene cyanol in 50% glycerol and the DNA-protein complexes formed was analysed on native polyacrylamide gel (8.0%) in 0.5 × Tris-borate EDTA running buffer at 10V/cm. After electrophoresis, the gel was dried and exposed to X-ray film overnight in dark. The following day the X-ray film was developed and OsMre11-DNA nucleoprotein complexes were visualised.

2.2.22: OsRad52 DNA binding assay:

DNA binding assay of OsRad52 protein was carried out according to procedure mentioned by Rajanikant et al. (37). A 20 µl of reaction mixture containing increasing amounts of OsRad52 protein, 20 mM Tris-HCl, 0.1 mM DTT was incubated at 37˚C for 5.0 min and reaction was started by adding 100 pmol single- stranded DNA or 100 pmol double stranded DNA. Samples were incubated at 37˚C for 20 min. The samples were mixed with DNA loading sample buffer containing 30% glycerol and the DNA-protein complexes formed were analysed on gradient native polyacrylamide gel (4- 15 %) in 0.5 X Tris- borate- EDTA running buffer at 10 V/cm.

DNA in the complexes were visualized by staining the gel with SyBr green. The gel-shifted complex was quantified using Image J software.

2.2.23: Nuclease assays:

OsMre11 protein mediated exonuclease and endonuclease activities and the requirement of co-factors for both types of activities were explained below.

a) Exonuclease assay:

A 10.0 µl reaction containing 1.0 µl of (50 pmol/µl) of pSK^+ plasmid linearized with *Bam*H1 was incubated with increasing amounts of OsMre11 protein in a buffer containing 20 mM Tris-Cl, 0.1 mM DTT in presence and absence of 5.0 mM $MnCl_2$. After incubation at 37˚C for 30 minutes, the reactions were terminated by the addition of 10 mM EDTA, 1% SDS and 10 mg/ml proteinase K. Samples were incubated for another 15 min at 37˚C and analysed on 2% agarose gel electrophoresed in Tris-borate buffer (pH 8.3) at 100 V/cm. After electrophoresis, the gel was stained with ethidium bromide and observed under UV- illumination.

b) Endonuclease assay:

To perform the endonuclease assay, a 10.0 µl reaction containing 1.0µl of (50pmol/ µl) of ΦX174 RF I DNA (super coiled form) was incubated with increasing amounts of OsMre11 protein in a buffer containing 20 mM Tris-Cl, 0.1mM DTT in presence and absence of 5.0 mM $MnCl_2$. After incubation at 37˚C for 20 minutes, the reactions were terminated by the addition of EDTA, SDS and proteinase K to a final concentration of 10 mM, 1% and 10 mg/ml respectively, and incubated for another15 min at 37˚C. Samples were analysed on 0.8% agarose gel and electrophoresed in Tris-borate buffer (pH 8.3) at 100V/cm.

2.2.24: Requirement of Co- factor for nuclease activity:

To test the requirement of metal ion co-factors, the above experiment was carried out with following modifications. A 15.0 µl reaction containing 50pmol/ µl of linearized pSK^+ plasmid

(for exonuclease activity) or 50 pmol/µl of ΦX174 RF I DNA (for endonuclease activity), was incubated with indicated concentrations of OsMre11 protein in buffer containing 20mM Tris-Cl, 0.1mM DTT and5.0 mM $MnCl_2$ or 5.0 mM $CaCl_2$ or 5.0 mM $ZnSO_4$or 5.0 mM$MgCl_2$or 5. 0 mM KCl. After incubation at 37˚C for 20 minutes, the reactions were terminated by the addition of 10 mM EDTA, 1.0% SDS and 10 mg/ml proteinase K and incubated for another15 min at 37˚C. Samples were analysed on2% agarose gel.

2.2.25: Time- point for nuclease activity:

Time point assay was carried out to determine the efficiency of nuclease property of OsMre11 protein. A 150 µl reaction containing 10.0 µl (50 pmol/µl) of linearized pSK^+ plasmid (for exonuclease activity) or 10.0 µl (50 pmol/µl) of ΦX174 RF I DNA (for endonuclease activity), was incubated with a fixed concentration of OsMre11 protein in buffer containing 20mM Tris-Cl, 0.1mM DTT,5.0 mM $MnCl_2$ and 15 µl aliquots were removed at fixed time points of 0, 20, 40, 60, 120 minutes from the main reaction volume. The reactions were terminated by the addition of EDTA, SDS and proteinase K to a final concentration of 10mM, 1.0% and 10 mg/ml respectively, and incubated for another15 min at 37˚C. Samples were analysed on 2% agarose gel.

2.2.26: Fluorescence assays:

To test the binding of OsMre11 protein to different forms of DNA, fluorescence based assays were carried out, where change in the tryptophan fluorescence of the OsMre11 protein upon binding to DNA was monitored. A 45µl reaction containing ssDNA, dsDNA, G4DNA (100 pmol/ µl) was incubated with increasing concentration of protein along with a buffer containing 20mM Tris-Cl, 0.1mM DTT and fluorescence was measured (excitation at 290 nm and emission at 300-450 nm) using FLS-980 fluorescence spectrophotometer (Edinburgh Instruments).

2.2.27: Renaturation assay using agarose gel electrophoresis:

Renaturation assay with OsRad52 was carried out according to the procedure mentioned by Rajanikant et al. (38) with the following modifications. A 2.0kb linear DNA fragment was heat denatured in water bath at 100°C for 10 min and immediately frozen in liquid nitrogen. A 20µl reaction mixture containing 20mM Tris-Cl, 0.1mM DTT and indicated amounts of OsRad52 was pre-incubated at 37°C. The reaction was started by adding 100ng of heat denatured DNA and incubated for 10min at 37°C. The reaction was stopped by adding 0.1% SDS and 2.0 units of proteinase K for deproteinization and further incubated at 37°C for 20 min and analysed on 0.8% agarose gel in 1X Tris-borate EDTA running buffer at 10 V/cm. Duplex DNA formed as a result of renaturation activity of OsRad52 was visualized by staining the gel with ethidium bromide.

2.2.28: Renaturation assay by FRET:

Demonstrating renaturation activity of OsRad52 using agarose gel involved a deproteinization step, which made it challenging to score the actual renaturation activity in real time. Hence, a straight forward FRET assay was carried out, which enabled the renaturation reaction to be measured in real time without protein removal from the system. FRET assay gave similar results wherein, the assay was designed to measure the decrease in emission intensity of fluorescein (donor) at 520 nm, upon excitation at 480 nm, as a function of time. Here, two complementary oligonucleotides Phi-W and Phi- C labelled with fluorescein and rhodamine at their 5' and 3' end respectively was used. The assay involves non radiative transfer of fluorescence energy from the donor molecule (fluorescein) to the acceptor molecule (rhodamine). Since the assay is distance dependent between the donor and acceptor molecule, it helps to reveal the status of physical re-union occurring during renaturation. As the renaturation occurs, both the complementary strands anneal together, which resulted in

juxtaposition of the donor and acceptor dyes, hence FRET condition was created. The decrease in fluorescence intensity was measured at 520 nm upon excitation at 480 nm.

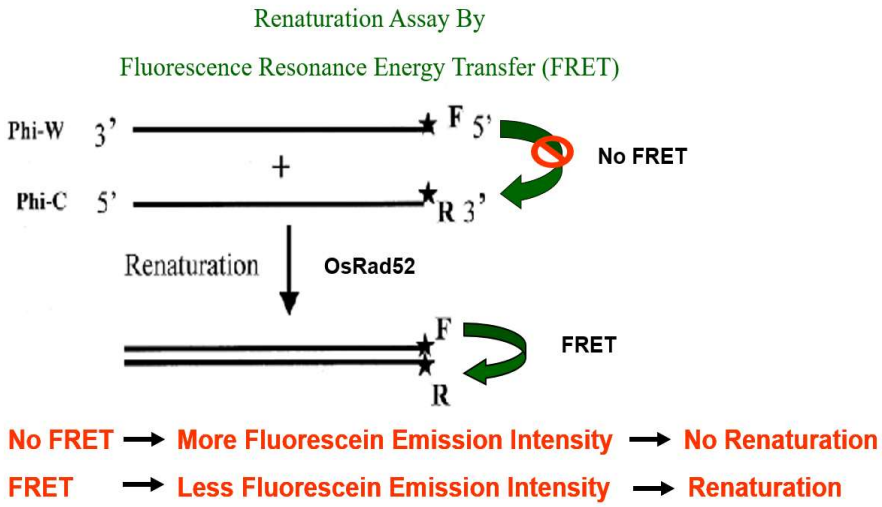

Figure 2.1: Diagrammatic representation of FRET principle

Renaturation assay by FRET was carried out according to the procedure mentioned by Rajanikant et al. (37). A reaction mixture containing 20 mM Tris- Cl, 0.1 mM DTT and increasing amounts of OsRad52 was pre-incubated at 37ºC for 5 min with Phi-C oligonucleotide (100 pico moles) labelled with rhodamine at 3' end. This was followed by addition of complimentary oligonucleotide Phi- W labelled at the 5' end with fluorescein. The decrease in fluorescence emission intensity of fluorescein at 520 nm after excitation at 480 nm as a result of renaturation was measured at 10 s interval for 5 minutes.

Chapter III

RESULT- I

BIOCHEMICAL CHARACTERIZATION OFOsMre11

3.0: INTRODUCTION:

The MRX/N complex which consists of Mre11, Rad50 and Xrs2 /Nbs1are the key, initial responders to DNA double strand breaks generated by different damaging events. This complex scans the entire DNA double strand for a break point, and binds to DNA at the break site (42). Mre11 protein of the MRN complex is highly conserved in eukaryotes, with the N- terminal end sharing several sequence motifs with *E. coli* SbcD protein, protein phosphatases and bacteriophage T4ngp46 nucleases (74). Mre11 protein has several enzymatic activities such as DNA binding, exonuclease and endonuclease in a Mn^{2+} dependent manner (50). Several studies have already been reported stating the biochemical characteristics of this protein in humans and yeast.

Significance of Mre11 proteins and their probable role have also been studied in plants like rice (*Oryza sativa*) and *Arabidopsis thaliana*, though most of the studies are limited to genetic and mutational analysis. In 2013, Samanic et al. performed comparative analysis of several Mre11 alleles to study genome stability and meiosis in *Arabidopsis thaliana.* The Mre11 mutants showed different phenotypes with respect to growth morphology (sterile and semi-dwarf with morphological abnormalities), genome stability (unstable genomes with chromosome fragmentation) and meiosis (prophase was absent and all subsequent stages of meiosis were severely impaired). In 2016, Samanic proposed that the N- terminal region of Mre11 is widely conserved and is most critical for meiosis. Level of OsMRE11 mRNA increased after 3 days of irradiation (30 watts UV-C light), therefore pointing to a possible role of OsMre11 protein in maintaining the stability of the rice chromosomes (53). However, no biochemical characterization of this important protein from plant systems been reported till date. Hence, we intended to study the biochemical properties of OsMre11 protein from rice (*Oryza sativa*, *Japonica*).

Previously in our lab, DNA repair proteins have been characterised from rice system, namely the OsDmc1, OsRad51, translin. In the present study, cDNA corresponding to the full length and N- terminal end of OsMRE11 was cloned into expression vector, followed by over expression in *E. coli* and purification. The OsMre11-N (N- terminal end of OsMre11) protein was used to study several predicted properties of Mre11 protein such as binding to different forms of DNA, endonuclease and exonuclease properties. The detailed results of the study are given below.

3.1: Cloning, over- expression of OsMre11 protein

Based on the *Arabidopsis thaliana* MRE11 sequence, analysis of the rice genome showed the presence of MRE11 homologue. The full length cDNA corresponding to OsMRE11 was available with Rice Genome Resource Centre, (RGRC), Japan (Accession number: AK070546, Clone name: J023054N24). The clone corresponding to full length OsMRE11 cDNA was procured from RGRC. OsMRE11 cDNA fragment (2115 bp) was amplified by PCR using a set of specific primers containing restriction enzyme sites for *Nde*1 and *Bam*H1 (OsMRE11 fwd and OsMRE11 rev) and the clone procured from RGRC as template (cloned in Lambda FLC vector).

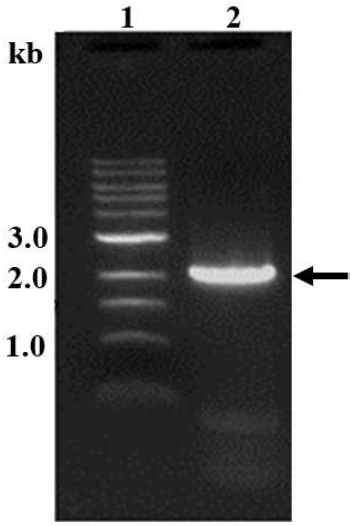

Figure 3.1: PCR amplification of OsMRE11 cDNA

A 2.1 kb OsMRE11 fragment was PCR amplified using primers specific for restriction sites Nde1 and BamH1. The amplified PCR product is indicated by an arrow.

Lane1: 1 kb marker

Lane 2: OsMRE11 amplicon indicated by an arrow

OsMRE11 full length cDNA was amplified successfully

pET28a expression vector and OsMRE11 amplicon were digested with *Nde*1 and *Bam*H1. The digested cDNA fragment was gel purified and ligated. This ligation mixture was used directly for transforming *E. coli* strain JM109 chemically competent cells and plated on LB plate containing 25µg/ml of kanamycin. Random colonies were checked for the presence of insert by colony PCR and were found to be positive.

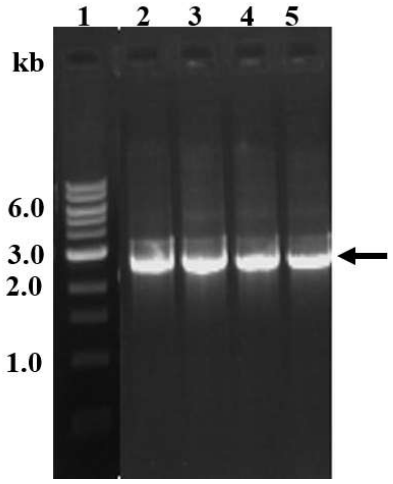

Figure 3.2: Colony PCR of E. coli strain JM109 harbouring pET28a with OsMRE11 cDNA insert

E. coli JM109 cells were transformed with pET28a- OsMRE11 plasmid. Random colonies were checked for the presence of the plasmid by colony PCR. Arrow indicates the amplified OsMRE11 fragment indicating successful transformation of pET28a plasmid harbouring the OsMRE11 cDNA.

Lane1: 1 kb marker

Lane 2- 5: positive clones of E. coli strain JM109 harbouring pET28a with OsMre11 cDNA

OsMre11 cDNA fragment was successfully cloned into pET28a expression vector

The recombinant plasmid was isolated from these positive colonies and subjected to restriction digestion with *Nde*1 and*Bam*H1. A 2115 bp OsMRE11 fragment was released.

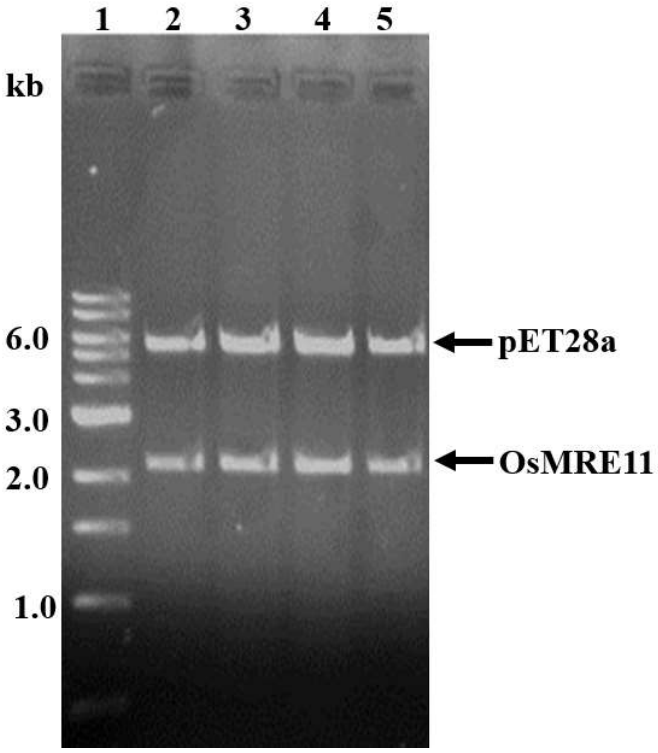

Figure 3.3: Restriction digestion of pET28a recombinant plasmids with BamH1 and Nde1

Plasmids isolated from the clones harbouring recombinant plasmid were subjected to restriction digestion by Nde1 and BamH1 enzymes to reconfirm the presence of the MRE11 insert.

Lane 1: 1 kb marker

Lane 2- 5: digested recombinant plasmids (OsMRE11 insert release)

Restriction digestion confirmed the presence of OsMRE11 fragment in the recombinant plasmids isolated from colony PCR positive clones

Randomly selected recombinant plasmid (plasmid 1) was sequenced for the confirmation of OsMRE11 sequence.

ATGGGAGACGAAAGCAACACACTCCGTGTACTTGTGGCAACCGATTGCCATCTCGGCTACATGGAAAAGGACGAGATTCGTAGGTTTGATTCATTTGAG
GCGTTTGAAGAGATATGCTCATTAGCAGAGCAGAATAAGGTTGATTTTGTGCTACTTGGTGGTGACCTTTTCCATGAGAACAAACCTTCACGTTCAACCT
TAGTTAAGACCATTGAGATTCTACGGCGTTACTGCTTGAATGACCAGCCAGTGAAGTTCCAGGTTGTCAGTGATCAGACAATCAACTTTCCAAACAGATT
CGGCCAAGTAAATTATGAAGATCCAAACTTCAATGTTGGATTGCCTGTGTTCACTATCCATGGAAACCATGATGATCCAGCTGGTGTGGATAATCTCTCTG
CAATTGATATTCTCTCAGCCTGCAATCTTGTAAATTATTTTGGGAAGATGGACCTTGGTGGCTCTGGTGTTGGTGAAATAGCAGTCTATCCTGTACTCGTA
AAAAAGGGTACAACTTTTGTTGCGCTCTATGGCCTTGGAAACATTAGAGATGAACGATTAAACAGGATGTTTCAGACACCTCATGCTGTACAATGGATGC
GACCTGAAACTCAAGATGGAATGTCAGTATCTGACTGGTTCAATATTCTTGTTCTTCATCAGAATAGGATAAAAACAAACCCTAAAAGCGCAATCAATGA
GCATTTCTTACCACGTTTTCTGGACTTTATAGTATGGGGCCATGAGCATGAATGCCTTATTGATCCCCAGGAGGTACCTGGGATGGGTTTTCACATCACTC
AACCAGGCTCATCAGTTGCAACCTCCCTGATTGATGGTGAAGCAAAACCAAAGCATGTTCTCTTGTTAGAAATCAAGGGAAACCAGTATAGGCCAACCA
AAATACCTTTGAGATCCGTTAGGCCCTTTCACTATGCTGAGGTTGTGTTAAAAGATGAAGTGGATGTTGATCCCAATGATCAGGCCTCTGTTCTTGAACAT
TTGGATAAAATTGTAAGAAATCTTATTAAGAAGAGTAGTCAGCCAACAGCCAGTAGACCAGAGACCAAACTTCCACTAATTAGAATCAAGGTAGATTAC
TCTGGGTTTTCAACAATTAATCCACAACGATTTGGTCAGAAGTATGTCGGGAAGGTTGCAAACCCCCAGGATATTCTTATCTTCTCAAAATCAGCAAAAA
AGCGTCAGACTACTGGAGTAGGCAATATTGATGACTCTGAGAAACTTCGTCCCGAGGAACTAAACCAACAAACTATTGAAGCTTTGGTTGCCGAAAATA
ACTTGAAAATGGAGATTCTTCCAGTTGATGACTTGGACATTGCATTGCATGATTTTGTGAGCAAGGATGACAAGATGGCGTTTTATGCCTGTTTGCAGCG
GAATCTTGAAGAAACCAGAACTAAATTGAATTCAGAGGCAGATAAATTTAAAATAGAGGAAGAAGATATAATAGTCAAAGTTGGCGAGTGCATGCAGGA
ACGTGTGAAGGAAAGGTCCCTCCGCTCCAAGGAAGATTCACGGTTCACGTCAAGCTCTCAAAACTTGGATACTGGAGGTAGATCTGTTACAGCTCAAA
GTAACCTGAACTCTTTCAGTGATGATGAAGACACCAGGGAGATGCTTCTCGGTGCAAGAACAACTAATGCTGGAAGGAAAGCATCTGGATTTACAAGA
CCCTCTAAAGATGCTACCGATGTTGCCAAAACTGGAACTTCCAGAAGGGGCAGGGGAAGAGGGACGGCCTCAATGAAGCAGACCACTCTGAATTTCAG
CCAATCAAGGTCAAGTGCTGCTATCCGCAGTGAGGAAGTGCAATCTTCTTCAGATGAGGAAAATGAGACTAATGAAGCAAACGAAGTTGTTGAATCTTC
AGAACCGGAGGAGAGTCCCCAACAAACTGGACGGGAAAGGGCAGCTCCTAGAGGTGGTAGAGGTAGGGGTAGAGGCGCTACTGCAAAAAGGGGTAG
AAAAGCAGATATTTCATCCATCCAGAGCATGCTGATGAGCAAAGATGATGATGACGATGATGAAGATGACAGGCCGAAGAAACCTCCACCTCGGGTCAC
TAGGAACTACGG AGCTGTTAGGAGGAGATGA

Figure 3.4: OsMRE11 cDNA sequence

MGDESNTLRVLVATDCHLGYMEKDEIRRFDSFEAFEEICSLAEQNKVDFVLLGGDLFHENKPSRSTLVKTIEILRRYCLNDQP
VKFQVVSDQTINFPNRFGQVNYEDPNFNVGLPVFTIHGNHDDPAGVDNLSAIDILSACNLVNYFGKMDLGGSGVGEIAVYPV
LVKKGTTFVALYGLGNIRDERLNRMFQTPHAVQWMRPETQDGMSVSDWFNILVLHQNRIKTNPKSAINEHFLPRFLDFIVWG
HEHECLIDPQEVPGMGFHITQPGSSVATSLIDGEAKPKHVLLLEIKGNQYRPTKIPLRSVRPFHYAEVVLKDEVDVDPNDQAS
VLEHLDKIVRNLIKKSSQPTASRPETKLPLIRIKVDYSGFSTINPQRFGQKYVGKVANPQDILIFSKSAKKRQTTGVGNIDDSEK
LRPEELNQQTIEALVAENNLKMEILPVDDLDIALHDFVSKDDKMAFYACLQRNLEETRTKLNSEADKFKIEEEDIIVKVGECM
QERVKERSLRSKEDSRFTSSSQNLDTGGRSVTAQSNLNSFSDDEDTREMLLGARTTNAGRKASGFTRPSKDATDVAKTGTSR
RGRGRGTASMKQTTLNFSQSRSSAAIRSEEVQSSSDEENETNEANEVVESSEPEESPQQTGRERAAPRGGRGRGRGATAKRG
RKADISSIQSMLMSKDDDDDDEDDRPKKPPPRVTRNYGAVRRR

Figure 3.5: Translated amino acid sequence of full length OsMre11 protein

Sequencing of recombinant pET28a- OsMRE11 confirmed that OsMre11 insert was devoid of any mutations

Randomly selected recombinant plasmid was transformed into *E. coli* expression host BL21 (DE3) PlysS and plated on LB plates containing antibiotics (25µg/ml of kanamycin and 34µg/ml of chloramphenicol). Colonies were checked for the presence of the recombinant plasmid by colony PCR.

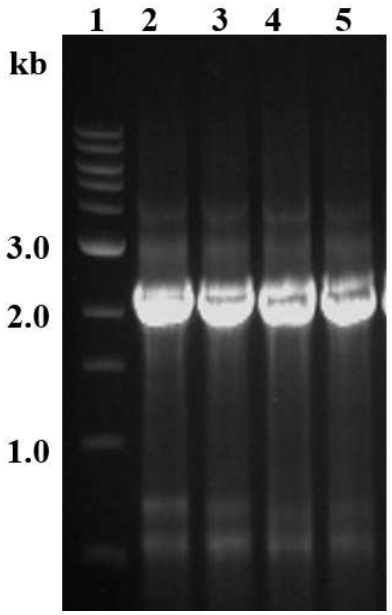

Figure 3.6: Colony PCR of E. coli expression host BL21 (DE3) pLysS harbouring pET28a with OsMRE11 cDNA

Recombinant pET28a plasmids harbouring OsMRE11 cDNA were transformed to E. coli expression host BL21 (DE3) pLysS cells. Random colonies were picked and colony PCR performed to confirm the presence of OSMRE11 fragment.

Lane 1: 1 kb ladder

Lane 2-5: Positive clones harbouring pET28a- OsMRE11 vector

Colony PCR confirmed the presence of pET28a-OsMRE11 plasmid in the expression host *E. coli* BL21 (DE3) pLysS

The recombinant *E. coli* BL21 (DE3) pLysS cells harbouring the recombinant plasmid construct pET28a with OsMRE11 cDNA, were grown at 37˚C in LB media supplemented with 25 µg/ml of kanamycin and 34 µg/ml of chloramphenicol until OD_{600} reached 0.6. Expression of protein was induced by adding 1.0 mM of IPTG (isopropyl-β-D-thiogalactoside) followed by shaking for another 3 hours at 37˚C. The cell extract of IPTG induced cells was resolved on 12% reducing SDS-PAGE and stained with coomassie brilliant blue. A ~80kDa over-expressed band of OsMre11 (705 amino acids) was observed.

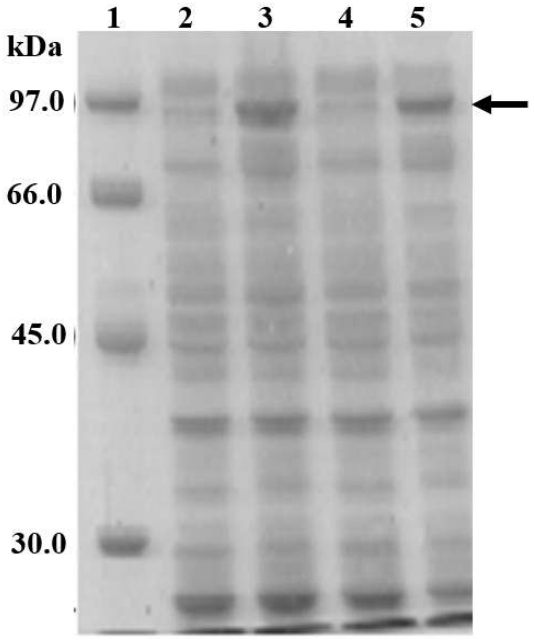

Figure 3.7: Over expression of OsMre11 protein in E. coli BL21 (DE3) pLysS

Over expression of OsMre11 protein after induction with IPTG was analysed. Total protein extracts from induced and uninduced cells were analysed on 12% SDS-PAGE for the presence of over- expressed OsMre11 protein.

Lane 1: Molecular weight markers from top to bottom, Phosphorylase B 94.0 kDa, BSA, 67.0 kDa, Ovalbumin, 43.0 kDa, Carbonic anhydrase 30.0 kDa, and Trypsin inhibitor 14.4 kDa
Lane2 and 4: Crude extracts form un-induced cells

Lane3 and 5: Crude extracts form induced cells (Presence of OsMre11 protein in the induced cell extract indicated by an arrow)

~80kDa OsMre11 protein was observed in the induced cells of *E. coli* BL21 (DE3) pLysS

Different concentrations of IPTG were tested and optimum induction was observed between 0 .5mM and 1.0mM of IPTG.

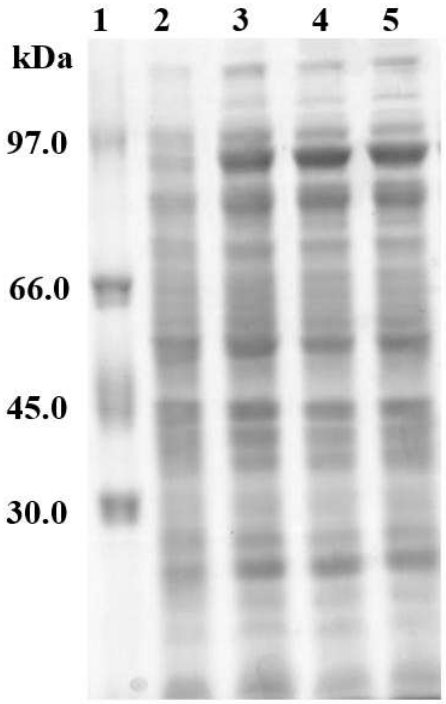

Figure 3.8: Over expression of OsMre11 protein in E. coli BL21 (DE3) pLysS with different concentrations of IPTG

Induction was carried out with different concentrations of IPTG to determine the optimum concentration of IPTG required by recombinant BL21 (DE3) pLysS cells for over- expression of OsMre11 protein.

Lane 1: Molecular weight marker

Lane 2: Lysate of uninduced cells

Lane 3, 4, 5: Lysate of induced cells (0.25, 0.5 and 1.0 mM IPTG respectively)

Maximum protein induction was observed with 1.0 mM IPTG

3.2: Western Blot analysis of purified OsMre11 protein:

Cloning of OsMRE11 cDNA in pET28a expression vector between *Nde*1 and *Bam*H1 restriction sites lead to the expression of a fusion protein with six histidine residues at the N-terminal end of the protein. The presence of the his-tagged protein was confirmed by Western blot using Anti-Histidine monoclonal antibody conjugated with alkaline phosphatase (AP). A major band of 80kDa, corresponding to OsMre11 was observed on the Western blot.

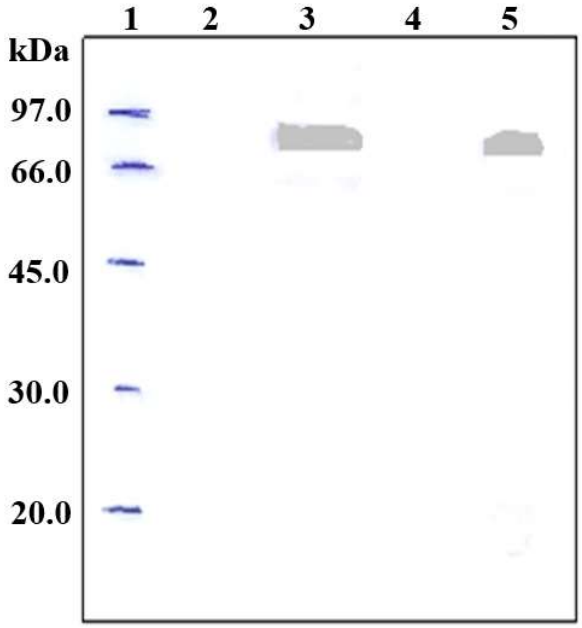

Figure 3.9: Western blot profile of OsMre11 protein

OsMre11 protein was over expressed as a fusion protein with histidine tag at N-terminal of the protein. Western blot of OsMre11 with monoclonal anti poly histidine antibody was carried out.

Lane 1: Molecular weight markers; Lane 2 and 4: uninduced cell lysate

Lane 3 and 5: induced cell lysates

Western blot analysis confirmed the expression of his- tagged OsMre11 protein upon induction with IPTG

To check for the solubility of the over- expressed protein, both the induced and un-induced cells were subjected to repeated freeze-thaw, sonication and centrifugation cycles to separate the supernatant and pellet fraction i.e. the soluble and insoluble fractions. The fractions were analysed on 12% reducing SDS-PAGE and stained with coomassie brilliant blue. A prominent induction band of ~80kDa was seen in the pellet fraction, as induced OsMre11 protein formed inclusion bodies.

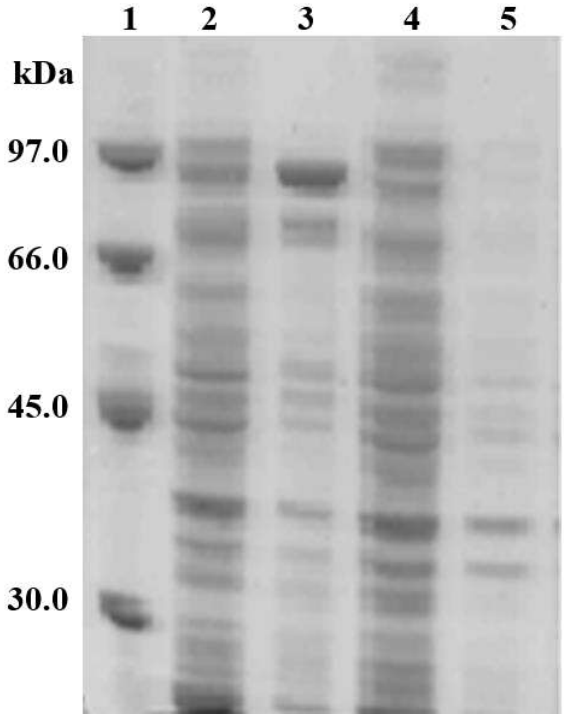

Figure 3.10: Localization of over- expressed OsMre11 protein

Supernatant and pellet fractions of induced and uninduced cells were analyzed on 12% SDS-PAGE to check the solubility of the OsMre11 protein.

Lane 1: Molecular weight markers; Lane 2: Supernatant fraction of the induced cell
Lane 3: Pellet fraction induced cells; Lane 4: Supernatant fraction of the uninduced
Lane 5: Pellet fraction of the uninduced cells

Over expressed OsMre11 protein was observed to form inclusion bodies upon induction.

3.3: Purification of OsMre11 protein

OsMre11 protein which was over- expressed as insoluble fraction was purified under denaturing conditions using 8M Urea. For this purpose, inclusion bodies were first isolated from the cell extract by repeated sonication in lysis buffer containing Triton X-100, sodium azide, PMSF, protease inhibitor cocktail and DTT followed by centrifugation. The histidine tag at the N-terminal end of the over-expressed protein was utilized to purify the protein by affinity chromatography using Ni-NTA affinity matrix under denaturing conditions (8M urea) at pH 8.0. Inclusion bodies were dissolved in buffer (100mM sodium dihydrogen phosphate, pH 8.0), centrifuged and clear supernatant was collected. Column was equilibrated with phosphate buffer (100mM sodium dihydrogen phosphate, pH 8.0), above supernatant was loaded and flow through was collected. Column was washed with same buffer at pH 8.0 and OsMre11 protein was eluted using the same buffer containing 8.0M urea at decreasing pH of 5.5 and 4.5. Eluted fractions were analysed on 12% SDS-PAGE. Several bands were observed on SDS-PAGE especially with lower molecular weight than OsMre11 (80 kDa).

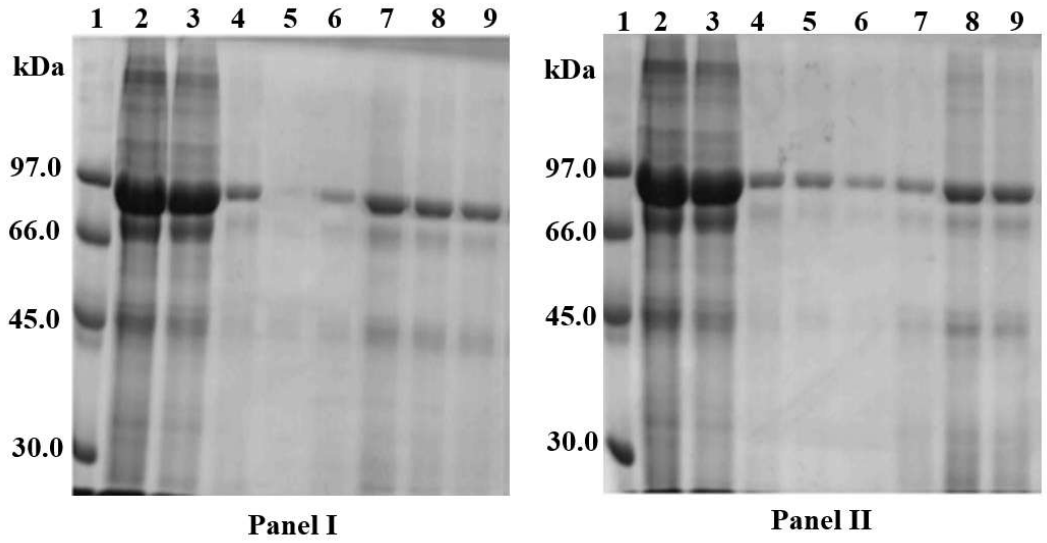

Figure 3.11: Purification of over- expressed OsMre11 protein

The over-expressed OsMre11 was purified using nickel chelating affinity matrix column (affinity chromatography), which binds to histidine tagged proteins and eluted with buffers with pH 5.5 and 4.5.

Panel I: Elution profile with 5.5 pH buffer; Panel II: Elution profile with 4.5 pH buffer

Lane 1: Molecular weight marker

Lane 2: Inclusion body preparation

Lane 3: Flow through

Lane 4: Wash with pH 6.3 buffer

Lane 5- 9 of panel I: Different elution fractions of OsMre11 eluted with pH 5.5 buffer

Lane 5-9 of panel II: Different elution fractions of OsMre11 eluted with pH 4.5 buffer

Partially pure fraction of OsMre11 protein was eluted from Ni-NTA column upon elution with decreasing pH

To determine whether these co- eluted proteins were degradation products or contaminating proteins which eluted out from the column, a western blot was carried out using anti-His monoclonal antibody conjugated with alkaline phosphatase (AP). It was observed that all bands below the 80 kDa OsMre11 protein also cross reacted with anti-histidine antibody, thereby revealing that these were indeed degradation products of OsMre11 rather than being any contaminating proteins which eluted along with the OsMre11 upon elution.

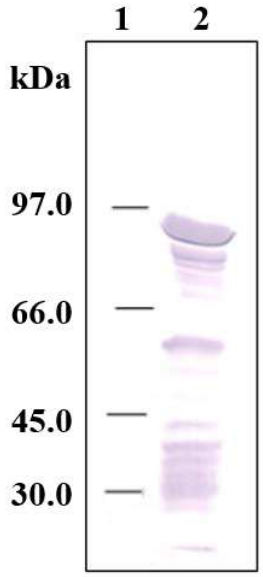

Figure 3.12: Western Blot analysis of purified OsMre11 protein

OsMre11 protein purified at 5.5 pH was subjected to western blot analysis using monoclonal anti poly- histidine antibodies.

Lane1: Molecular weight markers

Lane 2: Eluted fraction of OsMre11 with pH 5.5 buffer

Western blot analysis confirmed the multiple bands to be the degradation products of OsMre11 protein

3.4: Attempts to purify OsMre11 full length protein:

Several strategies listed below were employed to either induce the protein in soluble fraction or to purify the same without any further degradation products. Results of these experiments is presented in appendix.

Serial Number	Experiment	Result
1.	OsMre11 protein induced at 20˚C	OsMre11 was not induced as a soluble fraction
2.	Cells harbouring OsMRE11- peT28a plasmid was grown in M9 media	OsMre11 was not induced as a soluble fraction
3.	N- lauroylsarcosine and arginine	OsMre11 precipitated upon removal of arginine
4.	Cloning into pColdTF vector	No OsMre11 observed upon purification
5.	Chaperone assisted co-expression	OsMre11 induction failed
6.	*K. lactis* expression system (yeast)	OsMre11 not produced by yeast K. lactis
7.	Shuffle T7 Express lysY competent *E. coli* system	No OsMre11 observed upon purification

Table 3.1: Experiments undertaken to purify native full length OsMre11 protein

Some of the probable methods to purify full length OsMre11 protein was carried out.

3.5: Domains of full length OsMre11 protein analysis:

Since all attempts to purify OsMre11 protein lead to either degradation of the full-length protein in both denatured and native form, or no induction of the protein, the OsMre11 protein sequence was subjected to secondary structure prediction tools. It was found that the full length OsMre11 protein had around 42% random coils and a very high instability index of 54.11. Considering this as one of the factors contributing to the degradation of full length protein, functional domain analysis of OsMre11 protein was undertaken.

Domains of OsMre11:

OsMre11 protein sequence was subjected to domain analysis using the conserved domain database tool at NCBI website (www.ncbi.nlm.nih.gov), a resource for the annotation of functional domains in a protein. Analysis showed the presence of two major domains in the full length OsMre11 protein sequence, a nuclease domain and a DNA binding domain.

1.Mre11 nuclease, N-terminal metallophosphatase domain (9-277):

2. Mre11 DNA-binding domain (300-452):

Since two most important domains which are responsible for the functional properties of OsMre11 protein was present at the N- terminal end of OsMre11, the study of N- terminal domain of OsMRE11 (452 amino acids) was carried out.

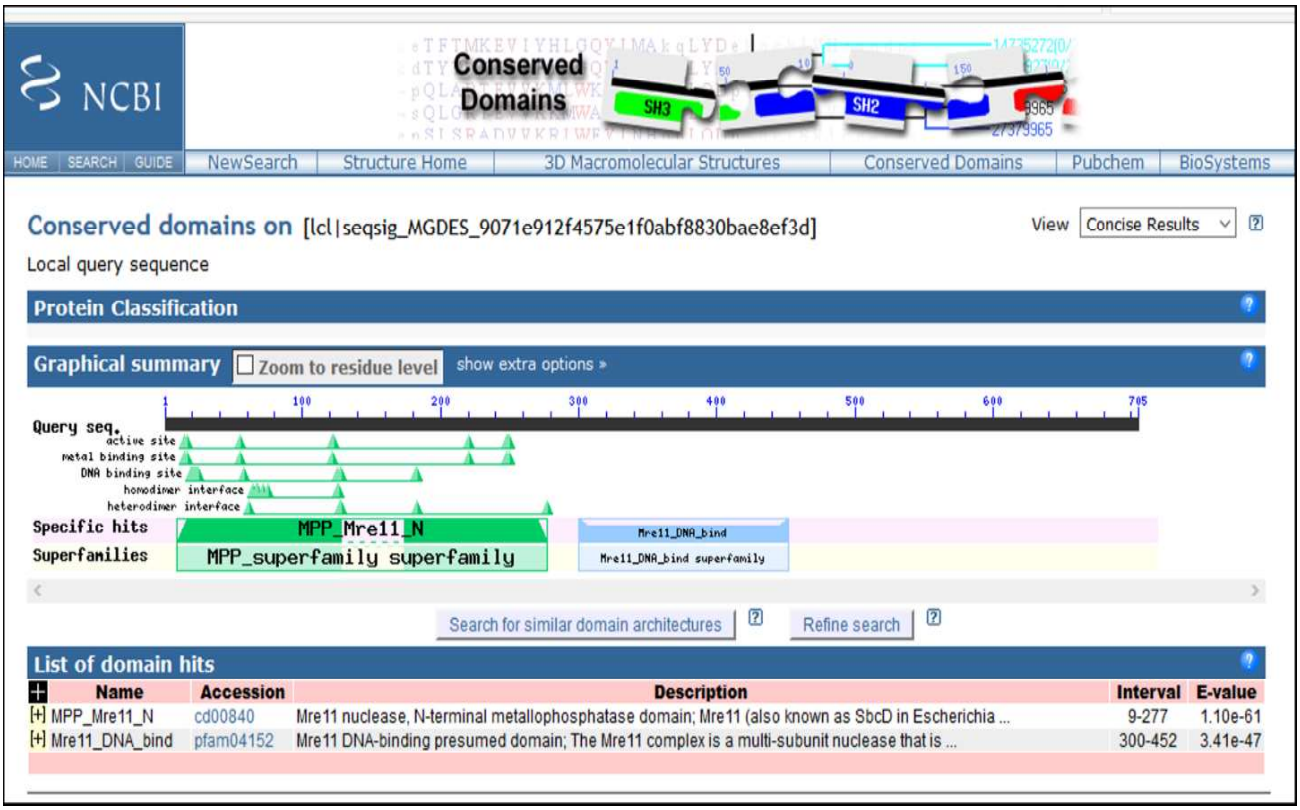

Figure 3.13: Functional domain analysis of OsMre11 protein

Upon analysis of conserved domains of full length OsMre11 protein, it showed the presence of two functional domains a) Mre11 nuclease, N-terminal metallophosphatase domain (9-277) and b) Mre11 DNA-binding domain (300-452).

Domain analysis showed the presence of two important domains at the N- terminal end of OsMre11 protein

3.6: Cloning, over- expression and purification of N- terminal end of OsMre11

cDNA fragment corresponding to OsMRE11 N-terminal domain (nuclease and the DNA binding domain; 1380 bp) was amplified using primers with *Bam*H1 and *Nde*1 restriction sites.

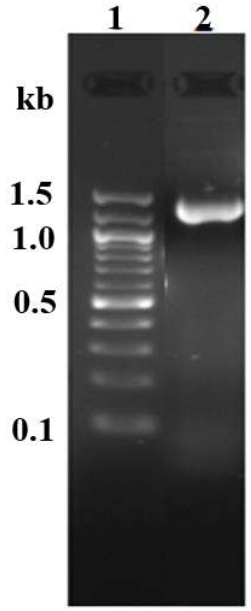

Figure 3.14: PCR amplification of cDNA corresponding to N- terminal end of OsMRE11

A 1.38 kb OsMRE11-N fragment was PCR amplified using primers specific for restriction sites Nde1 and BamH1.

Lane 1: 100 bp marker

Lane 2: cDNA corresponding to N- terminal end of OsMRE11 (1380 bp)

cDNA corresponding to N- terminal end of OsMre11 protein was amplified by PCR

Following PCR amplification, the amplicon and pET16b expression vector were digested with *Nde*1 and *Bam*H1. The cDNA fragment was then ligated into the digested pET16b expression vector. The above ligation mixture was then transformed into *E. coli* DH5α competent cells and plated on LB plate containing carbenecillin. Random colonies were checked for the presence of insert by colony PCR.

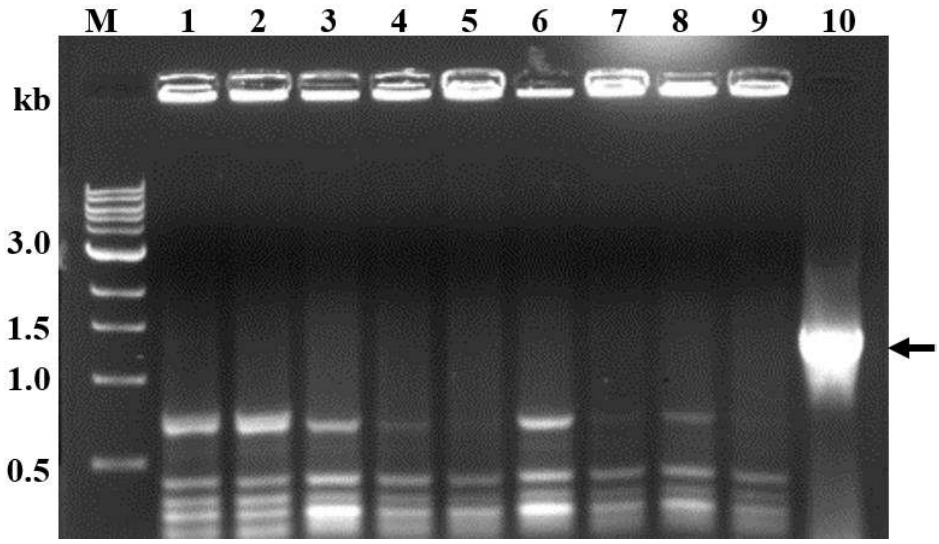

Figure 3.15: Colony PCR of E. coli DH5α recombinant colonies harbouring pET16b with cDNA fragment corresponding to N-terminus of OsMre11

E. coli DH5α cells were transformed with pET16b- OsMRE11-N plasmid. Random colonies were picked and checked for the presence of the plasmid. Arrow indicates the amplified OsMRE11-N fragment. Only clone number 10 showed the expected band.

Lane M: 1 kb Ladder; Lanes 1-9: Recombinant clones without OsMre11 insert

Lane 10: Recombinant positive clone with OsMre11 cDNA insert (indicated by an arrow)

cDNA fragment corresponding to N-terminus of OsMre11 was successfully cloned in pET16b expression vector

Plasmid was isolated from a single colony and sequenced and confirmed that no mutations were introduced during PCR amplification and cloning process.

ATGGGAGACGAAAGCAACACACTCCGTGTACTTGTGGCAACCGATTGCCATCTCGGCTACATGGAAAAGGACGAGATTCGTAGGTTTGATTC
ATTTGAGGCGTTTGAAGAGATATGCTCATTAGCAGAGCAGAATAAGGTTGATTTTGTGCTACTTGGTGGTGACCTTTTCCATGAGAACAAACC
TTCACGTTCAACCTTAGTTAAGACCATTGAGATTCTACGGCGTTACTGCTTGAATGACCAGCCAGTGAAGTTCCAGGTTGTCAGTGATCAGA
CAATCAACTTTCCAAACAGATTCGGCCAAGTAAATTATGAAGATCCAAACTTCAATGTTGGATTGCCTGTGTTCACTATCCATGGAAACCATG
ATGATCCAGCTGGTGTGGATAATCTCTCTGCAATTGATATTCTCTCAGCCTGCAATCTTGTAAATTATTTTGGGAAGATGGACCTTGGTGGCTC
TGGTGTTGGTGAAATAGCAGTCTATCCTGTACTCGTAAAAAAGGGTACAACTTTTGTTGCGCTCTATGGCCTTGGAAACATTAGAGATGAACG
ATTAAACAGGATGTTTCAGACACCTCATGCTGTACAATGGATGCGACCTGAAACTCAAGATGGAATGTCAGTATCTGACTGGTTCAATATTCT
TGTTCTTCATCAGAATAGGATAAAAACAAACCCTAAAAGCGCAATCAATGAGCATTTCTTACCACGTTTTCTGGACTTTATAGTATGGGGCCAT
GAGCATGAATGCCTTATTGATCCCCAGGAGGTACCTGGGATGGGTTTTCACATCACTCAACCAGGCTCATCAGTTGCAACCTCCCTGATTGAT
GGTGAAGCAAAACCAAAGCATGTTCTCTTGTTAGAAATCAAGGGAAACCAGTATAGGCCAACCAAAATACCTTTGAGATCCGTTAGGCCCTT
TCACTATGCTGAGGTTGTGTTAAAAGATGAAGTGGATGTTGATCCCAATGATCAGGCCTCTGTTCTTGAACATTTGGATAAAATTGTAAGAAA
TCTTATTAAGAAGAGTAGTCAGCCAACAGCCAGTAGACCAGAGACCAAACTTCCACTAATTAGAATCAAGGTAGATTACTCTGGGTTTTCAA
CAATTAATCCACAACGATTTGGTCAGAAGTATGTCGGGAAGGTTGCAAACCCCCAGGATATTCTTATCTTCTCAAAATCAGCAAAAAAGCGT
CAGACTACTGGAGTAGGCAATATTGATGACTCTGAGAAACTTCGTCCCGAGGAACTAAACCAACAAACTATTGAAGCTTTGGTTGCCGAAA
ATAACTTGAAAATGGAGATTCTTCCAGTTGATGACTTGGACATTGCATTGCATGATTTTGTGAGCAAGGATGACAAGATGGCG

Figure 3.16: cDNA sequence corresponding to the N- terminal domain of OsMRE11

MGDESNTLRVLVATDCHLGYMEKDEIRRFDSFEAFEEICSLAEQNKVDFVLLGGDLFHENKPSRSTLVKTIEILRRYCLNDQPV
KFQVVSDQTINFPNRFGQVNYEDPNFNVGLPVFTIHGNHDDPAGVDNLSAIDILSACNLVNYFGKMDLGGSGVGEIAVYPVL
VKKGTTFVALYGLGNIRDERLNRMFQTPHAVQWMRPETQDGMSVSDWFNILVLHQNRIKTNPKSAINEFLPRFLDFIVWGHE
HECLIDPQEVPGMGFHITQPGSSVATSLIDGEAKPKHVLLLEIKGNQYRPTKIPLRSVRPFHYAEVVLKDEVDVDPNDQASVLE
HLDKIVRNLIKKSSQPTASRPETKLPLIRIKVDYSGFSTINPQRFGQKYVGKVANPQDILIFSKSAKKRQTTGVGNIDDSEKLRP
EELNQQTIEALVAENNLKMEILPVDDLDIALHDFVSKDDKMA

Figure 3.17: Translated amino acid sequence of N- terminal end of OsMre11 protein

Sequencing of recombinant pET16b- OsMRE11 confirmed, the plasmid to be devoid of any mutations

Recombinant pET16b harbouring cDNA corresponding to the N-terminal domain of OsMre11, was transformed into chemically competent *E. coli* expression host Shuffle T7 Express lysY competent cells. These cells possess the enhanced ability to correctly fold proteins with multiple disulphide bonds in the cytoplasm, thereby producing soluble native recombinant proteins. Clones harbouring the recombinant plasmid was selected on carbenecillin-chloramphenicol (Cb- Cm) plates. Positive clones were grown in LB medium supplemented with Cb-Cm antibiotics and the OsMre11-N protein was induced by adding 0.4mM IPTG and allowed to grow overnight at 20˚C, with continuous shaking at 150 rpm. Cells were harvested and crude extract prepared in lysis buffer. Crude extract was analysed on 12% SDS-PAGE and a 55kDa over- expressed band of OsMre11 (indicated by an arrow) is observed.

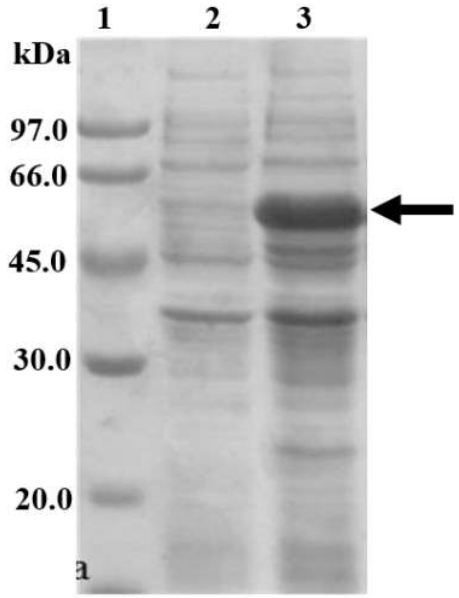

Figure 3.18: Over expression of N- terminal end of OsMre11 protein in E. coli Shuffle T7 Express lysY cells

Induced and uninduced cells were analysed on 12% SDS-PAGE for the presence of over-expressed OsMre11-N protein.

Lane 1: Molecular weight markers; Lane2: Crude extracts form un-induced cells

Lane3: Crude extracts form induced cells

An over expressed protein band around ~55.0 kDa indicated by an arrow, was observed in the induced cell lysate.

Induced cells were harvested after induction and subjected to repeated freeze-thaw cycles in lysis buffer and cell free extract was prepared by subjecting the cell to brief sonication, followed by centrifugation at 12,000 x g. The supernatant and pellet fractions of induced lysate was collected and analysed on 12% SDS-PAGE. A 55 kDa band was observed to be present in the soluble fraction.

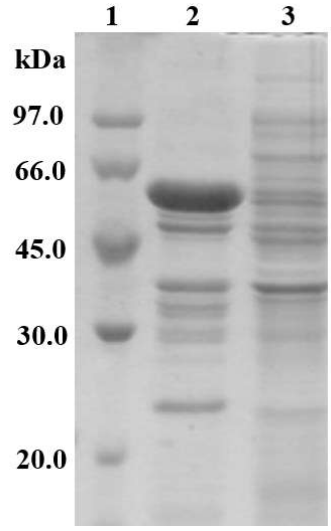

Figure 3.19: Analysis of pellet and supernatant fractions of E. coli Shuffle T7 Express lysY cells over- expressing OsMre11-N protein

Supernatant and pellet fractions of induced cells were analysed on 12% SDS-PAGE to check the solubility of the over expressed protein.

Lane 1: Molecular weight markers

Lane 2: Supernatant of induced E. coli cells

Lane 3: Pellet fraction of induced E. coli cells

The over expressed OsMre11-Nprotein was present in the soluble fraction of recombinant *E. coli* Shuffle T7 Express lysY.

Since the induced OsMre11-N band was present in soluble fraction, we used native buffer conditions (devoid of any form of denaturing agents) to purify the protein and subsequently used the same for biochemical characterization.

OsMre11-N cDNA was cloned into pET16b expression vector, which results in the induction of a fusion protein with six histidine residues at N-terminal of the induced protein. This N-terminal histidine tag aids in the purification of the overexpressed OsMre11 protein over nickel affinity matrix, which is supposed to bind specifically to histidine tagged proteins. Proteins with his-tag are known to bind to the column at basic pH (pH 8.0) and are eluted with increasing concentration of imidazole, which is known to elute the histidine tagged proteins bound to the nickel resin. The OsMre11 protein was purified under native conditions using Ni-NTA column. Fractions of each stage of purification was collected and subjected to 12% SDS- PAGE analysis and stained with coomassie brilliant blue. Fractions eluted with 200 and 300mM imidazole shows a relatively homogenous pure fraction of OsMre11-N protein devoid of any degradation or contaminating proteins. The purified protein fractions were collected, dialyzed, concentrated and used for further biochemical characterization.

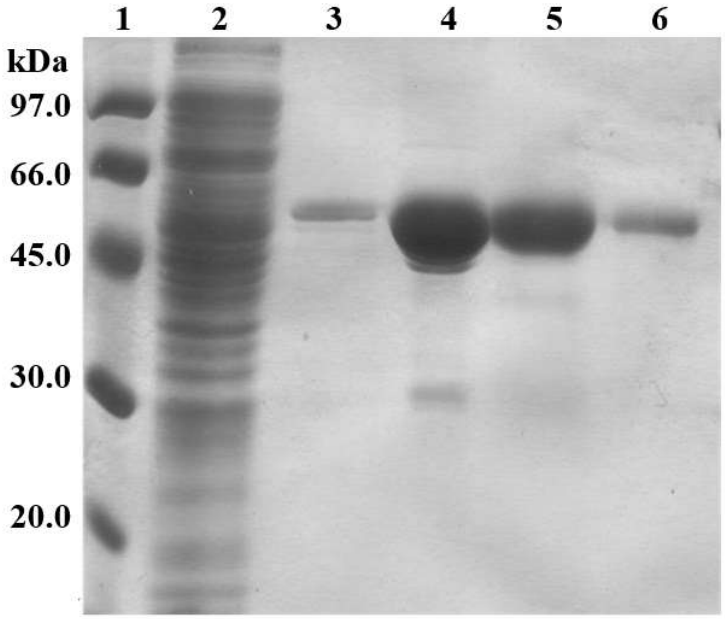

Figure 3.20: Purification of over expressed OsMre11-N under native conditions

The over-expressed OsMre11-N protein was purified using nickel chelating affinity matrix column (affinity chromatography), which binds to histidine tagged proteins and eluted with increasing concentration of imidazole (100- 400 mM).

Lane 1: Marker

Lane 2: Flow through

Lane 3: Column wash with wash buffer (pH 8.0)

Lanes 4,5, 6: OsMre11-N eluted buffer containing increasing amount of imidazole (100,200, 300 mM imidazole respectively)

Purification under native conditions resulted in a pure fraction of OsMre11-N protein.

3.7: Identity of the over- expressed OsMre11- N protein:

The purified 55 kDa OsMre11- N protein was subjected to western blot analysis using mouse anti-poly histidine monoclonal antibodies. On western blot, the purified protein showed a cross reacting band with anti-histidine antibodies.

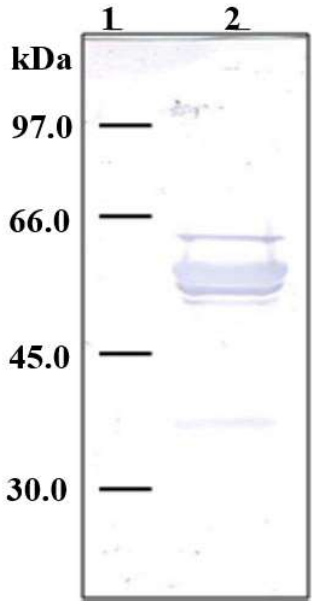

Figure 3.21: Western Blot analysis of purified OsMre11-N protein

OsMre11-N protein was over expressed as a fusion protein with histidine tag at N-terminal of the protein. Western blot of OsMre11-N with monoclonal anti poly histidine antibody was carried out.

Lane1: Molecular weight markers

Lane 2: Purified OsMre11-N protein

Western blot analysis showed the presence of his- tagged OsMre11-N protein

Identity of the 55 kDa over- expressed protein was further confirmed by MALDI-TOF analysis of OsMre11-N protein. The purified OsMre11 protein was resolved on 12% SDS-PAGE and the protein band excised and subjected to in-gel tryptic digestion. PMF spectra of the trypsin digested peptides were obtained on a Brukker MALDI-TOF/TOF system in reflectron mode. Peptide fragments corresponding to the peaks from MALDI spectrum were used to search the database, which resulted in identification of the protein as a OsMre11-N protein with 41% protein sequence coverage.

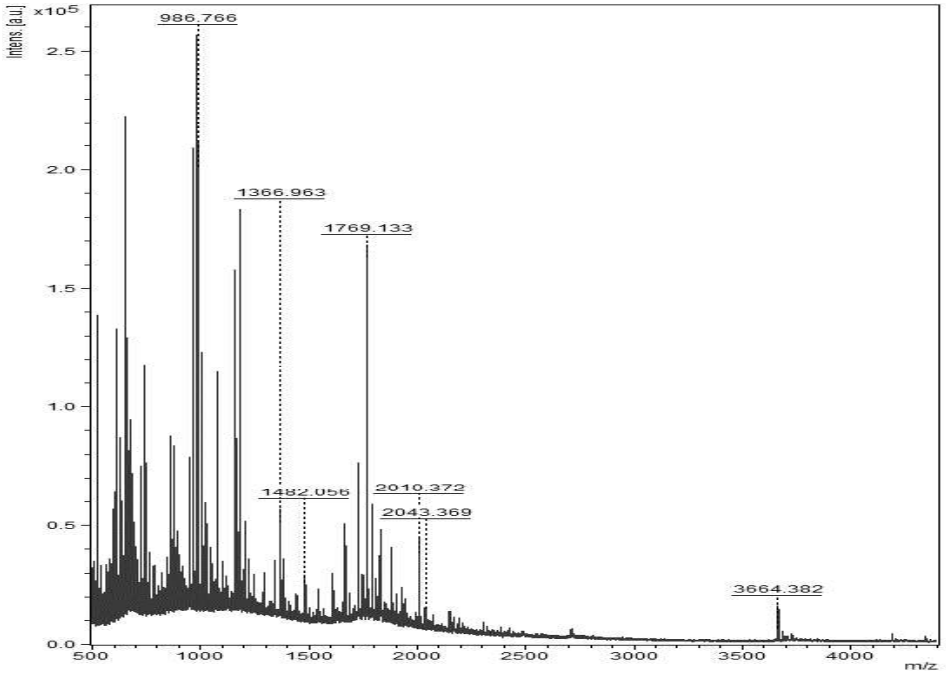

Figure 3.22a: MALDI –TOF analysis of OsMre11-N protein

Purified OsMre11-N protein was separated on 12% SDS-PAGE gel and a 55kDA band was processed for analysis by MALDI. m/z values are given on X-axis and relative intensity of peaks is shown on Y-axis.

Spectra showed very intense peaks with less back ground indicating the purity and identity of the protein.

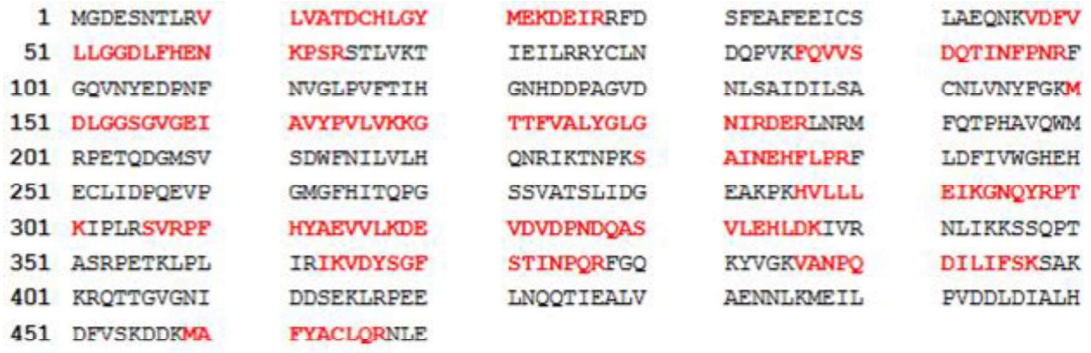

Figure 3.22 b: Protein sequence coverage upon MALDI –TOF analysis of OsMre11-N protein

MALDI-TOF analysis suggested the identity of purified protein as OsMre11 with 41 % sequence identity.

3.8: Circular Dichroism and native PAGE analysis of the purified OsMre11-N protein:

The CD spectrum of the purified OsMre11-N protein was determined in the wavelength range of 200- 260 nm to determine the secondary structure of the protein. The CD spectrum showed a negative peak at 200 nm, typical of helical proteins, confirming the theoretical prediction that purified rice Mre11 protein has helical structures in its native state.

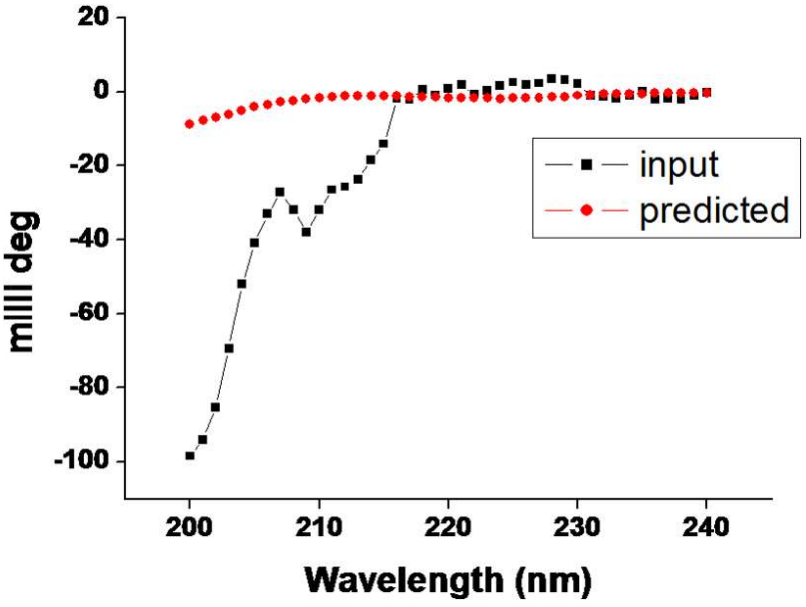

Figure 3.23: CD spectra of N- terminal end of OsMre11 protein

The observed ellipticity measured in milli-degree units was converted to molar ellipticity i.e. Delta epsilon (M−1 cm−1) and plotted against the wave length.

Negative peak at 200 nm indicates that protein has mostly alpha helical structures

Purified OsMre11-N protein was analysed on native PAGE to determine the oligomeric state of the protein in the native condition. The purified OsMre11-N protein upon native PAGE analysis, showed the presence of a band with an approximate molecular weight of 110kDa (dimer) and a band close to ~155 kDa probably corresponding to a trimer.

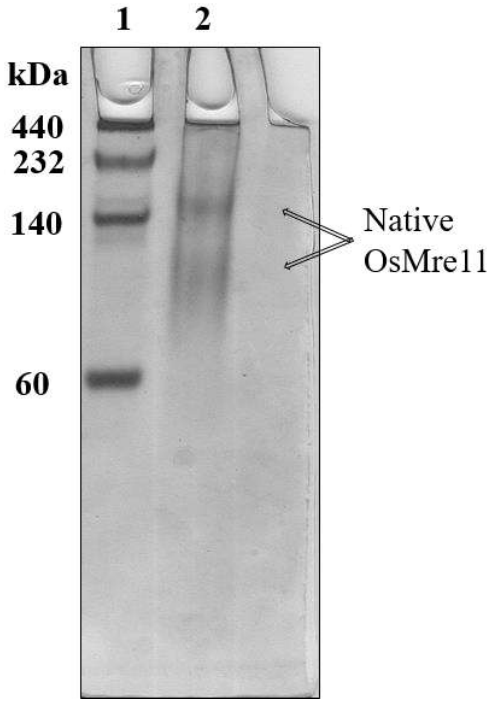

Figure 3.24: Native PAGE analysis of OsMre11-N protein

Purified OsMre11-N protein was subjected to native 8% PAGE analysis, under non- reducing conditions to determine the probable polymeric state of the protein in its native state.

Lane 1: Molecular weight marker

Lane 2: Native OsMre11-N protein

OsMre11 in native condition is shown to have a molecular weight of ~110KDa and 150kDa, which might suggest a native dimeric/ trimeric state.

3. 9: Biochemical characterization of OsMre11-N protein:

Since the identity of overexpressed and purified protein was established as OsMre11-N, we further investigated the biochemical properties of this protein. We studied the DNA binding properties and nuclease properties using different techniques.

3.9.1: DNA binding assays:

Single stranded DNA binding properties of OsMre11 protein was studied by electrophoretic mobility shift assays (EMSA). A^{32}P-labelled 3 pmol/µl single- stranded (4G3, 34 mer) DNA along with increasing concentrations of OsMre11-N protein was used in the assay. It was observed that OsMre11 protein bound ssDNA.

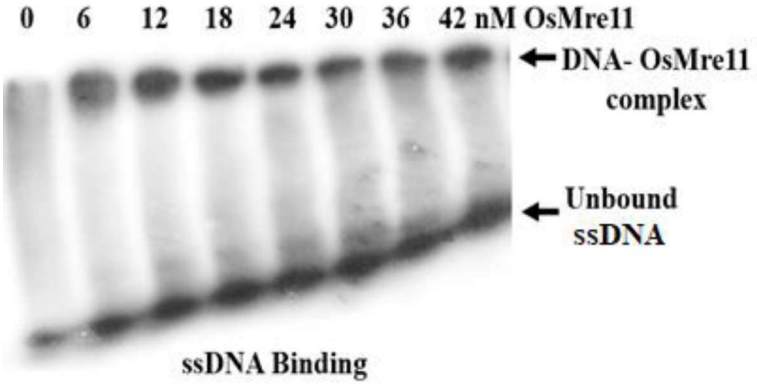

Figure 3.25: ssDNA binding of OsMre11-N by EMSA

ssDNA binding activity of OsMre11-N was monitored by electrophoretic mobility shift assay (EMSA). Indicated amounts of OsMre11-N (0- 42 nM) were added to ^{32}P-labelled 3 pmol/µl ssDNA in reaction mixture. Samples were subjected to electrophoresis on 8.0 % PAGE for 4 hours. Top bands correspond to OsMre11-ssDNA complexes whereas bands at the bottom of the gels correspond to the unbound naked ssDNA.

OsMre11-N was able to bind to ssDNA in a protein concentration dependent manner.

dsDNA binding activity of OsMre11-N was monitored by electrophoretic mobility shift assay (EMSA) using radiolabelled dsDNA. Indicated amounts of OsMre11-N (0- 42 nM) were added to 3 pmol of radiolabelled double-stranded DNA- annealed using 4G3* and 4G3C (34 mer) in reaction mixture. Samples were subjected to electrophoresis on 8 % native polyacrylamide gel for 4.0 hours.

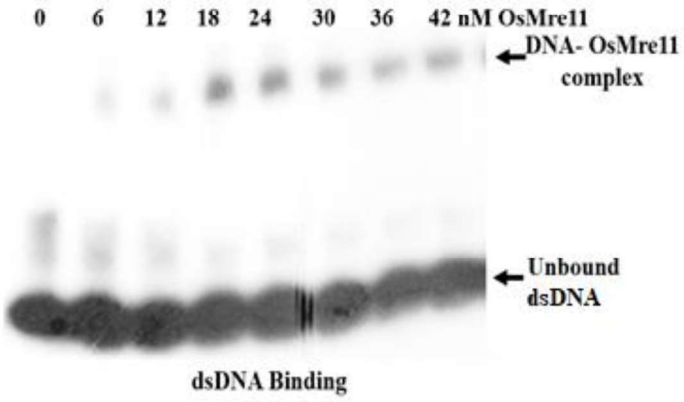

Figure 3.26: dsDNA binding activity of OsMre11-N by EMSA

dsDNA binding activity of OsMre11-N was monitored by electrophoretic mobility shift assay (EMSA). Indicated amounts of OsMre11-N (0- 42 nM) were added to ^{32}P-labelled 3 pmol/µl dsDNA in reaction mixture. Samples were subjected to electrophoresis on 8.0 % PAGE for 4 hours. Top bands correspond to OsMre11-dsDNA complexes whereas bands at the bottom of the gels correspond to the unbound naked dsDNA.

OsMre11-N was able to bind to dsDNA in a protein concentration dependent manner.

G4 DNA binding activity of OsMre11-N was monitored by electrophoretic mobility shift assay (EMSA) using radiolabelled DNA. Indicated amounts of OsMre11-N (0-42 nM) were added to 3 pmol of radiolabelled G4 DNA (34 mer) in reaction mixture. Samples were subjected to electrophoresis on 8 % native polyacrylamide gels for 4 hours.

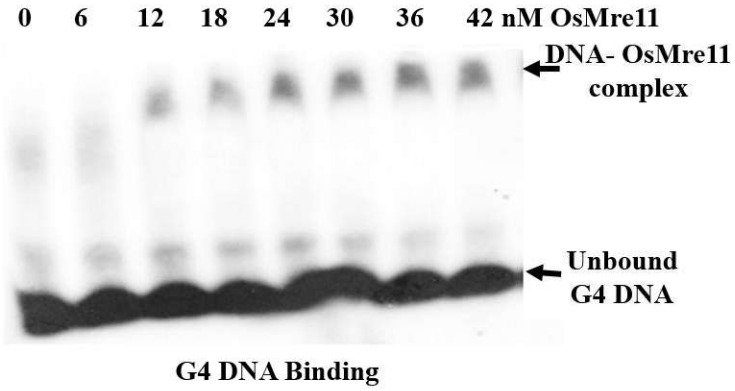

Figure 3.27: G4 DNA binding of OsMre11-N by EMSA

G4 DNA binding activity of OsMre11-N was monitored by electrophoretic mobility shift assay (EMSA). Indicated amounts of OsMre11-N (0- 42 nM) were added to ^{32}P-labelled 3 pmol/µl G4 DNA in reaction mixture. Samples were subjected to electrophoresis on 8.0 % PAGE for 4 hours. Top bands correspond to OsMre11-G4 DNA complexes whereas bands at the bottom of the gels correspond to the unbound naked G4 DNA.

OsMre11-N was able to bind to G4 DNA in a protein concentration dependent manner.

3.9.2: Fluorescence based DNA binding assay:

For analysing the binding of OsMre11-N protein to different forms of DNA we carried out fluorescence based assays, wherein changes in tryptophan fluorescence of the OsMre11-N protein upon binding to DNA was measured. It was observed that there was an increase in tryptophan fluorescence upon binding of the purified protein to different DNA substrates such as dsDNA, ssDNA and G4 DNA.

Indicated amounts of OsMre11-N (0-120 nM) were added to 100pmoles of 4G3, 34 mer oligonucleotide in reaction mixture. Samples were incubated for 20 minutes at room temperature and fluorescence was measured (excitation at 295 nm and emission at 305-360 nm). An increase in tryptophan fluorescence upon binding of the purified protein to ssDNA substrate is observed.

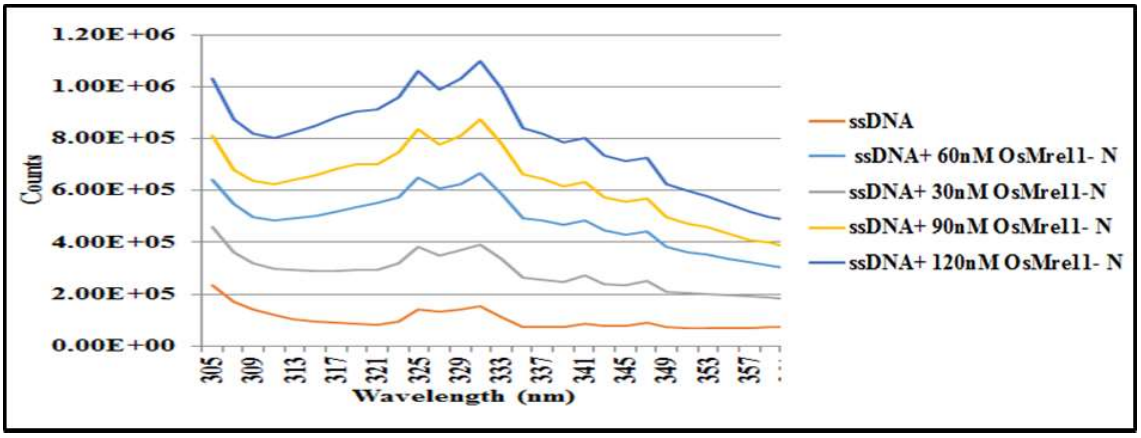

Figure 3.28: ssDNA binding of OsMre11-N monitored by change in the tryptophan fluorescence

ssDNA binding activity of OsMre11-N was also monitored by change in tryptophan fluorescence. Indicated amounts of OsMre11-N (0- 120 nM) were added to 100 pmoles of ssDNA in reaction mixture. Samples were incubated for 20 minutes at room temperature and fluorescence was recorded (excitation at 295 nm and emission at 305-360 nm).

OsMre11-N was able to bind to ssDNA in a protein concentration dependent manner.

dsDNA binding to OsMre11-N was monitored by fluorescence based assays, where the change in tryptophan fluorescence of the protein OsMre11-N upon binding to DNA was measured, thereby showing that the protein is undergoing a conformational change. Indicated amounts of OsMre11-N (0-120 nM) were added to 100pmolesof dsDNA formed by annealing equimolar concentration of 4G3 and 4G3C (34 mer oligonucleotides) in reaction mixture. Samples were incubated for 20 minutes at room temperature and fluorescence was measured, with excitation at 295 nm and emission from 305 nm to360 nm. An increase in tryptophan fluorescence upon binding of the purified OsMre11-N protein to dsDNA substrate was observed.

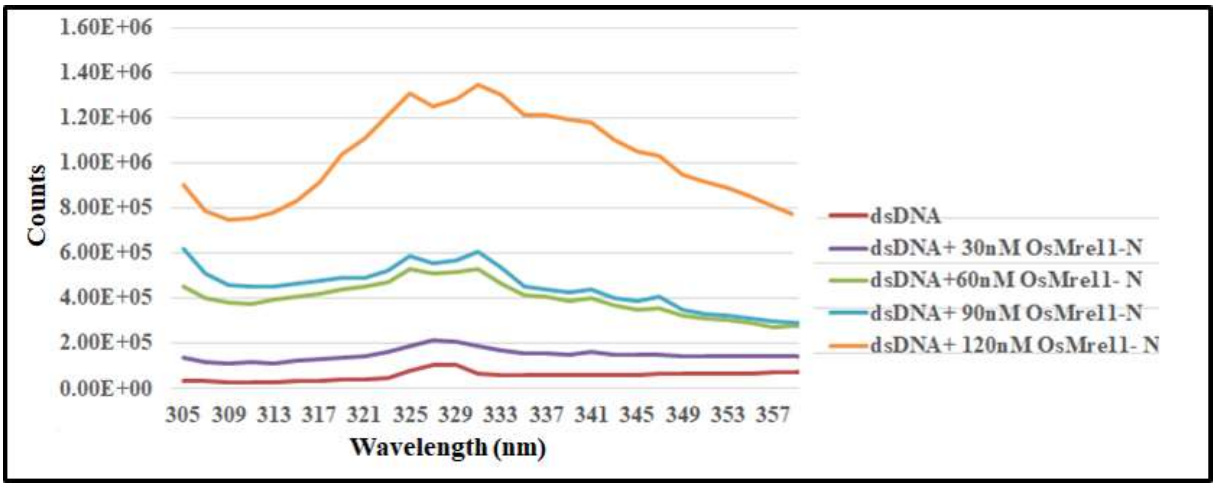

Figure 3.29: dsDNA binding of OsMre11-N monitored by change in the tryptophan fluorescence

dsDNA binding activity of OsMre11-N was also monitored by change in tryptophan fluorescence. Indicated amounts of OsMre11-N (0- 120 nM) were added to 100 p moles of dsDNA in reaction mixture. Samples were incubated for 20 minutes at room temperature and fluorescence was recorded (excitation at 295 nm and emission at 305-360 nm).

OsMre11-N was able to bind to dsDNA in a protein concentration dependent manner.

G4 DNA binding to OsMre11-N was monitored by fluorescence based assays, where the change in tryptophan fluorescence of the protein OsMre11-N upon binding to DNA was measured, indicated amounts of OsMre11-N (0-120 nM) were added to 100 p moles of G4 DNA formed from 4G3(34 mer oligonucleotides) in reaction mixture. Samples were incubated for 20 minutes at room temperature and fluorescence was measured, by exciting the sample at 295 nm and monitoring the emission between 305-360 nm. An increase in tryptophan fluorescence of OsMre11-N upon binding to G4 DNA substrate was observed.

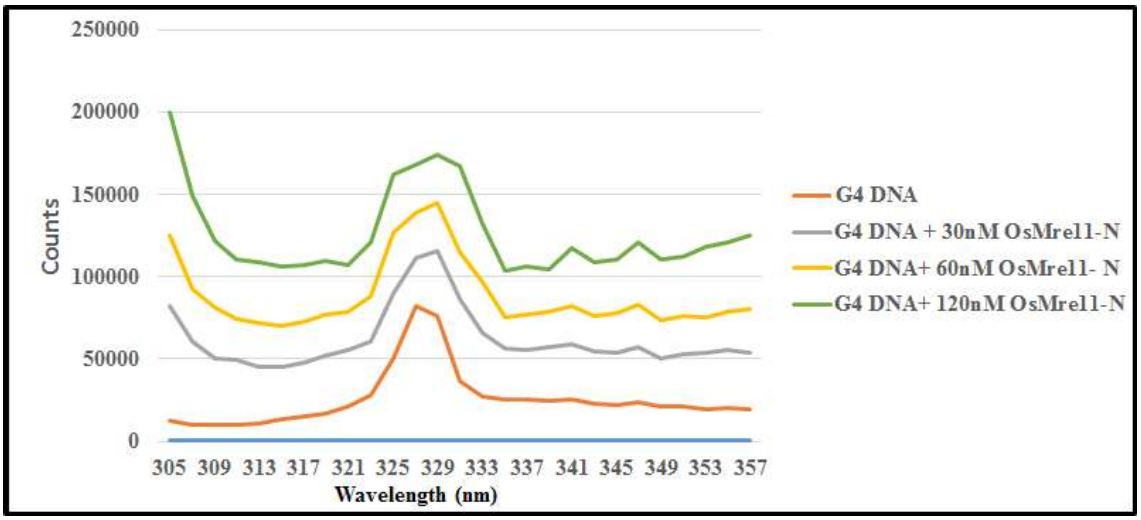

Figure 3.30: G4 DNA binding of OsMre11-N monitored by change in the tryptophan fluorescence.

G4 DNA binding activity of OsMre11-N was also monitored by change in tryptophan fluorescence. Indicated amounts of OsMre11-N (0- 120 nM) were added to 100 p moles of G4 *DNA in reaction mixture. Samples were incubated for 20 minutes at room temperature and fluorescence was recorded (excitation at 295 nm and emission at 305-360 nm).*

OsMre11-N was able to bind to G4 DNA in a protein concentration dependent manner.

3.9.3: Nuclease assays:

3.9.4: Exonuclease activity:

To demonstrate the exonuclease property of OsMre11 protein, a linearized pSK^+ plasmid was used as substrate. Ability of the OsMre11-N protein to digest the linearized fragment in presence and absence of Mn^{2+}, to form degraded products was monitored on agarose gel. It was observed that OsMre11-N could not facilitate the degradation of the dsDNA in absence of Mn^{2+}. However, the protein catalysed the degradation of linear pSK^+ plasmid in presence of 5.0 mM Mn^{2+}.

Increasing amounts of OsMre11-N (0-50 nM) were added to 100ng of linearized plasmid in reaction mixture in absence of Mn^{2+} ions (Panel I) and in presence of 5.0 mM Mn^{2+} (Panel II) and incubated for 20 minutes at 37˚ C. Samples were subjected to electrophoresis on 0.8 % agarose gels for 3 hours.

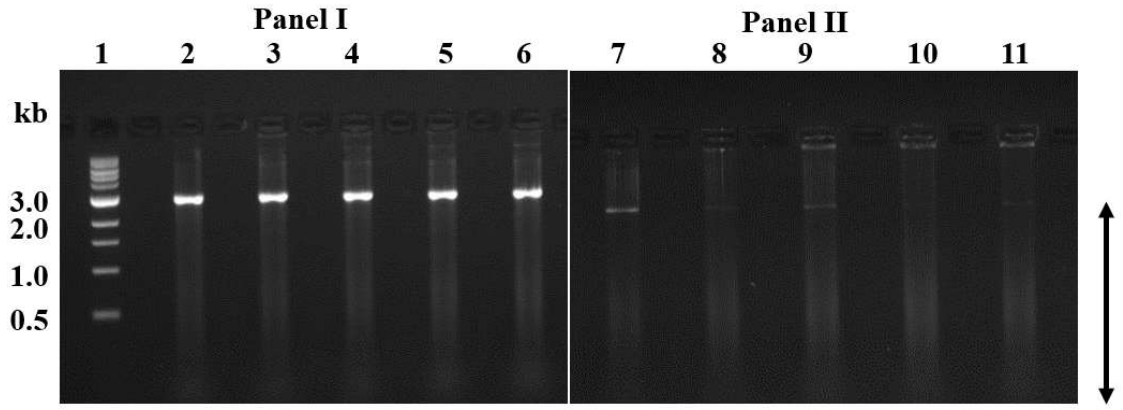

Figure 3.31: Exonuclease activity of OsMre11-N in absence (Panel I) and presence (Panel II) of Mn^{2+}

Exonuclease activity of OsMre11-N protein was monitored by incubating increasing concentration of protein (0- 50 nM) with linearized pSK^{+} plasmid in absence (Panel I) and presence (Panel II) of Mn^{2+}. Arrow represents the DNA degradation products formed.

Lane 1: 1 kb ladder

Lane 2-6: 0, 12, 25, 38, 50 nM OsMre11-N in absence of Mn^{2+} respectively

Lane 7-11: 0, 12, 25, 38, 50 nM OsMre11-N in presence of Mn^{2+}respectively

OsMre11-N protein exhibited exonuclease activity in a protein concentration dependent manner only in presence of Mn^{2+}

3.9.5: Endonuclease activity:

To demonstrate the endonuclease property of OsMre11-N, the ability of the protein to convert RF-I DNA (CCC form) to RF-II DNA (Nicked Circular), upon incubation with increasing amount of OsMre11-N protein in presence and absence of Mn^{2+} was tested. It was observed that OsMre11-N could not facilitate the conversion of RF-I DNA to RF-II DNA in the absence of Mn^{2+}. However, OsMre11 protein facilitated the conversion of RF-I DNA to RF-II DNA in presence of 5.0 mM Mn^{2+}. Increasing amounts of OsMre11-N (0-63 nM) were added to 1.0 µg of RF-I DNA in reaction mixture in absence of Mn^{2+} (Panel I) and in presence of 5.0 mM Mn^{2+} (Panel II) and incubated for 20 minutes at 37˚C. Samples were subjected to electrophoresis on 0.8 % agarose gels for 3 hours.

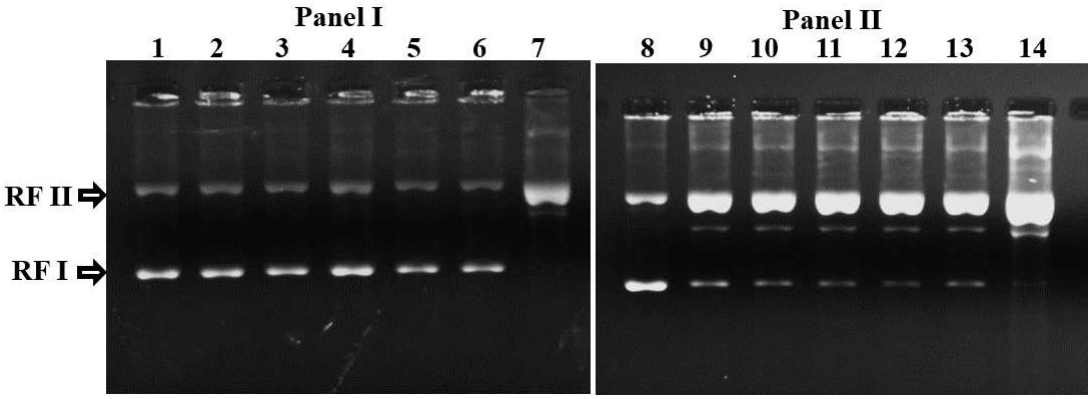

Figure 3.32: Endonuclease activity of OsMre11-N in absence (Panel I) and presence (Panel II) of Mn^{2+}

Endonuclease activity of OsMre11-N protein was monitored by incubating increasing concentration of protein (0- 63 nM) with PhiX-174 RF I DNA in absence (Panel I) and presence (Panel II) of Mn^{2+}. Arrow represents the conversion of RF-I to RF- II DNA formed by the protein upon incubation, resolved on 0.8% agarose gels.

Lane 1-6: 0, 12, 25, 38, 50, 63 nM OsMre11 in absence of Mn^{2+}

Lane 8-13: 0, 12, 25, 38, 50, 63 nM OsMre11 in presence of Mn^{2+}

Lane 7 and 14: RF-II DNA (control)

OsMre11-N protein exhibited endonuclease activity in a protein concentration dependent manner only in presence of Mn^{2+}

3.9.6: Co- factor requirement:

Ability of OsMre11-N protein to convert RF-I DNA (CCC form) to RF-II DNA (Nicked Circular) in presence of different cations was tested. RF-I DNA was incubated with different cations as shown in the image in reaction buffer and a fixed concentration of protein was added (38 nM) followed by incubation for 20 minutes at 37° C. Samples were subjected to electrophoresis on 0.8 % agarose gels for 3.0 hours. In presence of Mg^{2+} and Ca^{2+}, the protein OsMre11-N showed endonuclease activity and no activity was detected in presence of $Zn^{2+}$and K^{+} ions.

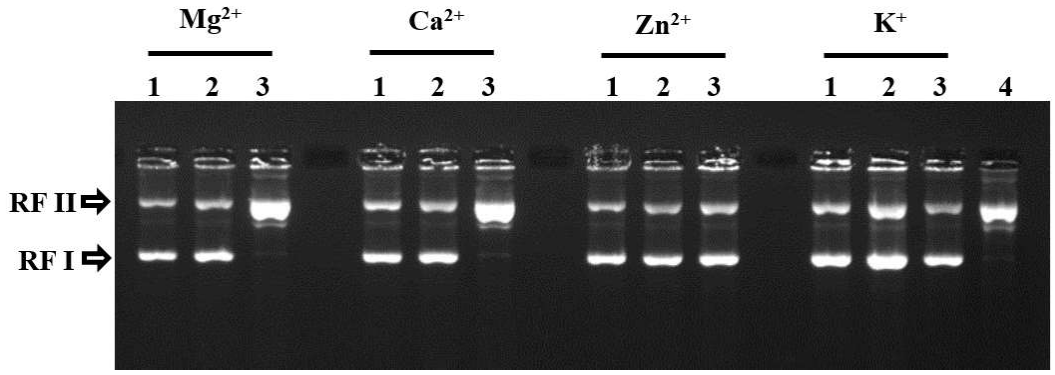

Figure 3.33: Co- factor requirement assay for endonuclease activity of OsMre11-N in presence of different metal ion cofactors

The co- factor requirement assay was carried out by incubating a fixed concentration of OsMre11-N protein with different co-factors, in a reaction mixture with RF-I DNA and resolved on 0.8% agarose gels.

Lane 1: Only RF I DNA (No protein, no metal ion); Lane 2: RF-I DNA with the mentioned cation (No protein); Lane 3: RF-I DNA with mentioned cation along with 38 nM OsMre11- N; Lane 4: RF-II DNA (control)

Both Mg^{2+} and Ca^{2+} ions are able to promote the endonuclease activity of OsMre11-N protein

100 ng of linearized pSK^+ plasmid was incubated with 38 nM of OsMre11-N of protein along with different cations and incubated for 20 minutes at 37° C. Samples were subjected to electrophoresis on 0.8 % agarose gels for 3.0 hours. Only in presence of Mn^{2+}, the protein OsMre11-N exhibited exonuclease activity as clearance of 3.0kb band was observed and no activity was detected in presence of Mg^{2+}, Ca^{2+}, Zn^{2+} and K^+ ions.

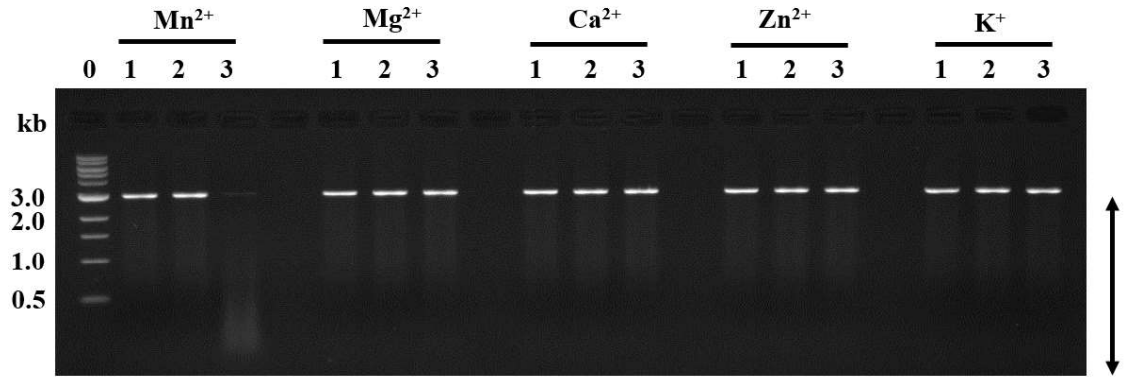

Figure 3.34: Co- factor requirement assay for exonuclease activity of OsMre11-N in presence of different metal ions

The co- factor requirement assay for exonuclease activity was carried out by incubating a fixed concentration of OsMre11-N protein with different co-factors, in a reaction mixture with linearized pSK^+ DNA and resolved on 0.8% agarose gels.

Lane 0: 1 kb ladder

Lane 1: Only linearized pSK^+ plasmid (No Protein, no metal ion)

Lane 2: linearized pSK^+ plasmid with mentioned cation (No protein)

Lane 3: linearized pSK^+ plasmid with mentioned cation along with 38 nM OsMre11- N

Unlike endonuclease activity, only Mn^{2+} is able to exhibit the exonuclease activity of OsMre11-N protein

3.9.7: Time point assay for maximum nuclease activity:

RF-I DNA was incubated with 38.0 nM OsMre11- N and 5.0 mM Mn^{2+}in reaction buffer and incubated at 37˚C. Aliquots were collected at different time intervals, deproteinized and stored at 4˚C until the final time point. Samples were subjected to electrophoresis on 0.8 % agarose gels for 3.0 hours. Within 20 minutes of reaction start time, the protein OsMre11-N is able to catalyse the conversion of RF-I DNA to RF-II DNA.

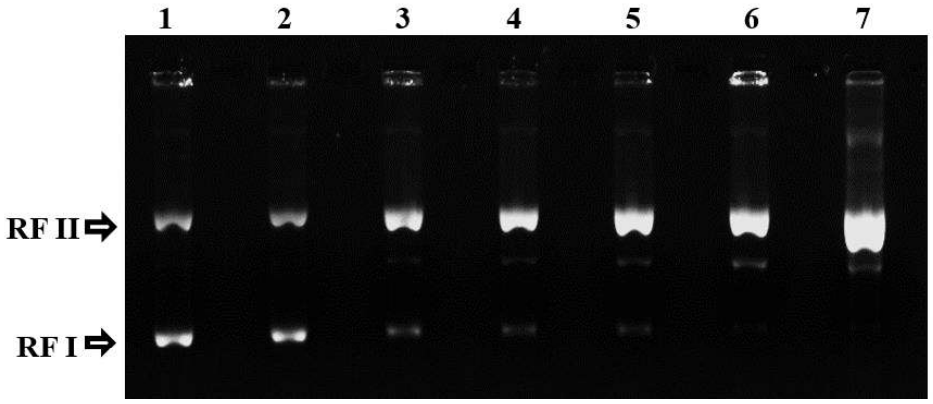

Figure 3.35: Time dependant endonuclease activity of OsMre11-N

Time dependent endonuclease activity was carried out by incubating 38.0 nM of OsMre11 protein with RF-I DNA in the reaction mixture. Aliquots were taken at every 20 minutes, deproteinized and resolved on 8.0% agarose gel.

Lane 1: RF-I DNA

Lane 2- 6: samples from 0, 20, 40, 60, 120 minutes of the reaction respectively

Lane 7: RF-II DNA (control)

Time dependent endonuclease assay showed that within 20 minutes of reaction start time, the protein OsMre11-N exhibited endonuclease activity.

100 ng of linearized pSK^+ plasmid was incubated with 38.0 nM of OsMre11-N and 5.0 mM of Mn^{2+} in reaction buffer and incubated at 37˚C. Aliquots were collected at different time intervals, deproteinized and stored at 4˚C until the final time point sample was collected. Samples were then subjected to electrophoresis on 0.8 % agarose gels for 3.0 hours. Within 20 minutes of reaction time, the protein OsMre11-N is able to catalyse the degradation of linearized pSK^+ plasmid (indicated by an arrow).

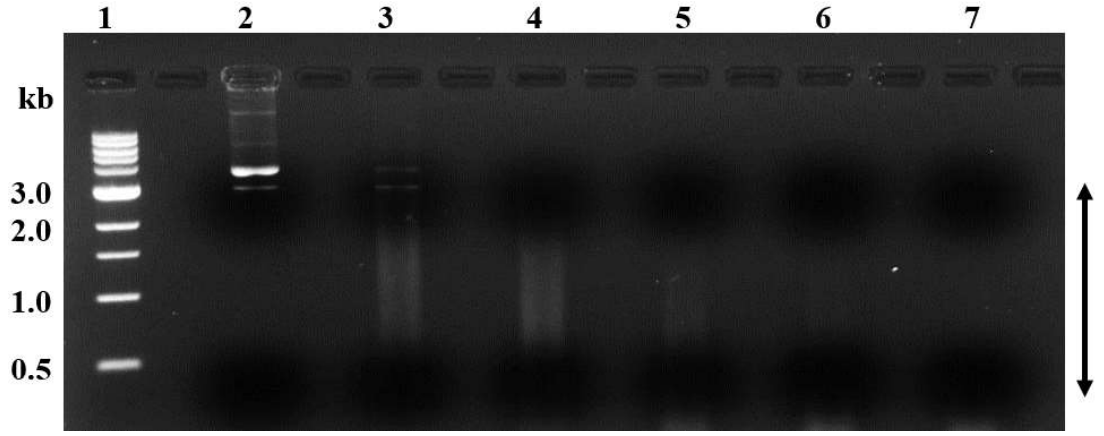

Figure 3.36: Time dependant exonuclease activity of OsMre11-N

Time dependent exonuclease activity was carried out by incubating 38.0 nM of OsMre11 protein with linearized pSK^+ DNA in the reaction mixture. Aliquots were taken at every 20 minutes, deproteinized and resolved on 8.0% agarose gel.

Lane 1: 1 kb Ladder

Lane 2: linearized pSK^+ plasmid

Lane 3-7: samples from 0, 20, 40, 60, 120 minutes of the reaction respectively

Time dependent exonuclease assay showed that within 20 minutes of reaction time, the protein OsMre11-N exhibited efficient exonuclease activity.

CHAPTER IV

RESULTS- II

BIOCHEMICAL CHARACTERIZATION OF OsRad52

4.0: INTRODUCTION:

Several genes have been reported to be involved in promoting HR, of which Rad52 (**Rad**iation sensitive **52**) is an important gene, found to be highly conserved across different species. It was believed that RAD52 is absent in plant systems until lately. However, recent genetic studies have shown the presence of RAD52 homologues in plants, though they are yet to be characterized biochemically. In 2011, Samach et al. reported that Rad52 homologs are present in all plants whose genome has been sequenced. It was suggested that due to Rad52 gene duplication, it is localised in all DNA containing organelles in plants. This study provided a clear indication for the presence of functional Rad52 homologs in plants which has high sequence similarity to oligomerization and DNA binding N- terminal domain of well-studied Rad52 proteins. In yet another study by Janicka et al. (72), it was suggested that organellar DNA binding 1 protein (ODB-1) assumes functions similar to nuclear Rad52 protein. ODB1 and Rad52 had similar DNA binding structural domains indicating that both these proteins might be functional homologs.

In the present study, the biochemical properties of rice Rad52 protein were investigated. OsRad52 was over- expressed in *E. coli* BL21 (DE3) cells and the protein purified. The identity of purified OsRad52 protein was confirmed by peptide mass fingerprinting. Gel filtration and native PAGE analysis indicated that the OsRad52 protein in its native state probably forms an undecameric (11 subunits) structure. Purified OsRad52 protein showed binding to single stranded DNA and double stranded DNA. Protein also mediated the renaturation of complementary single strands into duplex DNA in both agarose gel and FRET based assays. Detailed results of these experiments were presented in this chapter.

4.1: Cloning, over- expression and purification of OsRad52 protein:

Based on the *Arabidopsis* RAD52 sequence, analysis of rice genome showed the presence of RAD52 gene in *Oryza sativa* (Japonica sp). cDNA corresponding to OsRAD52 was obtained from RGRC, Japan. Attempts were done to amplify the cDNA by set of gene specific primers using the plasmid obtained from RGRC, Japan as template DNA. However, due to presence of very high percentage of GC content, PCR amplification was not successful. Different methods involving use of chemicals like DMSO, betaine, the Roche GC rich PCR amplification kit etc. for amplifying OSRAD52 cDNA were carried out. However, these methods also failed to amplify the cDNA for OsRad52. Hence it was decided to clone the OsRAD52 cDNA by sub-cloning method. OsRAD52 cDNA obtained from RGRC, Japan was sub-cloned into pBluescript SK^+ plasmid between*Sac*1 and *Eco*R1 restriction sites. Subsequently OsRAD52 cDNA fragment was subcloned between *Sac*1 and *Xho*1 restriction sites of pET28a plasmid for its over-expression.

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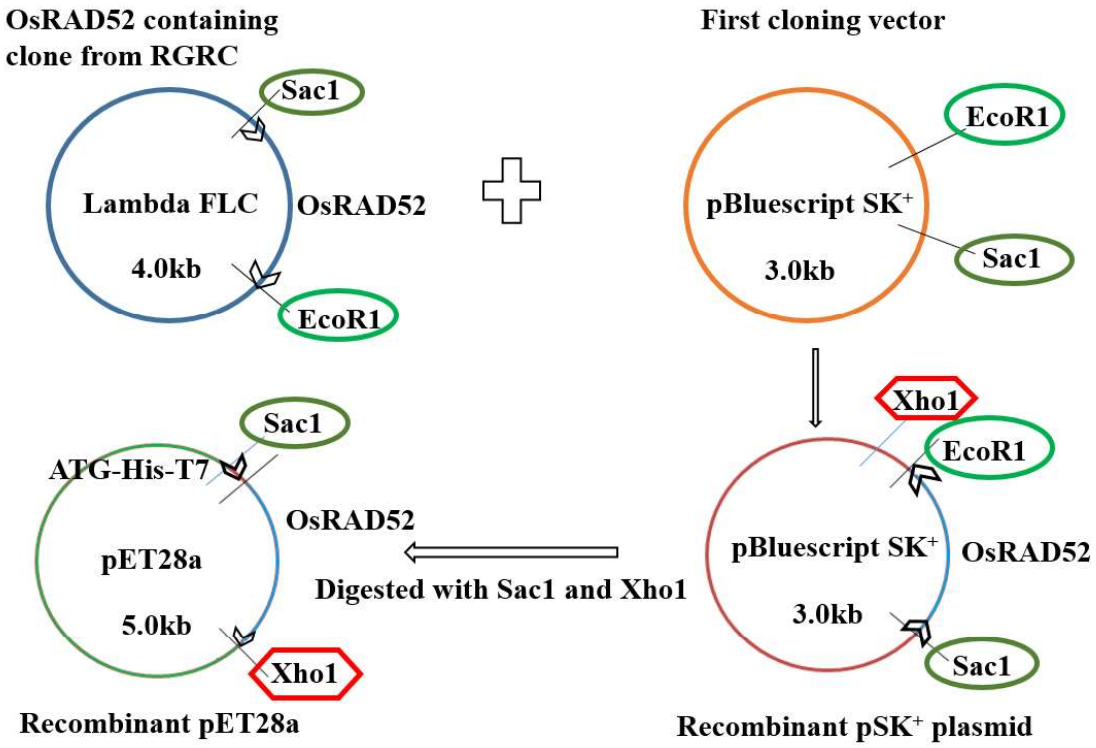

Figure 4.1: Diagrammatic representation of OsRAD52 cDNA cloning into pET28a vector.

Initially the OsRAD52 cDNA cloned in Lambda FLC vector was digested with *Sac*1 and *Eco*R1 restriction enzymes. The released OsRAD52 fragment was ligated with the similarly digested pSK^+ plasmid.

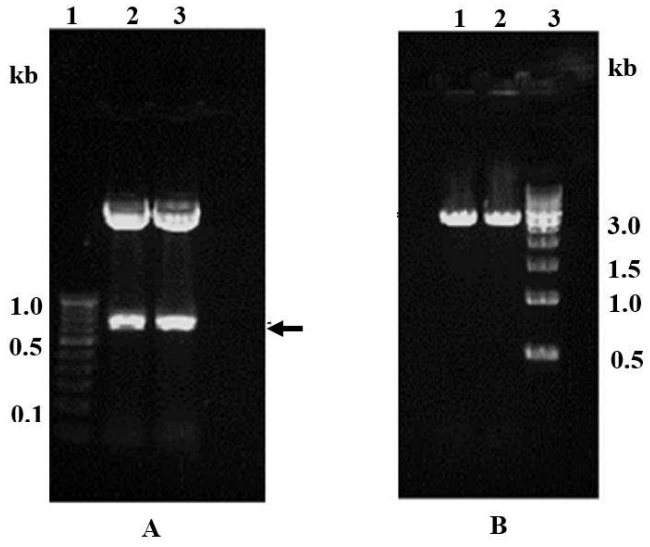

Figure 4.2: Restriction Digestion of Lambda FLC vector carrying OsRAD52 cDNA and pSK^+ plasmid with Sac1 and EcoR1

Panel A represents restriction digestion of Lambda FLC vector with Sac1 and EcoR1 restriction enzymes to release the OsRAD52 cDNA (indicated by an arrow) and panel B represents pSK^+ plasmid restriction digestion with Sac1 and EcoR1 enzymes.

Panel A: Lane 1: 100 bp ladder; Lane 2 and 3: digestion of Lambda FLC vector to release OsRAD52 cDNA

Panel B: Lane 1 and 2: pSK^+ plasmid restriction digestion, Lane 3: 1 kb ladder

OsRAD52 cDNA (~700 bp) was released from Lambda FLC vector and cloned into pSK^+ plasmid

The resulting recombinant plasmid was transformed into *E. coli* DH5α cells and random clones checked for the presence of OsRAD52 cDNA by restriction analysis by digesting the plasmid with *Sac*1 and *Eco*R1.

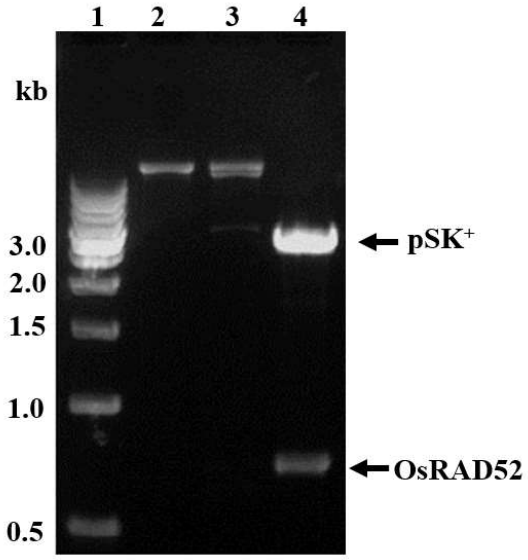

Figure 4.3: Restriction digestion of plasmid isolated from random colonies of E. coli DH5α harbouring pSK+ with RAD52 cDNA

E. coli DH5α cells were transformed with recombinant pSK+-OsRAD52 plasmid. Plasmids from random colonies were isolated and subjected to restriction digestion. Arrow shows the OsRAD52 insert release upon digestion with Sac1and EcoR1.

Lane 1: 1 kb ladder

Lane 2 and 3: Plasmids without OsRAD52 cDNA

Lane 4: Positive clone (~700bp OsRAD52 fragment released from recombinant pSK+ plasmid)

Positive clone of *E. coli* DH5 alpha harbouring pSK+ with Rad52 cDNA was successfully generated.

The recombinant pSK$^+$-RAD52 plasmid was isolated from the positive clones and digested with *Sac*1 and *Xho*1 restriction enzymes. The RAD52 fragment released was ligated into similarly digested pET28a plasmid.

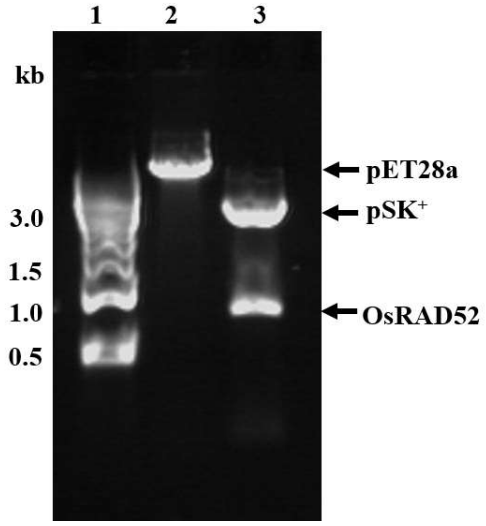

Figure 4.4: Cloning of OsRAD52 cDNA to pET28a plasmid

pET28a plasmid and recombinant pSK$^+$ plasmid harbouring the OsRad52 cDNA were digested with restriction enzymes Sac1 and Xho1, resulting in release of OsRAD52 cDNA from the recombinant pSK$^+$ plasmid (indicated by an arrow).

Lane 1: 1 kb ladder

Lane 2: pET28a plasmid digested with Sac1 and Xho1

Lane 3: recombinant pSK$^+$ plasmid digested with Sac1 and Xho1 resulted in the release of OsRAD52 cDNA

OsRAD52 cDNA was released from pSK$^+$ plasmid and ligated into similarly digested pET28a vector and transformed

The resulting recombinant plasmid was transformed into *E. coli* DH5 alpha cells and random clones checked for the presence of OsRAD52 cDNA by restriction analysis by digesting the plasmid with *Sac*1 and *Eco*R1.

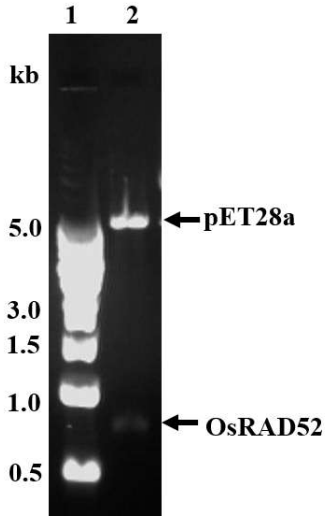

Figure 4.5: Restriction digestion of pET28a plasmid with Rad52 cDNA isolated from random colony

Random colony was picked and plasmid isolated, followed by restriction digestion with Sac1 and Xho1to check for the presence of OsRAD52 cDNA (indicated by an arrow).

Lane 1: 1 kb ladder

Lane 2: plasmid digested with Sac1 and Xho1 (~ 700 bp OsRAD52 fragment released) representing the positive clone

OsRAD52 fragment was successfully cloned into pET28a vector

Plasmid isolated from a randomly selected single colony was sequenced to confirm that no mutations were introduce during PCR amplification and cloning process.

GACTTTCCGAGCTCGATCGATCGAGCATGGAAGCCACAACCTCCAGCTTGCTCGTGCGCCCCGTCGACACGCGGCTCTCC
GCCGCCTCCCTCCCCATCGTCGTCCGCGCCCGCCGGCGCGTGGCCGTCGTCACGGCGGCGGCGCCGGAGCGGAAGCCG
GCCGCGGCGGCGAGCAGCAGCAACTACGTGGTGGTGCCGCTGGACGCGGCGCCGTCGGGGATCACGCGGCCGCTGGTG
GAAATCCTGCGCGACCTCAACAAGCGCGTCCCCGACACCGTCGTCCGTTCCTCCCGCCGCCGCGCGTCGCCCTCCGATCC
GGTCATCCCCTGGTATCATGCCAACAGGATGCTAAGCTTTTATGCTCCAGGCTGGTGTGGAGAGGTCCGTGATGTTATTTA
CACTGACAATGGGAAAGTGACAGTGGTATATCGTGTCACAGTACGAGGAACAGATGGAGAGGTTCATAGGGAGGCAGCTG
GAACAACATCACTGAATGATGCACGATTTGATGATCCCGTGGCTGCTGCAGAAGAGGCCGCATTCTGCAAGGCCTGCGCA
AGGTTTGGCTTTGGTCTGTATCTTTACCACGAAGACGAGACTCCGTAGGGAAGTGGCGCAGCATCCACTTCATAAGTTCTG
CGTTGGTTCTCGCCTGATTTGGTTCAAAAGGCTGTGGTTGATCCATCTCTCTGTTTATCAAGGTTTTCAGTCTGTATCAGG
AGTTGAAGTATCATTCATACTGGAATTC

Figure 4.6: Rad52 cDNA sequence cloned between Sac1 and Xho1 sites of pET28a vector

MEATTSSLLVRPVDTRLSAASLPIVVRARRRVAVVTAAAPERKPAAAASSSNYVVVPLDAAPSGITRPLVEILRDLNKRVPD
TVVRSSRRRASPSDPVIPWYHANRMLSFYAPGWCGEVRDVIYTDNGKVTVVYRVTVRGTDGEVHREAAGTTSLNDARFD
DPVAAAEEAAFCKACARFGFGLYLYHEDETP

Figure 4.7: Translated amino acid sequence of OsRad52 protein

Sequencing of recombinant pET28a- OsRad52 was confirmed to be devoid of any mutations

The resulting recombinant plasmid pET28a harbouring OsRad52 cDNA was sequenced and retransformed into *Escherichia coli* BL21 (DE3) cells for protein over expression.

Clones harbouring the recombinant plasmid was selected on LB kanamycin plates. Positive clones were grown in LB medium supplemented with 25µg/µl of kanamycin, till the OD 600 nm of the culture reached 0.6 and the OsRad52 protein was induced by adding 1.0 mM IPTG for 3.0 hours. Crude extract from the un-induced and induced cells was subjected 12% SDS-PAGE and induced proteins were visualised on gel by staining the gel with coomassie brilliant blue. A ~30 kDa over- expressed band of OsRad52 protein (indicated by an arrow) was observed.

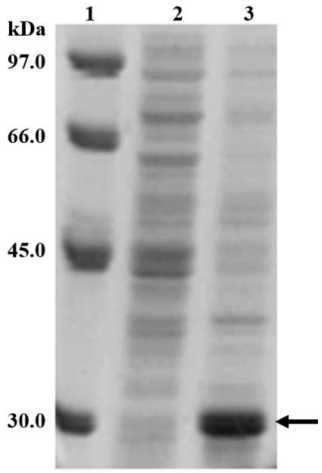

Figure 4.8: Over expression of OsRad52 protein in E. coli BL21 (DE3) cells

E. coli BL21(DE3) cells harbouring pET28a with Rad52 insert were induced with IPTG and were analysed on 12% SDS-PAGE for the presence of over- expressed OsRad52 protein.

Lane 1: Molecular weight markers; Lane2: Crude extracts form un-induced cells

Lane3: Crude extracts form induced cells

An over expressed protein band around ~30.0 kDa indicated by an arrow, was observed in the induced crude extract.

Induced cells were harvested and subjected to repeated freeze-thaw cycles in lysis buffer and cell free extract was prepared by subjecting the cell to brief sonication, followed by centrifugation at 12,000 x g. The supernatant and pellet fractions of induced cells were collected and analysed on 12% SDS-PAGE. A 30 kDa band was observed to be present in the pellet fraction.

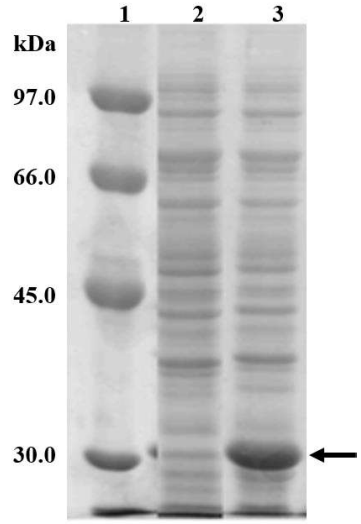

Figure 4.9: Solubility of OsRad52 protein in E. coli BL21 (DE3) cells

Supernatant and pellet fractions of induced cells were analyzed on 12% SDS-PAGE for checking the solubility of the induced protein.

Lane 1: Molecular weight markers

Lane 2: Supernatant of induced E. coli BL21 (DE3) cells

Lane 3: Pellet fraction of induced E. coli BL21 (DE3) cells

The over expressed protein was present as insoluble fraction in *E. coli* BL21 (DE3) cells.

It is known that recombinant over-expressed proteins are often present in inclusion bodies which cannot be extracted in native buffers. Hence OsRad52 was purified under denaturing conditions of 8.0 M urea as mentioned in the methods section. The purified OsRad52 protein was refolded by step-wise dialysis for 4 hours each against of dialysis buffer containing 6.0, 3.0, 1.5, 0 M urea at 4˚C. Purified OsRad52 protein was subjected to 12% SDS- PAGE at room temperature at constant voltage of 90 V for stacking and 110 V for resolving gel and stained with coomassie brilliant blue. Pure fraction of OsRad52 protein was observed in the fraction eluted with 400- 500mM imidazole.

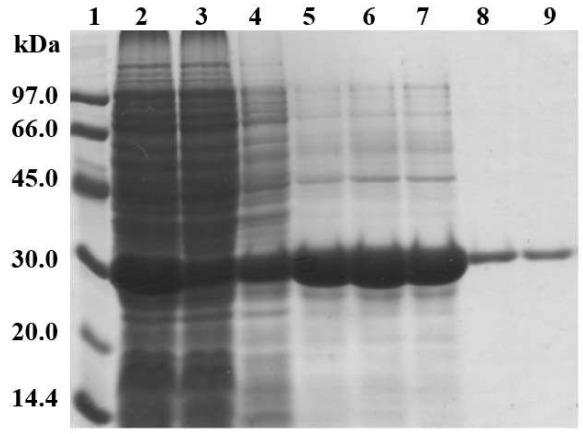

Figure 4.10: Purification of over expressed OsRad52 protein under denaturing conditions

The over-expressed OsRad52 was purified using nickel chelating affinity matrix column (affinity chromatography) and eluted with increasing concentration of imidazole.

Lane 1: Marker, Lane 2: Crude extract, Lane 3: Flow through, Lane 4: Column wash, Lanes5 - 9: OsRad52 protein eluted with increasing concentrations of imidazole (100, 200, 300, 400, 500mM respectively).

Purification under denaturing conditions of 8.0 M urea resulted in a pure fraction of OsRad52 protein with 400 and 500mM imidazole concentration.

4.2: Identification of OsRad52 protein:

Purified recombinant OsRad52 protein was subjected to western blot analysis using mouse anti poly-histidine antibodies as protein was expressed as a fusion protein, with six histidine residues at the N- terminal end of the over- expressed protein. On western blots, only single band was observed indicated that the eluted protein preparation was devoid of any degradation products.

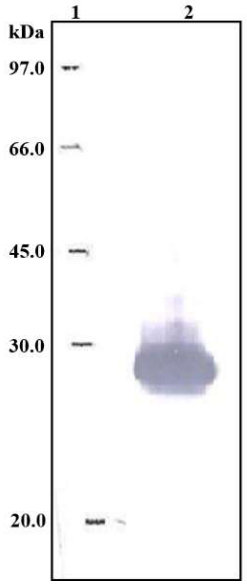

Figure 4.11: Western blot analysis of purified OsRad52 protein with anti-poly histidine antibody

OsRad52 protein was over expressed as a fusion protein with histidine tag at N-terminal of the protein. Western blot of OsRad52 with monoclonal anti poly histidine antibody was carried out.

Lane 1: Molecular weight markers; Lane 2: Purified OsRad52 protein

Histidine tagged OsRad52 protein was identified by western blot analysis using Anti-poly histidine antibodies

We further confirmed the identity of the purified protein by subjecting the protein for in-gel trypsin digestion followed by MALDI-TOF analysis. As the sequence of the OsRad52 protein is not available in SwissProt and NCBI, the data was analysed using the Masssorter v3.1 program (74) available at http://services.cbu.uib.no/software/massSorter, using the known protein sequence as the target. A total of 12 peptides were identified out of total 42 predicted peptides, giving sequence coverage of 66 %, thus confirming that the over-expressed protein was indeed OsRad52 protein.

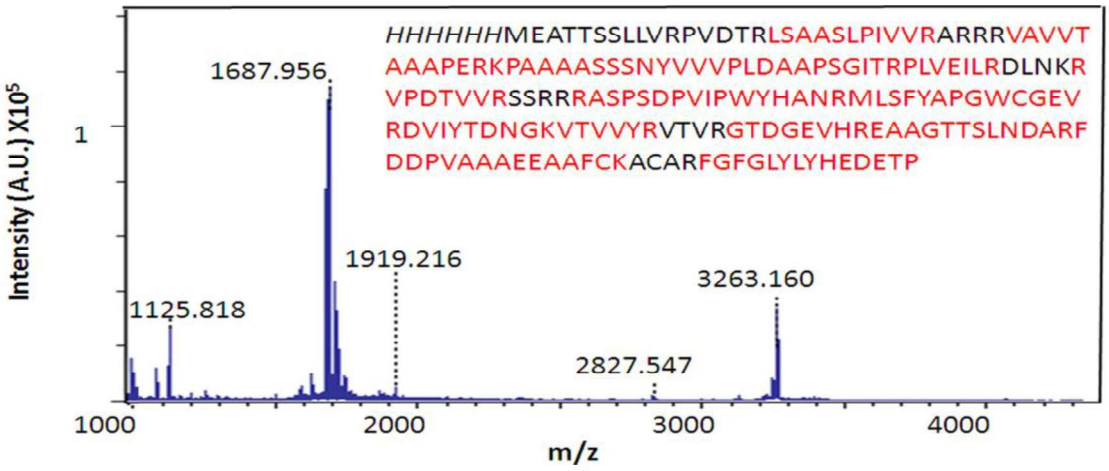

Figure 4.12: MALDI –TOF analysis of OsRad52 protein

Purified OsRad52 protein was separated on 12% SDS-PAGE gel and a 30kDA band was processed for analysis by MALDI. m/z values are given on X-axis and relative intensity of peaks is shown on Y-axis.

Spectra showed very intense peaks with less back grounds indicating the purity of the protein. MALDI-TOF analysis suggested the identity of purified protein as OsRad52 with sequence coverage of 66%.

4.3: Circular dichroism and Gel filtration of OsRad52 protein:

The far UV circular dichroism spectra of the purified OsRad52 protein was recorded in the wavelength range of 200- 260 nm to determine the secondary structure of the protein. The CD spectrum of OsRad52-2a showed two negative peaks at 209 and 225 nm, typical of helical proteins.

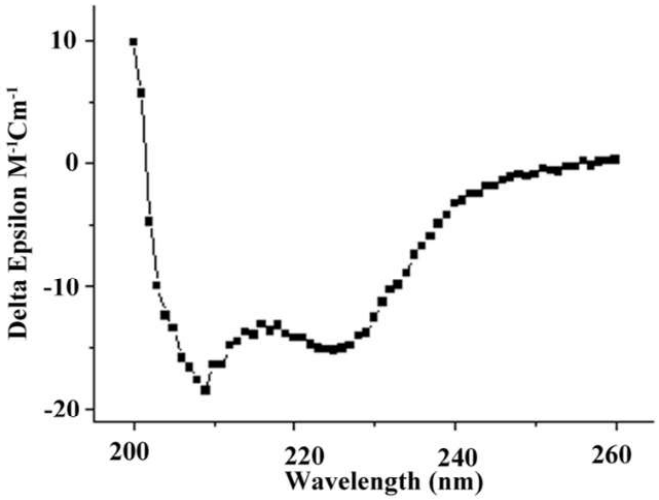

Figure 4.13: Circular dichroism analysis of OsRad52 protein

The observed ellipticity measured in milli-degree units was converted to molar ellipticity i.e. Delta epsilon (M−1 cm−1) and plotted against the wave length.

Negative peak at 209 and 225 nm indicates that protein has mostly alpha helical structures.

Native PAGE analysis of purified OsRad52 protein was carried out to determine the possible multimeric state of the protein in its native state. It appeared as a single band with an approximate molecular weight of 226 kDa, which suggests to an undecameric state of the protein.

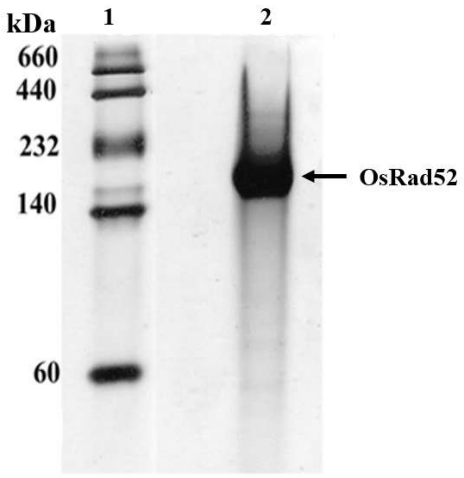

Figure 4.14: Native PAGE analysis of purified OsRad52 protein

Purified OsRad52 protein was subjected to native 8% PAGE analysis, under non- reducing conditions to determine the probable polymeric state of the protein in its native state.

Lane 1: Native Marker (Thyroglobulin- 669 kDa, Ferritin- 440 kDa, Catalase- 232 kDa, Lactate dehydrogenase- 140 kDa, Albumin- 60 kDa)

Lane 2: Native OsRad52 protein

On native PAGE, OsRad52 protein showed a band of ~226kDa protein which corresponds to a undecameric (11 mer) structure.

To further confirm the native state of OsRad52, gel filtration chromatography was performed. OsRad52 eluted as a minor peak (peak 1) and a major peak (peak 2) under native conditions. Peak 1 represented the aggregated Rad52 protein which eluted in void volume and peak 2 corresponded to an approximately 226 kDa protein, which confirmed an undecameric structure of the protein. The results indicated that OsRad52which has a theoretical molecular weight of 21.0 kDa, probably formed an undecameric structure in its native state with a molecular weight of ~226kDa.

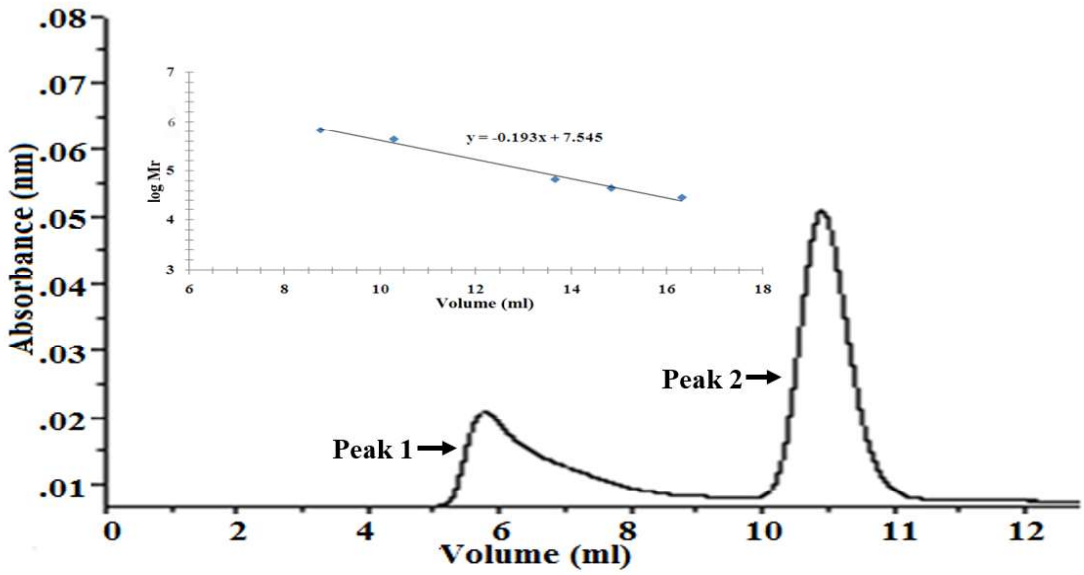

Figure 4.15: Gel Filtration analysis of purified OsRad52 protein

Gel filtration analysis of purified OsRad52 protein using Superdex™ 200 column chromatography. Inset picture shows the standard graph of elution volume vs molecular weight of the proteins (log Mr).

Peak 2 indicates a ~226kDa protein on native PAGE which corresponds to undecamer (11 mer) (~21.0kDa) form of OsRad52in its native state.

4.4: Biochemical characterization of OsRad52 protein:

4.4.1: DNA binding activity of OsRad52 protein:

The DNA binding properties of OsRad52 protein was studied by the electrophoretic mobility shift assays (EMSA), also known as the gel shift assay. A 31mer oligonucleotide Oligo1was used to study ssDNA binding assay. Assay was performed with a fixed concentration of oligonucleotide (100 pmol) and increasing concentration of OsRad52 protein. As protein concentration increased, DNA binding also increased, as more upward shift (as smear) of DNA-protein complexes was observed. Rad52 protein binds to ssDNA even at low concentration (3.7 nM).

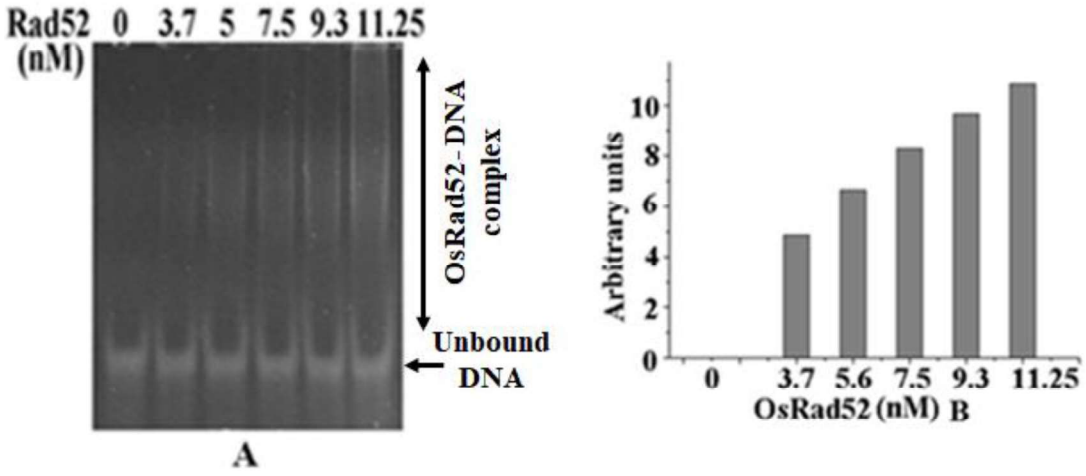

Figure 4.16: ssDNA binding activity of OsRad52 protein

A. *Indicated amount of OsRad52 protein (nM) was incubated with 100pmol of single stranded 31 mer oligonucleotide at 37°C for 20 min. The reaction mixtures were then resolved on gradient native poly acrylamide gel (4–5%). DNA in the complexes was visualized by staining the gel with SyBr green.*

B. *Quantification of gel-shifted complexes, shown in (A), using ImageJ software. DNA-protein complexes shift was expressed as arbitrary units against the OsRad52protein concentration used in the assay.*

A protein concentration dependent ssDNA binding was observed

Double stranded DNA synthesized by mixing equimolar concentration of NBS-1 and NBS-1 C followed by heat denaturation and slow renaturation at room temperature and was used as substrate for dsDNA binding assay. As seen with ssDNA, a very low concentration of rice Rad52 protein was required for binding to dsDNA and as the protein concentration increased, a larger complex was formed. The DNA-protein complexes were quantified by ImageJ software and a protein concentration dependant dsDNA binding was observed.

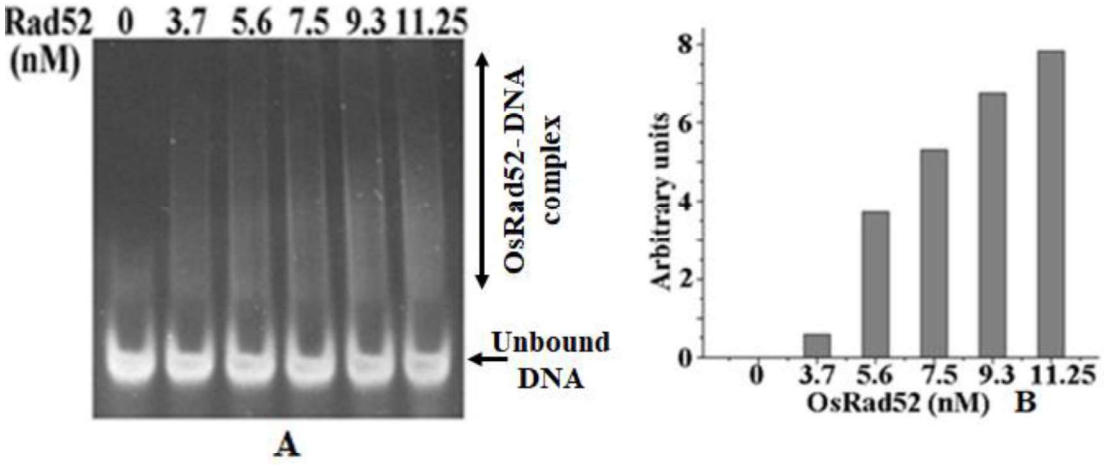

Figure 4.17: dsDNA binding of OsRad52 protein

A. Indicated amounts of OsRad52 protein (nM) was incubated with 100 pmol of double stranded oligonucleotides (annealed product of NBS-1 and NBS-1C) at 37°C for 20min. Reaction mixtures were resolved on gradient native polyacrylamide gel (4–5%). DNA in the complexes was visualized by staining the gel with SyBr green.

B. Quantification of gel-shifted complexes, shown in (A), using ImageJ software. DNA-protein complexes shift was expressed as arbitrary units against the protein concentration used in the assay.

A protein concentration dependent dsDNA binding was observed

Taken together, OsRad52 exhibited DNA binding properties similar to its homologues, viz. human and yeast Rad52.

4.4.2: Renaturation activity of OsRad52 protein:

Further, we carried out the renaturation assay of OsRad52 protein using a linear 2.0 kb dsDNA. 2.0 kb duplex DNA was heat denatured to form complementary single stranded DNA molecules and used for the assay. The ssDNA so formed were incubated with increasing concentration of OsRad52 protein and the formation of duplex DNA was monitored on agarose gels after deproteinization of the samples. A no protein control was included in the assay, which represented spontaneous renaturation under tested conditions. Protein mediated duplex DNA formation was higher when compared with control having no protein. However, we have observed marginal or no increase in the renaturation activity as protein concentration increased. The renaturation reached to a maximum level at a protein concentration as low as 3.7 nM.

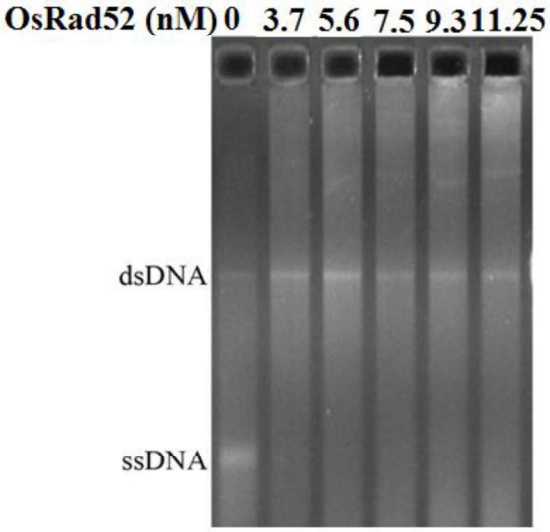

Figure 4.18: Renaturation activity of OsRad52 protein demonstrated using agarose gel

Indicated amount of OsRad52protein (nM) was incubated with 100ng of heat denatured DNA at 37°C for 10 min. The reaction was stopped by adding 0.1% SDS and 2.0 units of proteinase K for deproteinization and further incubated at 37°C for 20 min. The reaction mixtures were then resolved on 0.8% agarose gel. Duplex DNA formed as a result of renaturation activity of OsRad52 was visualized by staining the gel with ethidium bromide.

OsRad52 mediated duplex DNA formation was observed, thus protein mediated the renaturation of complementary single strands into duplex DNA

4.4.3: Renaturation activity of OsRad52 by FRET assay:

A straight forward FRET assay was carried out to measure the renaturation in real time without protein removal from the system. FRET assay gave similar results wherein, the assay was designed to measure the decrease in emission intensity of fluorescein (donor) at 520 nm, upon excitation at 480 nm, as a function of time. To measure renaturation, different concentrations of OsRad52 were pre-incubated with Phi-C oligonucleotide labelled with rhodamine at 3' end followed by addition of Phi-W labelled with fluorescein at 5' end. Upon addition of the complementary strand, annealing ensued which led to a time dependent decrease in fluorescein emission intensity at 520 nm. Here, the FRET efficiency was expressed as a function of time at different concentration of OsRad52. FRET efficiency of each reaction was calculated by subtracting the fluorescence emission intensity of the starting value (0 minute) from all the values following renaturation (5 minute). The rate of renaturation catalyzed by OsRad52 at different concentrations was plotted against time and was found to increase as the concentration of protein was increased. The extent and rate of renaturation was fairly high, i.e. within 100 sec the reaction did reach a steady state level at the highest protein concentration tested (3.7 nM). Under similar conditions, a control with no protein showed much slower spontaneous renaturation reaction. Thus, OsRad52 showed the renaturation activity on gel and FRET assays, which is a typical activity of Rad52 group of proteins.

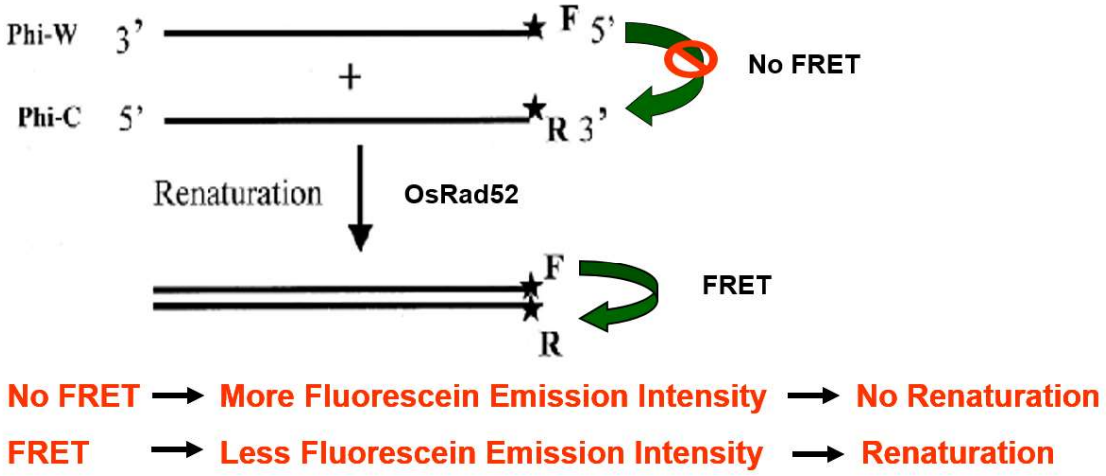

Figure 4.19: Diagrammatic representation of renaturation assay by FRET

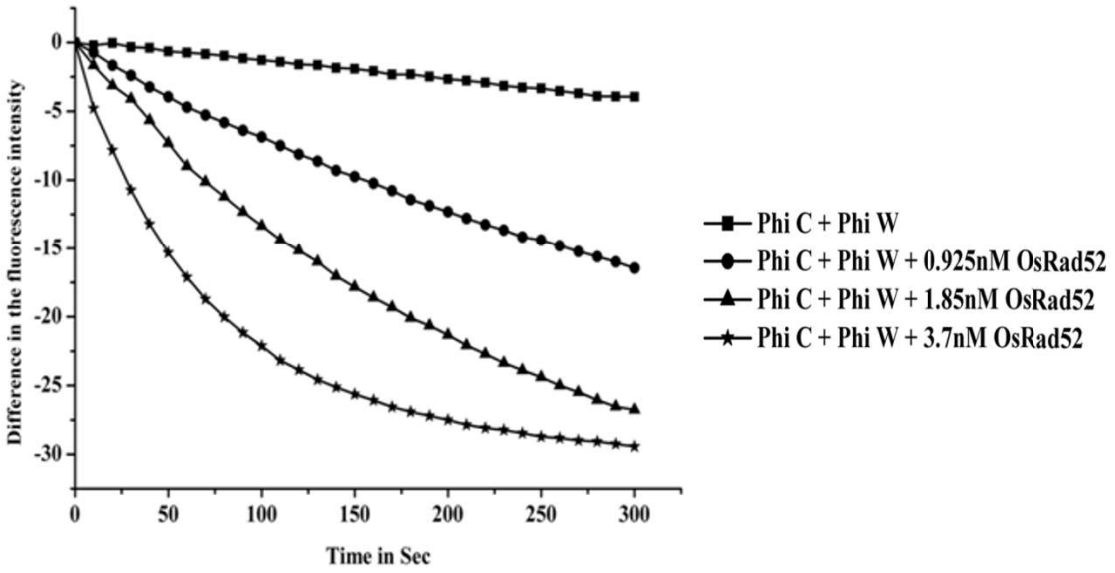

Figure 4.20: Time dependent renaturation activity of OsRad52 monitored by FRET

Time course of renaturation reaction was monitored and decrease in fluorescence intensity at 520 nm was expressed in arbitrary units. Zero second fluorescence value was adjusted to zero and decrease in the fluorescence emission intensity as a result of FRET was plotted against time.

The rate and extent of renaturation reaction catalyzed by OsRad52 was increased with increasing concentration of protein.

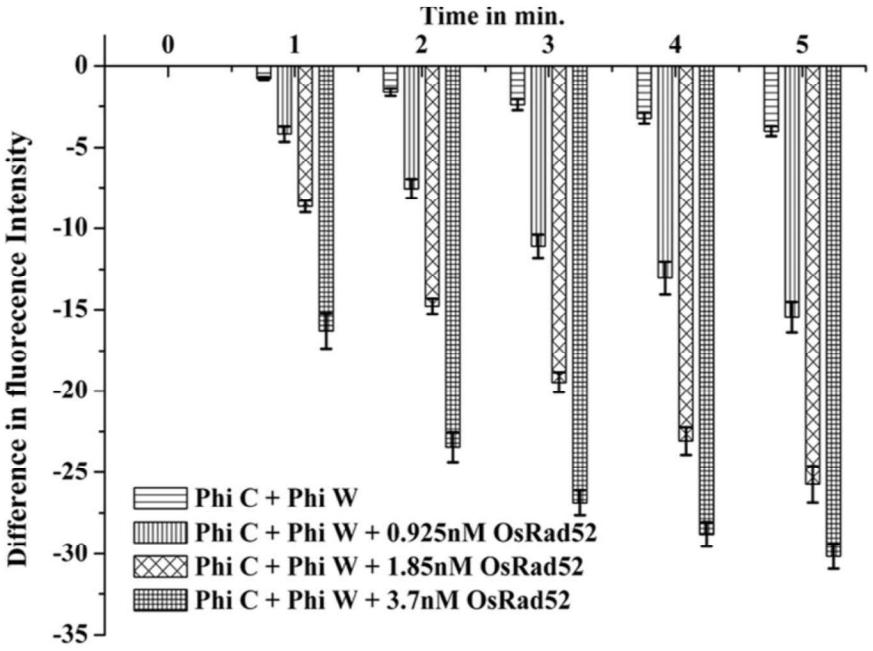

Figure 4.21: The extent of renaturation activity of OsRad52 at the end of reaction i.e. 5 minutes

Bar chart representing the decrease in fluorescein emission intensity at 520 nm of the Phi-W molecule upon increasing concentration of OsRad52 protein. Zero second fluorescence value was adjusted to zero and decrease in the fluorescein emission intensity after 5 min of the reaction as a result of FRET was plotted against protein concentration. The more the decrease, the more the activity.

Protein concentration dependent activity was observed. Control with no protein showed much slower spontaneous reaction.

Chapter V

DISCUSSION

The realization of inherent instability prevalent in DNA and its reactivity with several different agents, which ultimately leads to its damage, evolved late after the elucidation of the structure of DNA by Watson and Crick (2). Since, DNA contains all necessary information required for the survival of an organism, all living organisms have developed several mechanisms to overcome any damage to their genetic material. Whenever a eukaryotic organism encounters any damage to its DNA, it initiates the DNA damage response (DDR), which is a cellular network comprising of signal transduction leading to repair of damage, cell cycle arrest, leading to apoptosis if the lesion is irreparable.

In contrast to animals, plants being sessile organisms are unable to change their location, hence get exposed to several agents which cause harm to their genetic material. Plants require sunlight for their regular photosynthetic activity, and since sunlight contains UV, their chloroplasts continuously generate reactive oxygen species (ROS), which in turn is a continuous source of endogenous DNA damage. Moreover, unlike animal development which is embryonic in nature, plant development is postembryonic, i.e. their growth depends on division of meristematic cells, which divides throughout the life of a plant (75). Hence, it is necessary that plants develop a competent and precise DDR system to overcome any damage to its genetic material. Therefore, it would be imperative to understand the mechanisms of plant DDR system, but till date a plethora of reports is available of these mechanisms in yeasts and animals, and only recently have plant DDR systems begun to be studied in detail (76).

It has been widely reported that the plant homologs of prokaryotic DRR genes have very low sequence similarity but most of them retained the conserved domains probably suggesting the functional conservation of these proteins in plant systems (16). However, most of these prokaryotic and yeast specific genes are not well explored in plant genomes except the bacterial RecA gene. RecA has been found to be most conserved bacterial homolog in plants, which showed more than 40% amino acid identity in both rice and *Arabidopsis* genomes. The

Arabidopsis encoded RecA proteins are targeted to chloroplasts (77, 78) and mitochondria (79) and associated with DNA repair (80).

Since the homologous recombination would be relatively error-free repair pathway as compared to NHEJ, plants have refined ways of employing HR for DNA repair. However, plants will also have to balance the repair processes so as to avoid recombination between repeated sequences in their genome. Hence, it would be imperative to study the biochemical properties of proteins involved in HR in plants, which till date have only been functionally characterized at genetic level. Studies on plant counterparts of MRX complex has been reported for *Arabidopsis*, however, the functions of only AtMre11 and AtRad50 have been studied (81). These studies showed that mutations in *Arabidopsis* MRE11 and RAD50 genes lead to severe developmental defects, sensitivity to DNA damaging agents and lengthened telomeres in the mutants. In plants, RAD51 and RAD54 homologs have long been identified, leaving the question of whether plants contain RAD52 homologs or whether their absence is compensated by plant BRCA2 homologs. However, computational and experimental analyses provide clear evidence for the presence of functional RAD52 homologs in plants whose genomes have undergone extensive sequencing. In crop plants, knowledge about Mre11 and Rad52 is limited to only expression studies and biochemical studies have not yet been reported. Though these expression studies have duly shown the importance of both these genes in plants like *Arabidopsis* and rice, their biochemical characterization will help give a better and comparative understanding of the role carried out by these genes. Hence the objective of this thesis is to study the biochemistry of OsMre11 and OsRad52 from rice.

In this line, previously in our laboratory, genes of two types of meiotic recombinase, Type A and type B OsDMC1 were identified and sequenced from japonica and indica cultivars of rice (82). Similarly, the biochemical properties of purified OsDmc1 and OsRad51 protein have been studied and reported from our lab (36, 37, 38).

In the present study we report the biochemical characterization of purified OsMre11 protein and OsRad52 proteins from rice, *Oryza sativa*. The cDNA coding for OsMre11 protein was cloned into pET16b and transformed into expression hosts. However, due to the degradation of full length protein upon purification probably due to the presence of large amount of random coils, we cloned and over- expressed the N- terminal end of the OsMre11 protein and biochemically characterized it. Similarly, the OsRad52 cDNA was cloned, over- expressed purified and further protein was subjected to biochemical studies.

5.1: OsMre11:

In this work, we describe for the first time, the biochemical characterization of plant Mre11 protein from rice *Oryza sativa*, OsMre11. The results described here provide insight into the mechanism of plant Mre11 functioning, its binding characteristics and evidence for the presence of nuclease activity of plant Mre11 protein.

As already pointed out, information on plant Mre11 is limited to its genetic and mutational studies, where in importance of the protein in meiosis and fertility have been discussed in detail based on cytological and molecular characterization. Reports on AtMre11, claimed the importance of N- terminal region of Mre11 protein for its functioning (51) and that mutations in the MRE11 region causes severe defects in meiosis, DNA damage response and repair in *A. thaliana* plants (52). Further reports on OsMre11, demonstrated that it is essential for maintaining genetic stability of rice chromosome (53) and also for preserving the functioning of HR processes in rice tissue (54).

Present study mainly focuses on the biophysical and biochemical characterization of over-expressed and purified OsMre11 protein. cDNA of OsMRE11 gene was procured from RGRC, Japan. The obtained cDNA was cloned into Lambda FLC vector. The 2115 bp long MRE11 segment was PCR amplified using primers specific for restriction sites *Nde*1 and *BamH*1. The

amplicon cloned into pET28a vector and protein was over expressed by transforming the recombinant pET28a vector harboring the full length OsMRE11 fragment into BL21 (DE3) pLysS cells (Fig. 3.7). Upon studying the protein profile of induced cells, it was observed that OsMre11 protein formed inclusion bodies (Fig. 3.10). It is often known that the proteins over expressed in heterologous systems will form inclusion bodies especially plant proteins. Hence, attempts were made to purify the full length OsMre11 protein under denaturing conditions using buffers containing urea. To purify the protein, inclusion bodies were initially purified from the cell lysate and then protein purification was attempted under denaturing conditions by affinity chromatography. However, due to some unexplained reason, extensive degradation of purified OsMre11 was observed (Fig. 3.11, Fig. 3.12). Expression and purification OsMre11 was carried out by different methods reported in the literature, but full length protein either underwent extensive degradation upon purification or was not expressed at all (Table 3.1). It is relevant to point out that the presence of high degree of random coils will often contribute to the instability of the recombinant proteins, which will lead to their degradation (88, 89, 86). Upon subjecting the amino acid sequence of the full length protein to secondary structure prediction tool, it was understood that the protein had a large proportion (44 %) of random coils. Hence it was decided to characterize the functional domains of OsMre11 protein. An attempt was made to determine the conserved domains present in OsMre11, which revealed that the full length protein has two conserved domains namely, the DNA binding domain and the nuclease domain, both located at the N- terminal end of the full length OsMre11 sequence (Fig. 3.13).

The N- terminal end of Mre11 protein has been reported to be widely conserved. Petrini et al. in 1998 (86) reported that any modification in the N-terminal phosphoesterase motifs inactivated the yeast *Saccharomyces cerevisiae* Mre11. Similarly, it has been reported that human MRE11 shares close to 50% identity with its yeast counterpart at the N-terminal half of

the protein (87). Based on these findings and the fact that purification of full length protein underwent degradation and that most of the Mre11 activities was attributed to N-terminal domain, an attempt was made to purify the entire N- terminal domain of the protein which has both DNA binding and nuclease properties.

The N- terminal region of the MRE11 cDNA was cloned into pET16b plasmid, downstream of six histidine residues. The over- expressed protein was found to have a molecular weight of 55kDa on SDS-PAGE (Fig. 3.18). The protein was over- expressed in Shuffle T7 Express LysY competent *E. coli* cells, which produces recombinant proteins in the native state (Fig. 3.19). As expected, The N-terminal domain of the OsMre11 was expressed as soluble protein, hence it was subjected to purification protocol under native conditions. As the cloning strategy resulted in over- expression of a His-tagged protein, the over- expressed protein was purified by Ni-NTA chromatography with increasing concentration of imidazole. Upon SDS- PAGE analysis a pure fraction of OsMre11- N was observed (Fig. 3.20) i.e. the protein was devoid of any contaminating proteins as well as any degradation products like that of full length protein.

Human Mre11, bacterial Mre11and yeast Mre11 protein have all been shown to possess a dimer structure, which is essential for DNA end bridging and for carrying out its nuclease properties (89, 90, 91). Based on these reports we tried to analyse the probable oligomeric state of OsMre11- N protein. It was observed that the purified protein on native PAGE analysis, showed the presence of a band with an approximate molecular weight of 110kDa (dimer) and a band close to ~155 kDa probably corresponding to a trimer (Fig. 3.24). It was presumed that OsMre11 N-terminal domain might have formed an active structure, and hence this preparation was used for further biophysical and biochemical studies.

OsMre11 protein was also subjected to CD analysis to determine whether the recombinant protein is fully folded or not. As expected, the protein showed the presence of helical structures typical of refolded protein proving that OsMre11 protein had helical structures (Fig. 3.23).

Human and yeast Mre11 proteins have been shown to possess DNA binding and nuclease activities (50, 8). It is imperative that for mediating its biological function, OsMre11 must bind to ss and ds DNA substrates like that of its homologs from human and yeast. Present study also demonstrates that OsMre11 binds to both ss and dsDNA, in a protein concentration dependent manner (Fig. 3.25, Fig. 3.26). Hence OsMre11 probably mediating the biological function similar to those of human and yeast Mre11 proteins.

It has already been reported that Mre11 has a role to play in telomere maintenance, a functional entity in regulating the telomere length. It was shown that yeast Mre11 binds to G4 DNA, a conformation of DNA known to exist in telomeres (8). Similarly, human Mre11 have also been shown to be involved in fusion of short dysfunctional telomeres in human cells (91), and that depletion of MRN complex in human cells causes disruption of telomere maintenance (92). Taking note of these characteristics of Mre11 protein, we also tested the binding of OsMre11 protein to G4 substrates and we observed that consistent with these reports, OsMre11 also was able to bind to G4 substrates (Fig. 3.27) as well as to dsDNA or ssDNA, thereby hinting to a probable role in telomere maintenance in plants.

Fluorescence spectroscopy is a critical tool in biochemistry to study biomolecular interactions in real time. Tryptophan fluorescence is most widely used among all three fluorescent amino acids (tyrosine, tryptophan and phenylalanine) because the indole group of tryptophan is considered the most dominant source of UV absorbance at 295 nm and emission at 305- 400 nm. The photo-physical property of tryptophan is highly dependent on its immediate surroundings (92). Hence, any change in emission spectrum upon binding of a ligand to a

protein can be an indication of a probable protein- ligand interaction. We utilised this intrinsic property of tryptophan molecule present in OsMre11 to demonstrate DNA- protein interactions. As can be seen from the results, whenever a DNA molecule binds to protein, with increasing protein concentration the emission also increased (Fig. 3.28, Fig. 3.29, Fig. 3.30) suggesting that indeed OsMre11 is able to bind to different forms of DNA, with a significant increase in tryptophan emission intensity was seen, upon binding to G4 DNA substrates. It is interesting to note that increase in the fluorescence intensity upon binding to G4 and ss DNA was gradual. However, same was not observed in case of dsDNA and an sudden increase was observed. This may be due that the binding modes to these substrates may be different, and protein may be changing conformations accordingly.

Mre11 possesses nuclease activities which is widely conserved and has been studied in yeast (8, 93) and humans (42). Human Mre11 has been shown to have dsDNA exonuclease activity in buffers containing Mn^{2+}. The role of this activity in cell is highly debadrr, and still not clear but its biological relevance depends on the structure and context of DNA ends. In yeast, Mre11 nuclease activity is required for the resection of enzymatically generated DSBs and those DSBs which are bound by Spo11 protein (in meiosis). In humans, Mre11 nuclease activity is required for meiotic DSB repair where the DSBs are generated and bound by Spo11 protein. Based on these reports, we probed the nuclease activity of OsMre11 protein *in vitro*. Similarly, for OsMre11 the protein was able to degrade linear pSK^{+} plasmid- a dsDNA molecule efficiently in the presence of Mn^{2+}(Fig. 3.31) and not in the absence of Mn^{2+}or in presence of any other cations (Fig. 3.34), thereby indicating that rice Mre11 protein possesses similar characteristics as that of human Mre11, with manganese ion acting as the co- factor required to exhibit its exonuclease activity.

Mre11 also has endonuclease activity, thought to be involved in V(D)J recombination in mammals (50). Though VDJ recombination is absent in plants, OsMre11 is thought to act as a

probable endonuclease involved in NHEJ pathway which is more prevalent than HR in plants. In this report however, to test the endonuclease property, more authentic dsDNA substrate, RF-I DNA of PhiX-174 was used. Incubation with OsMre11 protein with this substrate converted RF-I to RF-II DNA, a nicked circular as a result of single strand endonucleolytic cutting (Fig. 3.32). And as hypothesised, the rice Mre11 protein did convert the RF-I to RF-II DNA, but only in the presence of divalent cations such as Mn^{2+}, Mg^{2+}, and Ca^{2+} ions (Fig. 3.33). Till date reports were available that Mre11 requires Mn^{2+}, as a co- factor to exhibit its nuclease activity, however in this study, for the first time, it was observed that the endonuclease activity of rice Mre11 was promoted not only by Mn^{2+}, but also by Mg^{2+}, Ca^{2+}ions, thereby inferring that the presence of any of these divalent metal ion as a co- factor is sufficient to initiate the endonuclease activity of OsMre11. Taken together, OsMre11 N-terminal domain binds to ssDNA, dsDNA, G4 DNA and mediates Mn^{2+}dependent exo and Mn^{2+}, Mg^{2+}, Ca^{2+} dependent endonuclease activities. From these data it was inferred that OsMre11 mediates it biological function similar to its counterparts from human and yeast systems.

Based on these findings, it was showed that OsMre11 from rice system is indeed a functional homolog of yeast or human Mre11, which shows all properties of the Mre11 protein and hence qualifies to be involved in DNA damage response as has been indicated by Hong et al. (53) and Jiuhan Ji et al. (34). For the first time, this report provides a detailed over-view of how the rice Mre11 protein might be working in vivo, with its binding and nuclease properties, and help in maintaining genetic stability in rice plants.

5.2: OsRad52:

In the present work, for the first time, the biochemical characterization of plant Rad52 protein from rice *Oryza sativa*, OsRad52 is reported. The results described here provide insight into the mechanism of plant Rad52 functioning, its binding characteristics and evidence for the presence of renaturation property of plant Rad52 protein.

As widely reported, DNA double strand breaks and single strand gaps are the efficient initiators of homologous recombination, and Rad52 mediates Rad51 loading onto ssDNA. This initiates HR, in which Rad51 promotes the strand exchange between the homologous DNA molecules (49). Additionally, Rad52 also catalyzes DNA annealing in Rad51 independent pathway. Rad52 protein is well characterized in both humans and yeast (57, 58). Most of the information on Rad52 is originated from studies on human and yeast homologues of Rad52. Information on plant Rad52 protein is sparse. Samach et al. (71) suggested that Rad52 protein plays an important role in both somatic and meiotic recombination. Janicka et al. (72) reported the presence of mitochondrial Rad52 called ODB1 and its role in mitochondrial DNA repair. In both these reports, the authors have proposed the presence of RAD52 homologues in plants and their role in DNA repair. Human and yeast Rad52 protein has been biochemically characterized and shown to bind to both ssDNA and dsDNA. Present study shows that rice Rad52binds ssDNA and dsDNA in a manner analogous to that of yeast and human Rad52 proteins, and mediates renaturation of complementary ssDNA into duplex DNA thereby supporting our conclusion that the rice Rad52 indeed belongs to Rad52 family proteins biochemically.

Although human and yeast Rad52 has 418 and 471 amino acids respectively, the plant Rad52 homologues first identified in *A. thaliana* was found to have (176 amino acids) and share 31% amino acid sequence similarity to the yeast Rad52 N- terminal end (71). This sequence was used to probe the sequences of all available land plant genomes. These plant proteins were

found to be 170-220 amino acids long with a weakly conserved N- terminal end and well conserved central and C- terminal ends. Since, Rad52 protein is highly conserved at the amino acid level in plants, Rad52 sequence from *Arabidopsis* was used to probe the rice proteome. Bioinformatics analysis showed the presence of Rad52 homolog in rice. Upon probing the RGRC cDNA library using the same sequence it was found that a full length cDNA clone for RAD52 was available with the RGRC library.

For OsRad52 characterization, the OsRad52 cDNA was procured from RGRC (Rice Genome Resource Centre), Japan. The cDNA obtained, was cloned in lambda FLC vector. Due to a very high proportion of GC content in the cDNA fragment, PCR amplification of OsRad52 cDNA was not successful, even with the help of chemicals like DMSO, betaine or the Roche high GC PCR amplification kit. It is known that high GC content creates the problem during PCR amplification. Even if the PCR amplification is successful, it is likely that some mutations are introduced during PCR reaction due to GC tracks in the template DNA. Hence a sub-cloning strategy was employed where in the OsRad52 fragment was cloned into pSK^+ plasmid and finally into the expression vector pET28a (Fig. 4.1). This recombinant pET28a plasmid harbouring the OsRAD52 fragment was then transformed into expression host *E. coli* BL21(DE3) cells for over- expression (Fig. 4.8). OsRad52 cDNA codes for 192 amino acids long protein and cloning in pET28a vector resulted in hexa-histidine tag at N- terminal. The over-expressed OsRad52 protein which has a theoretical molecular weight of 21.0 kDa was however observed to have a molecular weight of 30.0 kDa when resolved on SDSPAGE (Fig. 4.8). Nevertheless, it is not uncommon to observe this discrepancy between the theoretical and observed values during SDS-PAGE.

The over- expressed Rad52 protein was found to be present in the insoluble fraction of the cell lysate (Fig. 4.9), a common problem encountered with heterologous systems. It is relevant to point out that OsRad52 protein was predicted to be localized in the chloroplast with sorting

sequences, which are hydrophobic in nature. These hydrophobic sorting sequences might be the reason for inclusion bodies formation during over-expression in heterologous systems. Rice Rad52 protein was therefore, purified under denaturing conditions of 8.0 M urea by affinity chromatography (Fig. 4.10). Cloning strategy in expression vector resulted in a fusion protein with hexa-histidine tag at N-terminal of the protein. Presence of the histidine tag was confirmed by western blot analysis, where a single band was observed. The purified rice Rad52 protein was observed to be a stable protein and no degradation products was observed on both SDS-PAGE and western blots.

Purified OsRad52 protein was refolded by standard procedures under reducing conditions, to obtain the soluble protein. As the length of OsRad52 protein differs from Rad52 homologues of yeast and humans, it was interesting to investigate its native form to compare and contrast with that of other Rad52 proteins. The purified protein was subjected to CD (Fig. 4.13), MALDI mass spectrometry (Fig. 4.12) and gel filtration (Fig. 4.14) for protein identification and secondary structure prediction respectively. The secondary structure prediction method (SOPMA, https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) tool was initially used determine the secondary structure, which showed that the rice Rad52 protein had 21 % helical structures. This theoretical prediction was further confirmed by CD analysis of purified OsRad52 protein, which showed the presence of helical secondary structures (Fig. 4.13). The negative peaks at 209 nm and 225 nm are typical signatures of helical proteins thereby, confirming that the OsRad52 protein which was initially purified under denaturing conditions was properly refolded. MALDI mass spectrometry was carried out for rapid and sensitive identification of OsRad52 protein by peptide mass fingerprinting (95; 96). MALDI- TOF mass spectrometry results showed 66 % sequence coverage based on the 12 peptides identified of the 41 predicted peptides, thereby confirming the purified protein sample to be indeed an OsRad52 protein (Fig. 4.12). Moreover, it was interesting to see that the

coverage of the sequence was not confined to a particular domain; rather it was spread over the entire length of the protein sequence.

Electron microscopy studies have showed that human (97) and yeast (61) Rad52 protein formed ring-like structures in vitro. Full length human Rad52 protein formed a heptameric ring structure (98), whereas, the N- terminal region of human Rad52 protein comprising of 1-209 amino acids formed an undecamer (11mer) (99). Since plant Rad52 proteins were found to be homologous to the N-terminal region of human Rad52 protein, we presume that the rice Rad52 also forms similar structures. To test this, we carried out native PAGE (Fig. 4.14) analysis and gel filtration chromatography (Fig. 4.15), which revealed that OsRad52, indeed showed a complex with molecular weight of ~226 kDa, which indicated an undecameric assembly. Presence of a band on native PAGE and an elution peak during gel filtration analysis which corresponded to ~226 kDa indicated an undecameric form of OsRad52 in its native state. In agreement with the well characterized human Rad52 N-terminal domain, we conclude that rice Rad52 also formed multimeric structure (undecamer). Though, sequence variation is observed across the different kingdoms, it appears that the Rad52 group of proteins conserves multimeric nature in their native state, which is important for its biological function.

Human and yeast Rad52 was found to bind both ssDNA and dsDNA (56, 57) and human Rad52 was shown to have a slight preference for dsDNA (100). It has been shown that in human Rad52 protein, Arg 55, Lys 152, residues are essential for ssDNA binding activity, whereas Tyr 65, Arg 153, Arg 156 are essential for both ss and dsDNA binding and in subsequent recombination function (101). We have analyzed the OsRad52 protein sequence for the conservation of these residues. It is interesting to point out that in OsRad52 also, these domains are conserved and may be essential for DNA binding. For understanding the in vitro properties of rice Rad52 protein, nucleic acid binding assays were performed. It was observed that OsRad52 protein was able to bind to both ssDNA (Fig. 4.16) and dsDNA (Fig. 4.17) and form

nucleoprotein complexes as that of human Rad52 (57). OsRad52 was able to bind to both forms of DNA at low concentrations (3.7 nM). Based on these results, we conclude that OsRad52 is a functional homologue of the yeast and human Rad52 protein and presume that it participates in proposed role of DNA damage repair and homologous recombination in rice.

Rad52 protein has been proposed to play a central role in recombination and DNA repair by catalysing the annealing of complementary ssDNA to form duplex DNA. The domain responsible for ssDNA annealing is believed to reside in the N-terminal region of the protein hence is highly conserved (99). Yeast Rad52 has been shown to promote HR through strand annealing probably in a complex with RPA (56,61). In the present study, annealing (renaturation) function of OsRad52 protein was analysed by incubating increasing concentration of protein with complementary single stranded DNA molecules. We could observe that OsRad52 annealed complementary ssDNA into duplex DNA in a concentration dependent manner (Fig. 4.18) as compared to zero protein control proving that OsRad52protein is capable of catalysing the annealing of ssDNA.

Renaturation activity studied using agarose gels had a deproteinization step which involved removal of DNA bound proteins and hence monitoring of the reaction in real time was not possible. To overcome this, we performed renaturation assay with FRET, i.e. Forster resonance energy transfer. Here, two complementary oligonucleotides Phi-W and Phi- C, labelled with fluorescein and rhodamine at their 5' and 3' end, respectively, was used. The assay involves non-radiative transfer of fluorescence energy from the donor molecule (fluorescein) to the acceptor molecule (rhodamine) and since the assay is distance dependent between the donor and acceptor molecule, it helps to reveal the status of physical re-union during renaturation. This assay is sensitive to the dynamics of the DNA strands and local motion of the fluorophores. As the renaturation occurs, both the complementary strands anneal together, which resulted in juxtaposition of the donor and acceptor dyes, hence FRET condition was

created. The decrease in fluorescence intensity was measured at 520 nm upon excitation at 480 nm (Fig. 4.20). We observed that in presence of OsRad52, there was a time dependent decrease in fluorescence emission intensity (Fig. 4.21). The rate and extent of renaturation catalyzed by OsRad52 was increased as protein concentration increases. The rate of the renaturation was high with the reaction reaching a steady state within 100 sec of addition of the protein at 3.7 nM of OsRad52. Whereas, in the no protein control, the rate of renaturation is much slower thereby indicating that the possibility of spontaneous renaturation of the complementary strands is much slower under the experimental conditions. Hence, OsRad52 protein is capable of catalyzing annealing of ssDNA, a pre-requisite to mediate DNA recombination and repair.

Taken together, biochemical activities of OsRad52 protein that we have presented here suggests how plant Rad52 interacts with ssDNA, dsDNA and catalyses annealing of complementary ssDNA to form duplex DNA. We infer that OsRad52 plays a role similar to its counterparts in yeast and humans during homologous recombination mediated DNA repair in plants.

5.3: Future directions:

Homologous recombination, a DNA repair process is important for all living organisms. Upon encountering a damage to its genetic material a living organism under favourable circumstances carries out HR mediated damage repair. The steps include recognition of the DSB site, processing, signalling to recruit the proteins, pre-synapses and synapses formation, strand exchange, junction resolution to form repaired DNA molecules. Different proteins participating in some of these processes include Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, Mre11, and Xrs2. Mutations in these genes result in impairing of DNA repair as well as meiosis. Hence it would be of immense use to know the biochemical properties of all the proteins and map the interactions among these proteins for the better manipulation of this process inplants. Homologous recombination is an important process for meiosis as well as for normal somatic development. The process is vital for maintaining genomic stability during repair as well as for introduction of genetic variability which is the basis for evolution. Since the amount of repetitive DNA is very high in plant species, plant genomes can be potentially recombinogenic. In spite of this, plants have less homologous recombination as compared to yeast and mammals (16). The under lying biochemistry of this process in plants may help to advance our understanding of regulation of this process. We believe that the biochemical characterization of the two important proteins participating in HR, the OsMre11 and OsRad52 from rice, will spur further interest on the studies related to biochemistry of homologous recombination in plants. It would be interesting to study the functions of these proteins in vivo using knockdown or knockout approaches to decipher the phenotypes of rice plants. Further, it will be useful to unravel the structure function relationship using high-resolution crystallographic data of OsMre11 and OsRad52 proteins. This fine structural details will provide more mechanistic insights about their function. All this information will enhance our

current understanding of the plant DNA repair, and useful in better manipulation of the plant genomes for creating varieties with enhanced traits.

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APPENDIX

BIOINFORMATIC ANALYSIS OF OsMre11:

Sequence comparison of Mre11 proteins from different sources was carried out to determine the conservation among the proteins from different sources. Amino acid sequences of *Pyrococcus furiosus* Mre11 (Accession Number Q8U1N9), *Saccharomyces cerevisiae*Mre11 (Accession Number P32829), *Saccharomyces pastorianus* Mre11 (Accession number A0SQ57), *Homo sapiens*Mre11 (Accession NumberP49959), *Mus Musculus*Mre11 (Accession NumberQ61216), *Arabidopsis thaliana* Mre11 (Accession Number Q9XGM2),*Physcomitrella patens*Mre11 (Accession Number G4XIR1),*Oryza sativa* Mre11 (Accession NumberAK070546)and Zea mays Mre11 (Accession Number A0A1P8W040) were compared using Clustal Omega multiple alignment program.

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PF      ----------------MKFAHLADIHLGYEQFHKPQREEEFAEAFKNALEIAVQENVDFI 44
SC      --------MDYPDPDTIRILITTDNHVGYNENDPITGDDS-WKTFHEVMMLAKNNNVDMV 51
SP      ------------------------------------------------MMLAKNNNVDMV 12
HS      ----MSTADALDDENTFKILVATDIHLGFMEKDAVRGNDT-FVTLDEILRLAQENEVDFI 55
MM      ----MSPTDPLDDEDTFKILVATDIHLGFMEKDAVRGNDT-FVTFDEILRLALENEVDFI 55
AT      -------MSREDFSDTLRVLVATDCHLGYMEKDEIRRHDS-FKAFEEICSIAEEKQVDFL 52
PP      ------------DTNTLRILVATDCHVGYLENDEIRRFDS-FNAFEEICSIASQRQVDFV 47
OS      ---------MGDESNTLRVLVATDCHLGYMEKDEIRRFDS-FEAFEEICSLAEQNKVDFV 50
ZM      MSEPAQPSGGEGDVNTLRILVATDCHLGYMEKDEIRRFDS-FQAFEEICALADKNKVDFI 59
                                                          :* :.:**::

PF      LIAGDLFHSSRPSPGTLKKAIALLQIP---------------------------------- 71
SC      VQSGDLFHVNKPSKKSLYQVLKTLRLCCMGDKPCELELLSDPSQVFHYDEFTNVNYEDPN 111
SP      LQSGDLFHVNKPSKKSLYQVLKSLRLSCMGDKPCELELLSDPSQVFHYDEFTNVNYEDPN 72
HS      LLGGDLFHENKPSRKTLHTCLELLRKYCMGDRPVQFEILSDQSVNFGFSKFPWVNYQDGN 115
MM      LLGGDLFHENKPSRKTLHSCLELLRKYCMGDRPVQFEVISDQSVNFGFSKFPWVNYQDGN 115
AT      LLGGDLFHENKPSRTTLVKAIEILRRHCLNDKPVQFQVVSDQTVNFQ-NAFGQVNYEDPH 111
PP      LLGGDLFHENKPSRSTLVRTIEILRKYCMNDKPIQFQVVSDQTINFP-NKFGVVNYEDPN 106
OS      LLGGDLFHENKPSRSTLVKTIEILRRYCLNDQPVKFQVVSDQTINFP-NRFGQVNYEDPN 109
ZM      LLGGDLFHENKPSRSTLVKTIEILRRYCLNDQPVKFQVVSDQTVNFP-NRFGKVNYEDPN 118
        : .***** .:**  :*   :  *:

PF      KEHSIPVFAIEGNHDRTQRGPS--VLN------LLEDFGLVYVIGMRKEKVENEYLTSER 123
SC      FNISIPVFGISGNHDDASGDSLLCPMDILHATGLINHFGKV----IESDKIKVVPL---- 163
SP      FNISIPVFGISGNHDDASGDSLLCPMDILHATGLINHFGKV----IESDKIKIVPL---- 124
HS      LNISIPVFSIHGNHDDPTGADALCALDILSCAGFVNHFGRS----MSVEKIDISPV---- 167
MM      LNISIPVFSIHGNHDDPTGADALCALDVLSCAGFVNHFGRS----MSVEKVDISPV---- 167
AT      FNVGLPVFSIHGNHDDPAGVDNLSAIDILSACNLVNYFGKMVLGGSGVGQITVYPI---- 167
PP      FNVGLPVFTIHGNHDDPAGVDNLSAIDILAACNLVNYFGKVALGGNGVGNIALHPI---- 162
OS      FNVGLPVFTIHGNHDDPAGVDNLSAIDILSACNLVNYFGKMDLGGSGVGEIAVYPV---- 165
ZM      FNVGLPVFTIHGNHDDPAGVDNLSAIDILSACNLVNYFGKMDLGGSGVGQIAVYPV---- 174
         : .:*** * ****          ::      ::: **          ::    :
```

```
PF      LGNGEYLVKGVYKDLEIHGMKYMSSAWFEANKEILKRLFRPTDN---------------- 167
SC      ------LFQKGSTKLALYGLAA-------VRDERLFRTFKDGG-VTFEVPTM----REGE 205
SP      ------LFQKGSTKLALYGLAA-------VRDERLFRTFKDGG-VTFEVPTM----REGE 166
HS      ------LLQKGSTKIALYGLGS-------IPDERLYRMFVNKK-VTMLRPKE----DENS 209
MM      ------LLQKGSTKLALYGLGS-------IPDERLYRMFVNKK-VTMLRPKE----DENS 209
AT      ------LMKKGSTTVALYGLGN-------IRDERLNRMFQTPHAVQWMRPEVQEGCDVSD 214
PP      ------LLRKGSTNVALYGLGN-------IRDERLNRMFQTPHAVQWIRPESTDDCPFSD 209
OS      ------LVKKGTTFVALYGLGN-------IRDERLNRMFQTPHAVQWMRPETQDGMSVSD 212
ZM      ------LVKKGMTSVALYGLGN-------IRDERLNRMFQTPHSVQWMRPGTQDGESASD 221
              *.:   . : ::*:           .* * * *

PF      --AILMLHQGVREVSEARGEDYFEIGLGDLPEGYL-----YYALGHIHKRYE----TSYS 216
SC      WFNLMCVHQNHTGHTN----------TAFLPEQFLPDFLDMVIWGHEHECIPNLVHNPIK 255
SP      WFNLMCVHQNHTGHTN----------TAFLPEQFLPDFLDLVIWGHEHECIPNLVHNPMK 216
HS      WFNLFVIHQNRSKHGS----------TNFIPEQFLDDFIDLVIWGHEHECKIAPTKNEQQ 259
MM      WFNLFVIHQNRSKHGN----------TNFIPEQFLDDFIDLVIWGHEHECKIGPIKNEQQ 259
AT      WFNILVLHQNRVKSNP----------KNAISEHFLPRFLDFIVWGHEHECLIDPQEVSGM 264
PP      WFNIFVLHQNRVKANP----------KNAINEHMLAKFLDFVVWGHEHECLVDPQEVLGM 259
OS      WFNILVLHQNRIKTNP----------KSAINEHFLPRFLDFIVWGHEHECLIDPQEVPGM 262
ZM      WFNILVLHQNRIKTNP----------KSAINEHFLPRFLDFIVWGHEHECLIDPQEVPGM 271
           :: :**.                  : *  *        ** *:

PF      GSPVVYPGSLERWDFGDYEVRYEW---DGIKFKERYGVNK-GFYIVEDFKPRFVEIKVRP 272
SC      NFDVLQPGSSVATSLCEAEAQPKYVFILDIKYGEAPKMTPIPLETIRTFKMKSISLQDVP 315
SP      GFDVLQPGSSVATSLCEAEAQPKYVFILEIKYGEAPKMTPIPLETIRTFKMRSISLQDVP 276
HS      LFYISQPGSSVVTSLSPGEAVKKHVGLLRIK-GRKMNMHKIPLHTVRQFFMEDIVLANHP 318
MM      LFYVSQPGSSVVTSLSPGEAVKKHVGLLRIK-GRKMNMQKLPLRTVRRFFIEDVVLANHP 318
AT      GFHITQPGSSVATSLIDGESKPKHVLLLEIK-GNQYRPTKIPLTSVRPFEYTEIVLKDES 323
PP      DFHITQPGSSVATSLIDGESKPKHVLLLEIK-GNEYRPTKVPLKSVRPFKYKNVSLQDAA 318
OS      GFHITQPGSSVATSLIDGEAKPKHVLLLEIK-GNQYRPTKIPLRSVRPFHYAEVVLKDEV 321
ZM      GFHITQPGSSVATSLIDGEAKPKHVLLLEIK-GNQYRPTKIPLRSVRPFEYAEVVLKDEA 330
           :  ***    .:   *   :      **  .        :  :. *    : :

PF      FIDV----------------KIK----GSEEEIRKAIKRLIPLIPKNAYVRLNIGWRKPF 312
SC      HLRPHDKDATSKYLIEQVEEMIRDANEETKQKLADDGEGDMVAELPKPLIRLRVDYSAPS 375
SP      HLRPHDKDVTSKYLIEQVEEMIEEANEETKRKLGDDADGDMISELPKPLIRLRVDYSAPP 336
HS      DIFNPDNPKVTQAIQSFCLEKIEEMLENAERE-----RLGNSHQPEKPLVRLRVDYSGG- 372
MM      NLFNPDNPKVTQAIQSFCLEKIEEMLDSAERE-----RLGNPQQPGKPLIRLRVDYSGG- 372
AT      DIDPNDQNSILEHLDKVVRNLIE-----KASK-----KAVNRSEIKLPLVRIKVDYS-G- 371
PP      DLDANDVQSVMDHLSQMVHDLIA-----EAAE-------EGHPDPMLPLVRLRVDYT-G- 364
OS      DVDPNDQASVLEHLDKIVRNLIK-----KSSQ-----PTASRPETKLPLIRIKVDYS-G- 369
ZM      DVNSNDQDSVLEHLDKIVRNLIE-----KSSQ-----PTASRSEPKLPLVRIKVDYS-G- 378
         :                   *        :                :*:.:.:

PF      DLTEIKELLNVEYLKIDTWRIKERTDEESGKIGLPSDFFTEFELKI-------------- 358
SC      NTQS-----PIDYQVENPRRFS---NRFVGRVANGNNVVQFYKKRSPVTRSKKSGINGTS 427
SP      NKQS-----PIDYQVENPRRFS---NRFVGRVANGNNVVQFYKRRSPATRSKNLGLNGTN 388
HS      ------------FEPFSVLRFS---QKFVDRVANPKDIIHFFRHREQKEKTGEEINFGKL 417
MM      ------------FEPFNVLRFS---QKFVDRVANPKDVIHFFRHREQKGKTGEEINFGML 417
AT      ------------FMTINPQRFG---QKYVGKVANPQDILIFSKASKKGR--SEAN----- 409
PP      ------------FTTINPQRFG---QKFVGKVANPHDILLFTKAVKRRP-TADGK----- 403
OS      ------------FSTINPQRFG---QKYVGKVANPQDILIFSKSAKKRQTTGVGN----- 409
ZM      ------------FSTINPQRFG---QKYVGKVANPQDILIFSKSAKKRQTTG-DH----- 417
                    :   .  *:    :.  .::.   :..   .

PF      --------IDIL--GEKDFDD--FDYIIKLITEGKV----EEEGPLEEAVKKVSEEKGKT 402
SC      ISDRDVEKLFSESGGELEVQTLVND------LLNKMQLSLLPEVGLNEAVKKFVDKDEKT 481
SP      ISDRDVEKLLNESGGELEVQTLVND------LLNKMQLSLLPEVGLNEAVKKFVDKDEKT 442
HS      ITK------P-SEGTTLRVEDLVKQYF--QTAEKNVQLSLLTERGMGEAVQEFVDKEEKD 468
MM      ITK------PASEGATLRVEDLVKQYF--QTAEKNVQLSLLTERGMGEAVQEFVDKEEKD 469
AT      ----------IDDSERLRPEELNQQNIEALVAESNLKMEILPVNDLDVALHNFVNKDDKL 459
```

```
PP      ----------IEDEEKLRPEELNQQNIEALLAESNLKMEILPVSELGLALHKFVSKDDKL 453
OS      ----------IDDSEKLRPEELNQQTIEALVAENNLKMEILPVDDLDIALHDFVSKDDKM 459
ZM      ----------IDDSEKLRPEELNQQTIEALVAESNLKMEILPVDDLDIALHDFVNKDDKM 467
                          :    :         ::         :  *::.. .:. *

PF      VRQ-KIESIPKKKRGTLD------------------------------------------- 419
SC      ALKEFISHEISNEVGILSTNEEFLRTDDAEE-----------------MKALIKQVKRA 523
SP      ALKEFISHEISNEVDILSTNNEFLQTEDTEE-----------------MKALIKQVKRA 484
HS      AIEELVKYQLEKTQRFLKERHIDALEDKIDEEVR--RFRETRQKNTNEEDDEVREAMTRA 526
MM      AIEELVKYQLEKTQRFLKERHIDALEDKIDEEVR--RFRESRQRNTNEEDDEVREAMSRA 527
AT      AFYSCVQYNLQETRGKLAKDSD---AKKFEEDDLILKVGECLEER------------LKD 504
PP      AFIECVQENLDDTQNKLAEEAL---ADKLQEDDVIVKVSEHMQ----------------- 493
OS      AFYACLQRNLEETRTKLNSEAD---KFKIEEEDIIVKVGECMQER------------VKE 504
ZM      AFYSCLQRNLEETRNKLSSEAD---KSKFEEEDIIVKVGECMQER------------VKE 512
        .    :.   ..    *

PF      ---------------SWLGGAR------------------------------------- 426
SC      NSVRPTPPKENDETNFAFNGNGLDSFRSSNREVRTGSPDITQSHV---DNES-------- 572
SP      NSVRPATPMENDEASLSLDNNRLSSFQPSNELRRDKPSSTAQSHV---SKEA-------- 533
HS      RALRSQSEES----ASAFSADDLMSIDLA-----------EQ-----MANDSDD--SISA 564
MM      RALRSQSETS----TSAFSAEDL-SFDTS-----------EQ-----TANDSDD--SLSA 564
AT      RSTRP---TG----SSQFLSTGLTSENLTK----GSSG----IANASFSDDEDTTQMSGL 549
PP      ---------------------------------------------------------- 493
OS      RSLRS---KE----DSRFTSSS---QNLDT----GGRSVTAQSNLNSFSDDEDTREMLLG 550
ZM      RSLHS---KD----GTRLTTGS---HNLDT----GGKSFTAQSNQNSFSDDEDTREMLLG 558


PF      ---------------------------------------------------------- 426
SC      --------------RITHISQAESSKPTSKPKRVRTATKKKIPAFS-------------- 604
SP      --------------EVDRTKEFESVKPTKKTRSTRTVRRKKTAAPS-------------- 565
HS      --ATNKGR---GRGRGRRGGRGQNSASRGGSQRGRADTGLETSTRS-----RNSKTAVSA 614
MM      --VPSRGR---GRGRGRRGARGQSSAPRGGSQRGR-DTGLEITTRG-----RSSKATSST 613
AT      A-PPTRGRRGSSTANT---TRGRAKAPTRGRGRGKASSAMKQTTLDSSLGFRQSQRSAS- 604
PP      ---------------------------------------------------------- 493
OS      ARTTNAGRKASGFTRPSKDATDVAKTGTSRRGRGRGTASMKQTTLNFS----QSRS---- 602
ZM      ARSTDVGRKSSGFTRPSKDTADVAKRGTSKRGRGRGTSSMKQTTLSFS----QSRS---- 610


PF      ---------------------------------------------------------- 426
SC      -DSTVISDAENELGDNNDA-QDDVDI---------DENDIIMVSTDEEDASYGLLNGRKT 653
SP      -DSIVISDAENEFEDDNDARVDDVDV---------NEDDIIMVSTDEEDASYGLISGRKT 615
HS      SRNMSIIDA---FKSTRQQPSR--------NVTTKNYSEVIEVD--ESDVEED------- 654
MM      SRNMSIIDA---FRSTRQQPSR--------NVAPKNYSETIEVD--DSD-EDD------- 652
AT      ---AAASAA---FKSASTIGEDDVDSPSSEEVEPEDFNKPDSSS--EDDESTK-GKGRK- 654
PP      ---------------------------------------------------------- 493
OS      ---------------SAAIRSEEVQSSSDEENETNEANEVVESS--EPEESPQ-QTGRK- 643
ZM      ---------------ATVIRSEDVASSEEEA----DANEVVENS---EEESAQ-QVGRK- 646


PF      ---------------------------------------------------------- 426
SC      KTKTRPAASTKTASRRGKGR--ASRTPKTDILGSL---LAKKR----------------- 691
SP      KRKSRSPAGTKASSTRGKGRAKATRTPKTDILGSL---LAKKR----------------- 655
HS      -----IFPTTSKTDQRWSSTSSSKIMSQSQVSKGV-DFESSEDD-------DDDPFMNTS 701
MM      -----IFPTNSRADQRWSGTTSSKRMSQSQTAKGV-DFESDEDD-------DDDPFMSSS 699
AT      -----RPATTKRGRGRGSGTSK--RGRKNESSSSLNRLLSSKDDDEDEDDEDREKKLNKS 707
PP      ---------------------------------------------------------- 493
OS      -----RAAPRG-GRGRGRGATA-KRGRKADI-SSIQSMLMSKDDDDD----DEDDRPKKP 691
ZM      -----RAAPRGRGRGRGGGSTA-KRGRKTDI-ASMQNMMSKDD---D----DSEDEPPKK 692
```

```
PF      -------------- 426
SC      -----K-------- 692
SP      -----NRYDKLL-- 662
HS      SLRRNRR------- 708
MM      CPRRNRR------- 706
AT      QPRVTRNYGALRR- 720
PP      -------------- 493
OS      PPRVTRNYGAVRRR 705
ZM      TPRVTRNYGAVRRR 706
```

Figure A. 01: Multiple sequence alignment of full length Mre11

It is observed that conservation of Mre11 protein among different species is more in the N-terminal region as compared to C-terminal region.

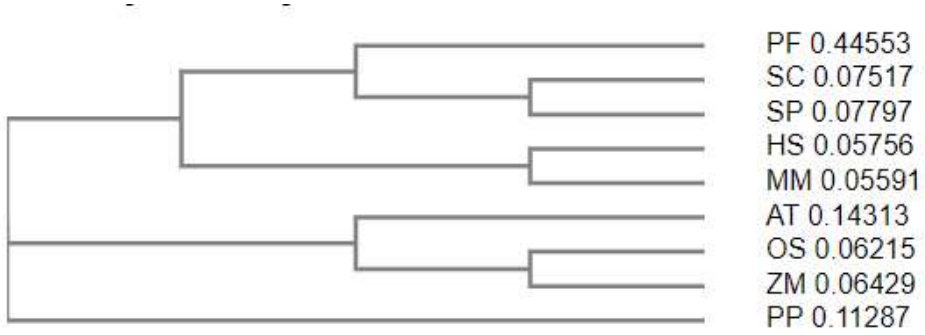

Figure A. 02: Phylogenetic tree of Mre11 sequences across different species

Phylogenetic tree analysis (dendrogram) of the same showed that plant Mre11 protein was diverged from other species Mre11 at early stage of evolution. Among plants, at later stages monocots (OS and ZM) were diverged from that of dicot plant (AT).

Multiple sequence alignment among the *Oryza sativa*, *Homo sapiens* and *Arabidopsis thaliana* N- terminal region of OsMre11 to drive home the point that these proteins have highly conserved DNA binding and nuclease domains, thereby promoting the argument that they might function similarly in exhibiting their respective DNA binding and nuclease properties.

Figure A. 03: Sequence alignment of the N- terminal end of Mre11 proteins

Sequences marked in green highlights the Mre11 nuclease domain, which is highly conserved across human, rice and Arabidopsis Mre11. Sequences marked in orange highlights the Mre11 DNA binding domain.

BIOINFORMATIC ANALYSIS OF OsRad52:

Sequence comparison of Rad52 proteins from different sources was carried out to determine conservation among the protein from different sources. Amino acid sequences of *Arabidopsis thaliana*Rad52 like protein 1, Mitochondria (Accession Number Q9FVV7), *Arabidopsis thaliana*Rad52 like protein 2, Chloroplastic (Accession Number Q9FIJ4), *Oryza sativa*Rad52 (Accession NumberAK070236), *Saccharomyces cerevisiae*Rad52 (Accession Number P06778), *Homo sapiens*Rad52 (Accession NumberP43351), *Mus Musculus*Rad52 (Accession NumberP43352) were compared using Clustal Omega multiple alignment program.

```
1       ----------------------MAGLGLRLKAA-----KWTLR-SGSGAVSREWSSEMGK 32
2       MALQVQQTSAAFTISSPST----AAARIKLSPFRTVAVNRGVRCSGGGVGGGDAGKKKAV 56
OS      ----MEATTSSLLVRPVDTRLSAASLPIVVRARRRVAVVT--------AAAPERKPAAAA 48
SC      -------------------------MN--------EI----------M-DMDEKKP--- 12
HS      ----------------------MSGTEE--------AI----------LGGRDSHP-AAG 19
MM      ----------------------MAGPEE--------AV----------HRGCDNHPPFVG 20
                                                           :

1       GVRR-FSTETENDVPTSGISRPLAEILKELNKKVPDSVIRTRVE---DGCSIKYIP-WH- 86
2       PNSNYV-VPIDKFSSSSSITRPLIEILRDLNKKIPDNIVKSHDPPSTSAATSGFIP-WY- 113
OS      SSSNYVVVPLD--AAPSGITRPLVEILRDLNKRVPDTVVRSSRRR--ASPSDPVIP-WY- 102
SC      -----------------VFGNHSEDIQTKLDKKLGPEYISKRVGF--GTSRIAYIEGWRV 53
HS      GGSVLC------FGQCQYTAEEYQAIQKALRQRLGPEYISSRMAG--GGQKVCYIEGHRV 71
MM      GKSVLL------FGQSQYTADEYQAIQKALRQRLGPEYISSRMAG--GGQKVCYIEGHRV 72
                               *   * :::    : .            *

1       --IVNRIMNMHAPEWSGEVRSVTYS----PDGNT---VTVAYRVTLYGTDAEIFRESTGT 137
2       --HANRMLSFYAPGWCGEVRDVIFS----ENGN----VTVVYRLTIRGSDGEAHRESTGT 163
OS      --HANRMLSFYAPGWCGEVRDVIYT----DNGK----VTVVYRVTVRGTDGEVHREAAGT 152
SC      INLANQI--FGYNGWSTEVKSVVIDFLDERQGKFSIGCTAIVRVTL--TSGTYR-EDIGY 108
HS      INLANEM--FGYNGWAHSITQQNVDFVDLNNGKFYVGVCAFVRVQL--KDGSYH-EDVGY 126
MM      INLANEM--FGYNGWAHSITQQNVDFVDLNNGKFYVGVCAFVKVQL--KDGSYH-EDVGY 127
           .*.:  :    *. .: .          :*:      .  :: :  ...    *  *

1       TSVD-----DKGYGDAVQKAEAMAFRRACARFG--LGLHLYHEDAL-------------- 176
2       VTTT-----DDHIEDPVTAAEEIAFCRACARFG--LGLYLYHE---------------- 199
OS      TSLN-----DARFDDPVAAAEEAAFCKACARFG--FGLYLYHEDETP------------ 192
SC      GTVENERRKPAAFERAKKSAVTDALKRSLRGFGNALGNCLYDKDFLAKIDKVKFDPPD-F 167
HS      GVSEGLKSKALSLEKARKEAVTDGLKRALRSFGNALGNCILDKDYLRSLNKLPRQLPLEV 186
MM      GVSEGLRSKALSLEKARKEAVTDGLKRALRSFGNALGNCILDKDYLRSLNKLPRQLPLDV 187
                         *   .: ::   **  :*  : .:

1       ------------------------------------------------------------ 176
2       ------------------------------------------------------------ 199
OS      ------------------------------------------------------------ 192
SC      DENNLFRPTDEISESSRTNTLHENQEQQQYPNKRRQLTKVTNTNPDSTKNLVKIENTVSR 227
HS      DLTKAKR-----------QDLEPSVEEARYNSCRPNMA-L---------GHPQLQQVTSP 225
MM      DLTKTKR-----------EDFEPSVEQARYNSCRQNEA-L---------GLPKPQEVTSP 226
```

```
1       ------------------------------------------------------------ 176
2       ------------------------------------------------------------ 199
OS      ------------------------------------------------------------ 192
SC      GTPMMAAPAEANSKNSSNKDTDLKSLDASKQ-----DQDD--LLDDSLMFSDDFQDDDLI 280
HS      SRPS--HAV-----IPADQDCSSRSLSSSAVESEATHQR---KLRQK-QLQQQFRE---- 270
MM      CRSS--PPHDSNIKLQGAKDISSSCSLAATLESDATHQRKLRKLRQK-QLQQQFRE---- 279

1       ------------------------------------------------------------ 176
2       ------------------------------------------------------------ 199
OS      ------------------------------------------------------------ 192
SC      NMGNTNSNVLTT----EKDPVVAKQSPTASSNPEAEQITFVTAKAATSVQNERYIGEESI 336
HS      RMEKQQVRVSTP--SAEKSEAAPPAPPVTHSTPVTVSEPLL--------EKDFLAGVTQE 320
MM      QMETRRQSHAPAEEVAAKHAAVLPA-PPKHSTPVTAASELL--------QEKV------V 324

1       ------------------------------------------------------------ 176
2       ------------------------------------------------------------ 199
OS      ------------------------------------------------------------ 192
SC      FDPKYQAQSIRHTVDQTTSKHIPASVLKDKTMTTARDSVYEKFAPKGKQLSMKNNDKELG 396
HS      LIKTLEDNSEK-------------------------------------------------WAVT 335
MM      FPDNLEENLEM-------------------------------------------------WDLT 339

1       ------------------------------------------------------------ 176
2       ------------------------------------------------------------ 199
OS      ------------------------------------------------------------ 192
SC      PHMLEGAGNQVPRETTPIKTN-ATAFPPAAAPRFAPPSKVVH-----PNGNGAVPAVPQ- 449
HS      PDAGDGVVKPSSRA-DPAQTSDTLALNNQMVTQNRTPHSVCHQKPQAKSGSWDLQTYSAD 394
MM      PDLED-IIKPLCRA-EPAQTSATRTFNN----QDSVPHIHCHQKPQEKPGPGHLQTCNTN 393

1       ---------------------------------     176
2       ---------------------------------     199
OS      ---------------------------------     192
SC      QR-----------STRREVGRPKINPLHARKPT     471
HS      QRTTGN---WESHRKSQDMKKRKYDPS------     418
MM      QHVLGSREDSEPHRKSQDLKKRKLDPS------     420
```

Figure A. 04: Multiple sequence alignment of full length Rad52 sequences

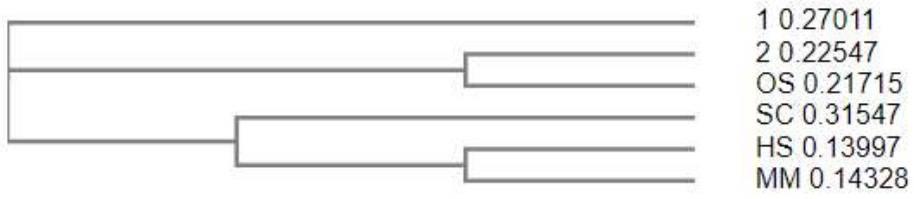

Figure A. 05: Phylogenetic tree of Rad52 sequences across different species

Since, it has already been reported that plant Rad52 proteins are homologous to the N- terminal end of human and yeast Rad52, we did a sequence alignment of the N- terminal end of human and yeast Rad52 to the full length of rice Rad52.

```
OS      MEATTSSLLVRPVDTRLSAASLPIVVRARRRVAVVTAAAPERKPAAAASSSNYVVVPLDA    60
HS      MSGTEE----------------------------AILGGRDSHPAAGGGSVLCF----GQ    28
SC      ----MN----------------------------EIMDMDEKKPV---------------    13
             .                                  : :*.

OS      APSGITRPLVEILRDLNKRVPDTVVRSSRRRASPSDPVIPWYHANRML-----SFYAPGW    115
HS      C-QYTAEEYQAIQKALRQRLGPEYISS---RMAGGGQKVCYIEGHRVINLANEMFGYNGW    84
SC      ----FGNHSEDIQTKLDKKLGPEYISK---RVGFGTSRIAYIEGWRVINLANQIFGYNGW    66
              .    *   * :::    : .   * . .   : : .. *::      *   **

OS      CGEVRDVI----YTDNGKVTVVYR--VTVRGTDGEVHREAAGTTSLNDA----RFDDPVA    165
HS      AHSITQQNVDFVDLNNGKFYVGVCAFVRVQLKDGSYHEDVGYGVSEGLKSKALSLEKARK    144
SC      STEVKSVVIDFLDERQGKFSIGCTAIVRVTLTSGTYREDIGYGTVENERRKPAAFERAKK    126
        . .: .         :**. :     * *  ..*  :.: .  .  .       ::

OS      AAEEAAFCKACARFGF--GLYLYHEDETP-------------------------------    192
HS      EAVTDGLKRALRSFGNALGNCILDKDYLRSLNKLPRQLPLEVDLTKA----------KRQ    194
SC      SAVTDALKRSLRGFGNALGNCLYDKDFLAKIDKVKFDPPDFDENNLFRPTDEISESSRTN    186
         *   .: ::   **   *  : .:*

OS      ----------------------- 192
HS      DLEPSVEEARYNSCR-------- 209
SC      TLHENQEQQQYPNKRRQLTKVTN 209
```

Figure A. 06: Sequence alignment of the N- terminal end of Rad52 proteins

Results of attempts made to purify OsMre11 full length protein:

As already discussed in Table 3.1, several experiments were carried out to purify the full length protein. Here, we have discussed a few results of the experiments undertaken.

Serial Number	Experiment	Result
1.	OsMre11 protein induced at 20˚C	OsMre11 was not induced as a soluble fraction
2.	Cells harbouring OsMRE11- peT28a plasmid was grown in M9 media	OsMre11 was not induced as a soluble fraction
3.	N- lauroylsarcosine and arginine	OsMre11 precipitated upon removal of arginine
4.	Cloning into pColdTF vector	No OsMre11 observed upon purification
5.	Chaperone assisted co-expression	OsMre11 induction failed
6.	*K. lactis* expression system (yeast)	OsMre11 not produced by yeast K. lactis
7.	Shuffle T7 Express lysY competent *E. coli* system	No OsMre11 observed upon purification

1. N- lauroylsarcosine is an ionic surfactant derived from sarcosine and is known to solubilize recombinant proteins that accumulate as inclusion bodies. Since, the full length OsMre11 protein localized as inclusion bodies upon induction, we attempted to solubilize the isolated inclusion bodies in sarcosine followed by Ni- NTA purification. Upon SDS- PAGE analysis of the elutes, no OsMre11 protein band was observed in the lysate.

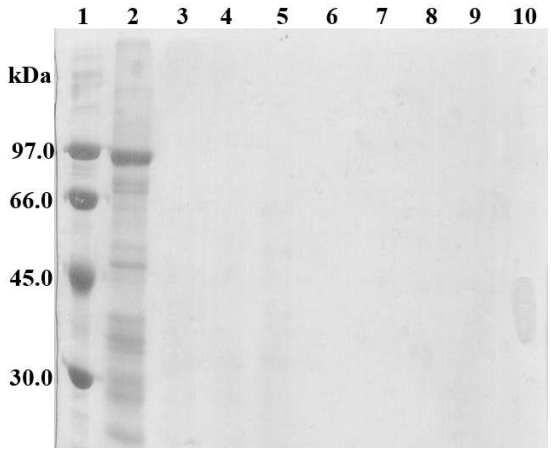

Figure A. 07: Ni-NTA purification of OsMre11 solubilized in 0.3% sarcosine

Lane 1: Molecular weight marker

Lane 2: Inclusion body preparation solubilized in 0.3 % sarcosine

Lane 3: Flow through

Lane 4: Wash with pH 8.0 buffer

Lane 5- 10: Elution with buffer containing increasing amount of imidazole (100,200, 300, 400, 500, 1000 mM imidazole respectively)

No OsMre11 protein was observed using N- lauroylsarcosine during purification process.

2. Arginine has been reported to aid in refolding of recombinant proteins by suppressing protein aggregation. Generally, 0.1- 1M arginine is used for refolding of recombinant proteins. OsMre11 was dissolved in 0.1 M arginine and purified. However, the over- expressed OsMre11 protein upon step- wise dialysis to remove the arginine, the protein again precipitated.

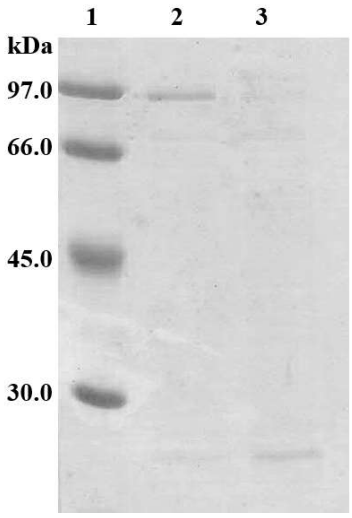

Figure A. 08: SDS-PAGE of OsMre11 full length protein after dialysing arginine

Lane 1: Molecular weight marker

Lane 2: OsMre11 protein resuspended in 0.1M arginine

Lane 3: OsMRe11 protein suspension after dialysing out arginine

Dialysing out arginine resulted in precipitation and degradation of OsMre11 protein

3. To obtain full length OsMre11 protein in the soluble fraction, the OsMre11 cDNA was cloned into pColdTF over- expression vector. pColdTF vector is known to improve the rate of solubilization and soluble expression of recombinant proteins and it uses trigger factor (TF) chaperone as soluble tag. However, even after using this method of cloning, upon SDS-PAGE analysis of the elution profile, no 132kDa band of OsMre11 with TF fusion protein is observed, thereby leading to abortion of this technique.

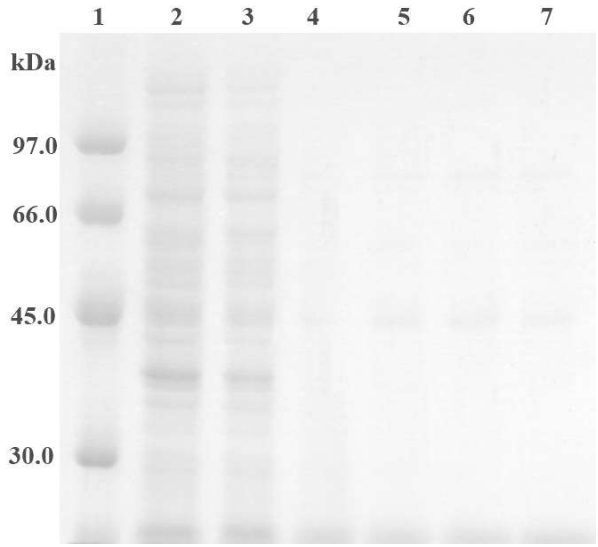

Figure A. 09: Purification of over expressed OsMre11-pColdTF protein

Lane 1: Molecular weight marker

Lane 2: Induced cell lysate

Lane 3: Flow through

Lane 4: Wash with pH 8.0 buffer

Lane 5- 7: Elution with buffer containing increasing amount of imidazole (100,200, 300 mM imidazole respectively)

Upon Ni- NTA purification, no band ~132kDa corresponding to OsMre11 with TF fusion protein is observed.

4. Chaperone assisted co- expression of full length OsMre11 was attempted in a bid to obtain the pure protein. The Chaperone plasmid set from Takara Biotech consists of five different types of chaperone plasmids, each designed to enable efficient expression of multiple chaperones that assists in the protein folding process. Co-expression of target protein with chaperones is known to increase the amount of expressed proteins in the soluble fraction.

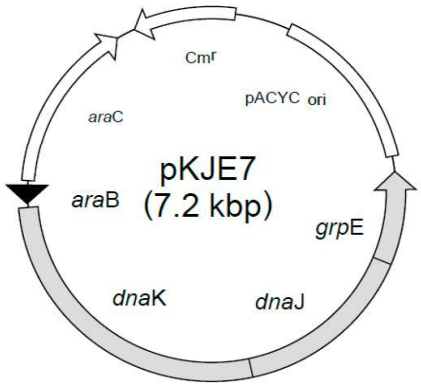

Figure A. 10: pKJE7 chaperone used for co- expression of OsMre11 protein

For OsMre11 over- expression we used the pKJE7 plasmid, which consists of dnaK-dnaJ-grpE chaperones, known to assist in the co-expression of target proteins in the soluble form.

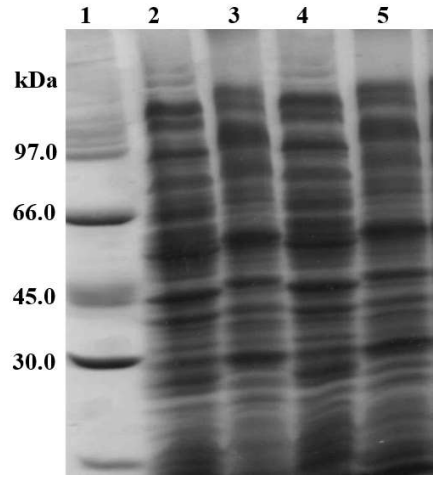

Figure A. 11: Over- expression profile of cells harbouring pET281- OsMre11 and pKJE7 chaperone

Lane 1: Molecular weight marker

Lane 2: Uninduced cell lysate (- IPTG, - arabinose)

Lane 3: Uninduced cell lysate (- IPTG, + arabinose)

Lane 4: Uninduced cell lysate (+ IPTG, - arabinose)

Lane 5: Induced cell lysate (+ IPTG, + arabinose)

Upon over- expression with both IPTG and arabinose, OsMre11 protein was not observed on gel.

5. Following the failure of all these trials, an eukaryotic system was used to over- express and purify rice Mre11 protein, to avoid problems of post translational modification of eukaryotic protein. Yeast *Kluveromyces lactis*, was used to purify OsMre11 protein. Here, the protein may be expressed intracellularly or be secreted from the cell using the expression vector pKLAC2. The gene of interest is cloned into pKLAC2 downstream of *K. lactis* α- mating factor domain to produce a α-MF fusion protein, which is then transported through the yeast secretory pathway and cleaved with the help of Signal peptidase in ER and the Kex protease in the Golgi leading to the secretion of native form of protein of interest into the growth medium.

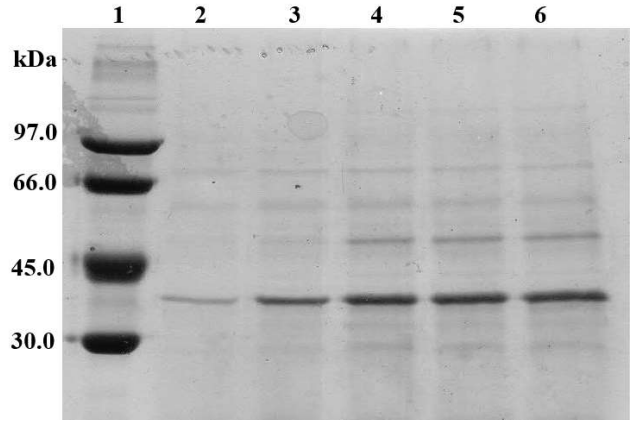

Figure A. 12: SDS- PAGE profile of yeast growth media harbouring K. lactis yeast cells

Lane 1: Molecular weight marker

Lane 2-6: Supernatant of yeast culture medium of days 1 to 5 respectively

OsMre11 was not secreted into the medium by yeast cells, even after 5 days of growth