Theor Appl Genet (2011) 122:251–262
DOI 10.1007/s00122-010-1440-4

ORIGINAL PAPER

The *Rad50* genes of diploid and polyploid wheat species. Analysis of homologue and homoeologue expression and interactions with *Mre11*

R. Pérez · A. Cuadrado · I. P. Chen · H. Puchta · N. Jouve · A. De Bustos

Received: 21 May 2010 / Accepted: 25 August 2010 / Published online: 9 September 2010

Abstract The MRN complex plays a central role in the DNA repair pathways of eukaryotic cells and takes part in many other processes, including cell cycle checkpoint signalling, meiosis, DNA replication and telomere maintenance. This complex is formed by the interaction of the products of the *Mre11*, *Rad50* and *Nbs1* genes. This paper reports the molecular characterization, expression and interactions of the *Rad50* gene in several wheat species with different levels of ploidy. The homoeologous *Rad50* wheat genes were found to show a high level of conservation. Most of the RAD50 domains and motifs previously described in other species were also present in wheat RAD50; these proteins are therefore likely to have similar functions. Interactions between the RAD50 wheat proteins and their MRE11 counterparts in the MRN complex were observed. The level of expression of *Rad50* in each of the species examined was determined and compared with those previously reported for the *Mre11* genes. In some cases similar levels of expression were seen, as expected. The expression of the RAD50 homoeologous genes was assessed in two polyploid wheat species using quantitative PCR. In both cases, an overexpression of the *Rad50B* gene was detected. Although the results indicate the maintenance of function of these species' three homoeologous *Rad50* genes, the biased expression of *Rad50B* might indicate ongoing silencing of one or both other homoeologues in polyploid wheat. To assess the consequences of such silencing on the formation of the MRN complex, the interactions between individual homoeologues of *Rad50* and their genomic counterpart *Mre11* genes were examined. The results indicate the inexistence of genomic specificity in the interactions between these genes. This would guarantee the formation of an MRN complex in wheat.

Communicated by E. Guiderdoni.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1440-4) contains supplementary material, which is available to authorized users.

R. Pérez · A. Cuadrado · N. Jouve · A. De Bustos (✉)
Department of Cell Biology and Genetics,
University of Alcalá, Alcalá de Henares, Spain
e-mail: alfredo.bustos@uah.es

I. P. Chen · H. Puchta
Karlsruhe Institute of Technology (KIT),
Botanical Institute II, 76133 Karlsruhe, Germany

Introduction

DNA double-strand breaks (DSBs) are one of the most cytotoxic kinds of DNA damage (Waterworth et al. 2007). DSBs can lead to mutations, chromosomal rearrangements and instability, cancer and cell death (Berkovich et al. 2007; Rupnik et al. 2008). They are produced by exogenous agents such as ionising radiation or genotoxic compounds but also occur during normal cell processes such as replication and meiosis (Steininger et al. 2008). Cells must urgently repair these DSBs to avoid their negative effects; the arrest of the cell cycle is one of first steps taken, followed by the activation of repair mechanisms. There are two main ways to undertake the repairs required: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR allows for repair without the loss of genetic information, whereas NHEJ can induce errors (Puchta, 2005). A myriad of proteins are involved in both types of repair, each with a specific role.

The MRN (*Mre11*, *Rad50* and *Nbs1* [*Xrs2* in yeast]) complex plays a central role in both the HR and NHEJ repair pathways (Borde and Cobb 2009). It also takes part

in many other processes, including cell-cycle checkpoint signalling, meiosis, DNA replication, and telomere maintenance (reviewed by Williams et al. 2007). This variety of functions is owed to the different enzyme activities and structural characteristics of the members of the complex (Assenmacher and Hopfner, 2004). The importance of the *Mre11*, *Rad50* and *Nbs1* genes to this complex is revealed by the fact that orthologues of *Mre11* and *Rad50* have been identified in all taxonomic kingdoms, and *Nbs1* is found in all eukaryotic cells (Rupnik et al. 2008). Further, the disruption of any of these genes is lethal in mammals (Stracker et al. 2004), and their hypomorphic mutation in humans leads to several diseases, including Nijmegen breakage syndrome (NBS), ataxia-telangiectasia-like disorder (ATLD), and NBS-like disorder (NBSLD) (for more information on mutations in *Nbs1*, *Mre11* and *Rad50*, see Waltes et al. 2009).

The *Rad50* gene is a member of the structural maintenance of chromosomes (SMC) protein family, which is involved in correctly organizing DNA in chromosomes (Kinoshita et al. 2009). RAD50 is a large protein that binds DNA in an ATP-dependent manner (Acharya et al. 2008), and is composed of several domains that conform the complex structure so characteristic of SMC proteins. Much of the work done on the *Rad50* gene has involved yeasts and mammals (especially Man). In plants, characterization and genetic studies have only been performed in model species (Gallego et al. 2001; Daoudal-Cotterell et al. 2002; Gherbi et al. 2001; Bleuyard et al. 2004).

Our group has characterized the homoeologous *Mre11* genes of the wheat genomes (De Bustos et al. 2007), determining their levels of expression in species of different ploidy. The present work characterizes the homoeologous *Rad50* genes of the same wheat species in order to examine how they interact with their genomic counterpart *Mre11* genes. The results could help clarify how the polyploid species resolve the silencing of duplicated genes. Bearing in mind the polyploid nature of wheat, the expression of *Rad50* homoeologous genes was analysed in tetraploid and hexaploid forms. It has been shown that duplicated genes can suffer changes in their expression leading to the silencing and even the elimination of some (for a review, see Adams and Wendel 2005a). In earlier work we showed that the duplicated *Mre11* gene belonging to the B genome was downregulated in tetra and hexapolyploid wheat, probably indicating a process of silencing after polyploidization (De Bustos et al. 2007).

The aim of the present work was to: (1) analyse the MRN complex genes in a group of wheat species, including diploid and alloploid forms, (2) determine whether the interactions between these genes were genome-specific or not, and (3) determine whether the silencing process observed in the *Mre11* genes is also taking place in the *Rad50* genes.

Materials and methods

Plant material

Rad50 genes were characterized in the diploid species *Triticum monococcum* L. (genome A) and *Aegilops tauschii* Coss. (genome D), and the tetraploid species *Triticum turgidum* L. cv. Vitron (genomes A and B). Expression analyses were also performed for the hexaploid wheat *Triticum aestivum* L cv. Chinese spring (genomes A, B and D). Nulli-tetrasomic lines of the hexaploid wheat cv. Chinese spring were used in PCR experiments to assign genes to genomes. All materials used came from our own plant stocks.

DNA and RNA purification

Genomic DNA was purified following standard protocols. RNA was isolated from pollen mother cells as previously described (De Bustos et al. 2007). Briefly, RNA was extracted from the anthers of immature spikelets at meiosis (confirmed microscopically) using the morphological development of the flower organs as an indicator of meiocyte development. Total RNA was purified using Tripure reagent (Roche) following the manufacturer's recommendations. The Turbo DNA-free Kit (Ambion) was used to eliminate contaminating DNA, and the RNA cleaned using the Megaclear kit (Ambion). RNA integrity was confirmed by gel electrophoresis; the concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Cloning of *Rad50* genes

Genomic sequence

Homoeologous *Rad50* genes were amplified by PCR using a set of eight pairs of primers (Online Resource 1). PCR reactions were performed in a T3 thermocycler (Biometra) in 50 µl volumes containing 100–200 ng of genomic DNA, 0.2 µM of each primer, 250 µM of each dNTP, 1 µl of *PfuUltra* II fusion HS DNA polymerase (Stratagene) and 1X *PfuUltra* II fusion HS DNA polymerase buffer. The reaction conditions consisted of one cycle of 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 50–60°C (the annealing temperature was adjusted for each pair of primers), 1 min at 72°C, and a final extension cycle of 5 min at 72°C. The amplified fragments were agarose purified and cloned into the BlueScript plasmid (Stratagene) following standard protocols. Sequencing of the clones was carried out in an ABI Prism 377 sequencer (Applied Biosystems) using designed primers (Online Resource 1). The analysis of the DNA sequences obtained was performed using the Sequence Navigator program (Applied Biosystem).

RNA sequence

The synthesis of first strand cDNA was performed using 1 μg of DNA-free RNA with the Transcriptor One-Step RT-PCR kit (Roche) using Oligo $(dT)_{15}$ as the primer (Roche), following the protocol provided by the manufacturer. *Rad50* cDNA sequences were obtained by PCR using 2 μl of first strand cDNA and the RadF8L and Rad F1R primers (Online Resource 1). The PCR reaction, cloning and sequence procedures were as indicated above. RACE experiments were conducted to obtain the complete cDNA sequences; the RACE kit (Roche) was used for this purpose, employing the designed primers Rad50SP1b, Rad50SP2b, Rad50SP3b and Rad50SP5 (Online Resource 1), following the manufacturer's recommendations.

Sequence alignment

DNA and deduced protein sequences were aligned using the Clustal W 1.5 program (Thompson et al. 1994). Phylogenetic trees were constructed as previously described (De Bustos et al. 2007).

Southern blot assay

Rad50 copy numbers were determined by digesting 20 μg of genomic DNA from the diploid, tetraploid and hexaploid wheat species. DNA digested with the *BamH*I and *Apa*I restriction endonucleases (which cut at only one place in the genomic sequence of these genes) was subjected to electrophoresis in a 1% agarose gel. Fragments were blotted onto nylon membranes (Roche). Primers RadSth3L2 and RadSth3R2 (Online Resource 1) were used to amplify a fragment of 448 bp (labelled with digoxigenin [Roche]) from the *Rad50* gene to provide the required probe. Hybridisation and detection were carried out as previously described (De Bustos et al. 2007).

Assignation of homoeologous genes to wheat genomes

The assignment of cloned genes to their genomes was performed via PCR using primers Rad50difL2 and Rad50difR2 (Online Resource 1) in a T3 thermocycler (Biometra). PCR reactions (volume 20 μl) were performed using 200 ng of genomic DNA from the diploid, tetraploid and hexaploid species of wheat and from the nulli-tetrasomic lines. All reactions involved 0.5 μM of primers, 100 μM of each dNTP and 2U Taq Polymerase in 1X buffer (Sigma). The thermocycler was programmed for a cycle of 5 min at 95°C followed for 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final extension step at 72°C for 5 min. 4 μl samples of the reaction mixture were then loaded onto an acrylamide gel (7%) and subjected to electrophoresis following standard procedures.

Protein interaction experiments

Interaction analysis was performed using the Matchmaker LexA Two-hybrid System (Clontech). Full-length cDNAs of the *Rad50* and *Mre11* homoeologous genes were cloned into the plasmids pLexA and pB42AD to create DNA binding and activation domain fusion proteins respectively. *Rad50* cDNA amplified with RadTHL/RadTHR2 primers (Online Resource 1) was digested with *EcoR*I endonuclease and cloned into both plasmids. Primers MreTH-L/MreTH-R (Online Resource 1) were used to amplify *Mre11* cDNA. The products obtained were cut with *BamH*I and *Not*I endonucleases and cloned into the LexA vector. The LexA clones were then digested with *EcoR*I and *Xho*I and the fragments cloned into pB42AD.

Interactions between proteins were assayed in a *Saccharomyces cerevisiae* EGY48 (p8op-lacZ) host strain in both directions (experiments performed in triplicate). The detection of interactions was carried out in two assays using the reporter genes LEU2 and LacZ provided by the manufacturer. Toxicity and self-activation assays were also performed involving negative and positive controls, as recommended by the manufacturer.

Expression analyses

Quantitative PCR (QPCR) experiments were conducted to examine the expression of the *Rad50* genes of all the examined wheat species using the Universal Probe-Library (UPL) System (Roche). The primers and probes used were selected according to the recommendations of the Universal ProbeLibrary Assay Design Center (Roche). For the homologous *Rad50* genes, the primer pair QRadL4/QRadR4 and the probe UPL #25 were used (Online Resource 1). As endogenous controls, wheat cell division control protein (bank accession EU267938) and a gene similar to the RNase L inhibitor-like protein (bank accession AY059462) were used, following the recommendations of Paolacci et al. (2009). For these controls, the primers and probes used were CDCPL/CDCPR, UPL #88 and RNILPL/RNILPR, UPL #39 respectively (Online Resource 1). All experiments were performed using the 7500 Fast Real Time PCR System (Applied Biosystem). Reactions were conducted in 20 μl volumes including 2 μl of cDNA obtained as described above (about 100 ng of input RNA), 10 μl of *Premix Ex Taq* (Takara), 0.4 μl of ROX Refence Dye II (Takara), 0.2 μM of each primer, and 0.1 μM of UPL probe. The thermocycler protocol consisted of one cycle of 30 s at 95°C, 40 cycles of 5 s at 95°C, and 34 s at 60°C. In each PCR reaction, samples were analysed

Table 1 Characterisation of the *Rad50* homoeologous genes

Rad50 genes (species)	gDNA (databank accession)	mRNA (databank accession)	Coding sequence	5′UTR	3′UTR	Exon–intron	Deduced protein
Rad50A (*T. monococcum*)	17,006 (EU159421)	4,508 (EU159417)	3,948	277	283	27–26	1,316
Rad50A (*T. turgidum*)	16,093 (EU159423)	4,466 (EU159419)	3,948	256	262	27–26	1,316
Rad50B (*T. turgidum*)	16,942 (EU159424)	4,473 (EU159420)	3,948	291	234	27–26	1,316
Rad50D (*Ae. tauchii*)	16,470 (EU159422)	4,387 (EU159418)	3,948	247	192	27–26	1,316

The length of the gDNA and mRNA sequences and the size of the coding region and 5-3 UTR are indicated. The number of exons, introns and amino acids for the deduced protein are shown.

in duplicate; all experiments were performed in triplicate. Prior to performing the QPCR reactions, the specificity of the primers was analysed by acrylamide electrophoresis (7%). The amplification efficiency of the primers for *Rad50* and those for the controls was assessed as described by Livak and Schmittgen (2001). The efficiency (E) was calculated from the equation $E = (10^{[-1/m]}–1) \times 100$, where m is the slope of linear regression (Paolacci et al. 2009). The results of the QPCR experiments were analysed using 7500 Fast System Software (Applied Biosystem). The relative changes in the expression of *Rad50* were calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001) employing the geometric mean of both endogenous controls as described by Vandesompele et al. (2002).

The expression of the *Rad50* homoeologous genes in the polyploid species of wheat was also examined by quantitative QPCR according to Nomura et al. (2005) using the Universal Probe-Library System (Roche). For each homoeologous gene, a specific combination of primers and probe was designed. In the case of *Rad50* from the A genome, the primers QRadAL2/QRadAR and the probe UPL #159 were used. For *Rad50* from the B and D genomes the same probe, UPL #118, was used. The primers QRadBL3/QRadBR4 specifically amplified the *Rad50B* gene and the primers QRadDL/QRadDR specifically amplified *Rad50D* (Online Resource 1). In all cases, plasmid DNA of each gene was used as a control. The cDNA used, the reaction conditions, the endogenous genes employed, and analyses undertaken were those indicated above.

Results

Molecular characterization

The genomic DNA and mRNA sequences of the *Rad50* genes were characterized by PCR in the diploid species *Triticum monococcum* L. (genome A) and *Aegilops tauschii* Coss. (genome D), and the tetraploid species *Triticum turgidum* L. cv. Vitron (genomes A and B). For this purpose, information available in sequence databases was used. Thus, cDNA *Rad50* sequences of *Arabidopsis thaliana* and rice were aligned with EST sequences of wheat, barley and rye, and primers were designed based on the most conserved regions (Online Resource 2a). Due to the large size of the *Rad50* gene and the difficulty in designing primers, the genomic sequence was amplified in eight fragments (Online Resource 2b). Each fragment was cloned and sequenced (2–3 clones/fragment) and the derived sequences were assembled in a consensus sequence of the whole gene. No variation was observed within sequences from the diploid species while two types of sequences were obtained for the tetraploid species that were assigned to the two different homoeologous genomes (see below).The mRNA sequences were obtained by PCR using the total RNA extracted for each species and employing primers designed from the extremes of the gDNA (see "Materials and methods"); this produced a single amplified fragment. The complete mRNA sequence was obtained using the 5′-3′ RACE technique. Table 1 shows the results obtained for each of the genes characterised.

All of the genes characterised showed a coding region of the same size, 3,951 bp distributed in 27 exons, ranging in length from 54 to 322 bp (data not shown), coding for a putative protein of 1,316 amino acids. No deletion or insertion differences were seen between the genes; indeed, only a few base substitutions were detected when sequences were compared, ranging from 13 SNPs in *Rad50D* to 26 in *Rad50A* of *T. turgidum*, resulting in 98–99% similarity between proteins (see below). The differences in the size of the gDNA sequences were mainly due to variation in insertions and deletions in the introns, accompanied by base substitutions. For example, an insertion of 485 bp was found in the 18th intron of *T. monococcum* and a deletion of 296 bp was detected in the 22nd intron of the *Rad50A* of *T. turgidum*. On average, deletions were more abundant, ranging from 12 in *Rad50A* of *T. turgidum* to 17 in *Rad50A* of *T. monococcum*. The number of insertions ranged from 2 in *Rad50A* of *T. monococcum* to 7 in *Rad50A* of *T. turgidum*. Overall, great differences were seen in the size of the introns for each gene, ranging from 54 to 4,094 bp (data not shown).

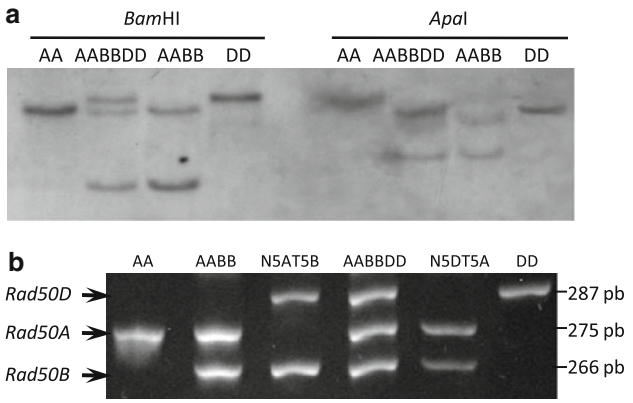

Fig. 1 **a** Assessment of the *Rad50* copy number by Southern blot analysis. Genomic DNA of *T. monococcum* (AA), *Ae. tauschii* (DD), *T. turgidum* (AABB) and *T. aestivum* (AABBDD) were digested with *BamH*I and *Apa*I and hybridised with a *Rad50* probe. **b** Assignment of cloned sequences to wheat genomes was carried out by PCR in the above mentioned wheat species using the nulli 5A-tetra 5B and nulli 5D-tetra 5A lines of wheat

The *Rad50* copy number was assessed by Southern blotting. The hybridisation of digested gDNA from the wheat species with a *RAd50* probe revealed wheat *Rad50* to be a single gene (Fig 1a). This was deduced from the fact that the number of bands observed was related to the level of ploidy of the species analysed. The nulli-tetrasomic lines of wheat confirmed the assignment of the cloned sequences to their wheat genomes. Using FISH we previously defined the chromosomal location of the *Rad50* gene (Pérez et al. 2009) as being on the short arm of chromosome 5 in wheat. Based on this result, two nulli-tetrasomic lines of chromosome 5 were used in PCR analysis. Primers Rad50 difL2/Rad50 difR2 were designed based on a region of the 13th intron of the gene that amplified three products in hexaploid wheat (Fig. 1b). Amplifications of the gDNA of the diploid and tetraploid species, along with that of the nulli-tetrasomic lines, allowed the assignation of the genes to the wheat genomes.

To determine the level of similarity between the sequences characterized, and to compare them with previously characterized sequences, the deduced proteins were aligned with those of rice, *A. thaliana*, Man and yeast (Online Resource 3). Overall, the wheat RAD50 proteins showed a high degree of conservation, with a maximum similarity of 99% between RAD50A (from both *T. monococcum* and *T. turgidum*) and RAD50D. The RAD50B protein showed a slight divergence with a similarity of 98% to the other wheat proteins. The RAD50 proteins of the above plant species included in the analysis were all of the same size (1,316 residues) and showed an appreciable degree of conservation, mainly in the characteristic domains and motifs. Thus, the RAD50 proteins of wheat

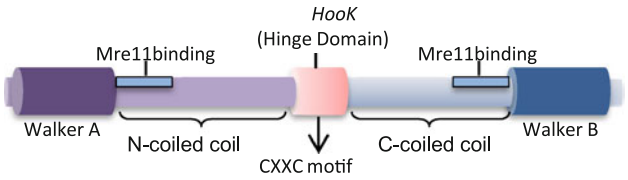

Fig. 2 Schematic representation of the main motifs and domains found in RAD50 proteins

and rice showed a similarity of 80%, descending to 64% in the case of the RAD50 protein of *A. thaliana*. Lower homology was detected with human and yeast proteins (30 and 25% respectively).

The alignment performed also permitted the identification of motifs and domains previously characterized in other RAD50 proteins (Fig 2). Those of the wheat proteins showed the most important characteristics of this class of protein (Online Resource 3), including the Walker A and B motifs, the signature motif and the P and D loops described by Hopfner et al. (2000), the hinge domain (Anderson et al. 2001), including the CXXC motif (Hopfner et al. 2002), and the Q loop (Moncalian et al. 2004). The boundaries of the N and C globular domains and the coiled coil were identified in agreement with De Jager et al. (2004).

Protein interaction analysis

Yeast two-hybrid analysis was used to investigate interactions implying the products of the wheat genes *Rad50* and *Mre11*. The main goal was to analyse how the interactions between homoeologous genes are regulated, assessing whether the interaction of each gene is restricted to the partner belonging to the same genome, or whether interactions between genes from different genomes occur. The dimerization of both proteins was also studied in order to analyse the structure of the MR complex in wheat.

The homoeologous *Rad50* genes characterized in this study and the previously characterized *Mre11* genes (De Bustos et al. 2007) were cloned into both the pLexA and pB42AD plasmids (DNA binding and activation vectors respectively) to perform the interaction analysis in both directions. Triplicate experiments were conducted for each interaction. Prior to the interaction experiments, toxicity analysis and self-activation assays were performed for each of the 16 hybrid proteins obtained. None resulted toxic to the yeast strain used nor produced any activation of the reporter genes. Interactions were detected using two different methods: a nutritional assay where the reporter gene LEU2 allows the yeast strain to grow when interaction occurs, and the β-galactosidase assay where activity of this enzyme is monitored after the expression of the lacZ reporter gene.

Table 2 Interactions between the homoeologous RAD50 and MRE11 proteins

	RAD50-A_m	*RAD50-A_t*	*RAD50-B*	*RAD50-D*	*MRE11-A_m*	*MRE11-A_t*	*MRE11-B*	*MRE11-D*
MRE11-A_m	+	+	+	+	+	+	+	+
MRE11-A_t	+	+	+	+	+	+	+	+
MRE11-B	+	+	+	+	+	+	+	+
MRE11-D	+	+	+	+	+	+	+	+
RAD50-A_m	−	−	−	−	+	+	+	+
RAD50-A_t	−	−	−	−	+	+	+	+
RAD50-B	−	−	−	−	+	+	+	+
RAD50-D	−	−	−	−	+	+	+	+

The homoeologous A genes of *T. monococcum* and *T. turgidum* are shown as A_m and A_t respectively. Interaction is scored as +; no interaction is scored as −

The study performed in yeast demonstrated that the products of the *Rad50* genes are able to interact with those of the *Mre11* independent of the coding genome (Table 2, Online Resource 4) suggesting that these proteins also could interact in wheat. Each of the RAD50 proteins was able to bind to any product of the *Mre11* genes. Strong signals were detected in all cases, indicating great affinity between the products of the *Rad50* and *Mre11* genes (data not shown). In the analysis of dimer formation, only interaction between the MRE11 proteins was detected. No dimerization was observed when RAD50 proteins were assessed. The dimerization of MRE11 proteins coded for by homoeologous genes from different genome was also observed.

Expression studies

The expression of the *Rad50* genes was analysed by quantitative PCR in all four wheat species examined in this study. To avoid differences in developmental stage, RNA was isolated from several plants of each species. Microscopic checking confirmed that all microspores were at the same stage of meiosis. In a first step, three of the endogenous controls suggested by Paolacci et al. (2009) were tested for the validation of reference genes in wheat quantitative PCR analyses. Several primer combinations and probes were assayed for the ADP-ribosylation factor (ADP RF), the cell division control protein (CDCP) and the RNase L inhibitor-like protein (RNI), along with the *Rad50* gene. Of the endogenous controls, CDCP and RNI showed the best results in the amplification efficiency assays (Online Resource 5); these were selected as housekeeping genes for the study.

Figure 3 shows the results of the expression analysis. The expression of the *Rad50* gene of the diploid *Ae. tauschii* was the lowest of all the wheat species examined, and was therefore used as a calibrator in the analysis. The *Rad50* gene of the hexaploid species *T. aestivum* showed the highest level of expression, with a fold change of 2.7 compared to *Ae. tauschii*. The other diploid species, *T. monococcum*, showed a slightly higher level of expression with respect to *Ae. tauschii* (1.3 fold change) whereas the *Rad50* expression of the tetraploid *T. turgidum* was higher, reaching 1.53 times the expression of the calibrator.

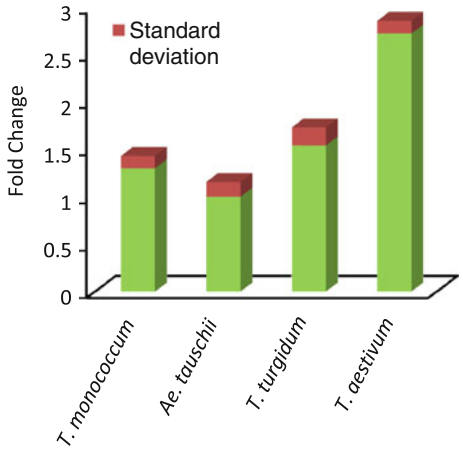

Fig. 3 Fold change in *Rad50* gene expression in the four wheat species analysed, calculated as indicated in "Materials and methods" using both RNI and CDCP endogenous control genes. *Dark box* indicate the standard deviation (three replicate)

The relative expression of each of the three homoeologous *Rad50* genes of wheat was analysed in the two polyploid species. Gene-specific QPCR was performed according to Shitsukawa et al. (2007), designing a set of specific primers and probes for each homoeologous *Rad50*. The specificity of amplification was confirmed using plasmids containing cloned cDNA sequences of each homoeologous gene. The accuracy of the technique was assessed using genomic DNA from each species, for which a similar level of amplification of the homoeologues should be expected (Adams et al. 2003). The results obtained with the cDNA showed different expression of the homoeologous genes (Fig 4) in both polyploid species. The *Rad50B* gene showed higher level of expression (72%) than the *Rad50A* gene (27%) in the tetraploid species *T. turgidum*. In the hexaploid *T. aestivum*, the *Rad50B* gene

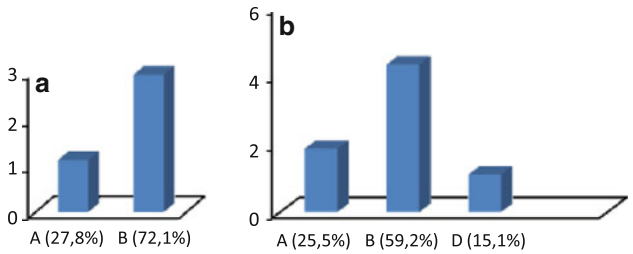

Fig. 4 Percentage expression of each *Rad50* homoeologous gene in the tetraploid *T. turgidum* (**a**) and in the hexaploid *T. aestivum* (**b**)

again showed higher expression (60%) with respect to the *Rad50A* and *Rad50D* genes that account for the 25 and 15% of the total expression.

To compare the level of expression of *Rad50* to that of its counterpart *Mre11* in the MR complex, the expression of both genes was analysed for each of the wheat species (Fig 5). Comparisons showed a similar level of expression of both genes in *T. monococcum* and *T. turgidum.* However *T. aestivum*, and especially *Ae. Tauschii*, showed differences in expression.

Discussion

Molecular characterization

Comparative genomic approaches are frequently used to isolate genes in species for which only partial sequence information regarding their genomes is available. This is the case of wheat, a hexaploid species with a very large genome (Paolacci et al. 2009) but for which the structural and functional analysis of the transcriptome is particularly important for economic reasons. The isolation and characterization of wheat genes is therefore usually performed via comparative genomics, using the information of conserved domains of sequenced plant genomes and EST databases (Johnson and Bhave 2004; Shitsukawa et al. 2007). Our group has used this type of methodology widely to characterize new HMW glutenin genes of *T. aestivum* (De Bustos et al. 2000) and related *Secale* and *Aegilops* species (De Bustos et al. 2001; De Bustos and Jouve 2003, 2006).

Although the recombination machinery is highly conserved between organisms (Hartung and Puchta 2004), the components of the MRN complex do not show the same level of conservation. Thus, whereas homologous *Mre11* and *Rad50* genes have been found in all taxonomic groups examined, conserving high sequence homology (D'Amours and Jackson 2002), the third component *Nbs1* (*Xrs* in yeast) is only present in eukaryotes and the genes characterized do not show sequence conservation. In previous work by our group (De Bustos et al. 2007), *Mre11* homologous wheat genes were characterized using a comparative genomic approach given the high level of conservation found in *Arabidopsis* and rice genes. A similar strategy was used to characterize the *Rad50* genome counterparts of *Mre11* in the present work. Information regarding these model species and the EST sequences of cereal crops was also used. The characterization of genes in polyploid species is usually impeded by their great similarity, and their donor diploid species are usually used in such work (Johnson and Bhave 2004). In this work the genes were isolated from the diploid species carrying the A and D genomes, as well as from the tetraploid species (AABB) since the donor diploid species of the B genome has not yet been identified (Huang et al. 2002). Although *T. urartu* (A^u) has been proposed the donor species of the A genome, *T. monococcum* (A^m) from our own stock collection was used since it has been successfully employed as a model for the A genome of hexaploid wheat (Stein et al. 2000), confirming the close relationship between the A^u and the A^m genomes. The diploid species *Ae. tauschii*, considered the donor of the D genome, was used for the characterization of this genome.

In wheat species, *Rad50* is found as a single copy gene, as confirmed by Southern experiments, in agreement with that found in other species including *A. thaliana* (Gallego et al. 2001). Recently we have reported the localization of *Rad50* wheat genes, using a FISH protocol employing tyramide for signal amplification (Tyr-FISH), on the short arm of the chromosomes of homologous group five (Pérez et al. 2009). This experimental approach was also used to localize the *Mre11* genes to homologous group two (data not published). Nulli-tetrasomic lines of wheat for the chromosomes of homologous group five were used to assign

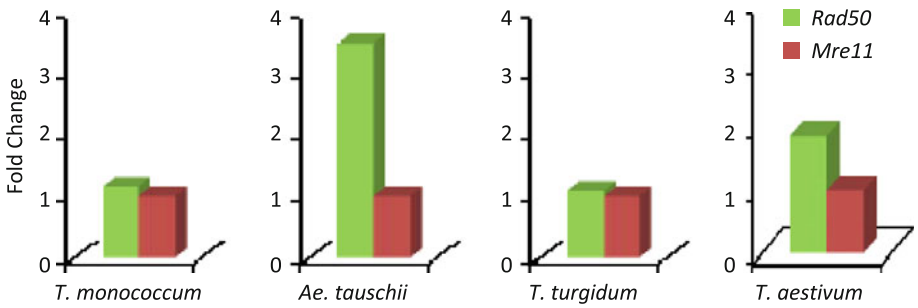

Fig. 5 Comparison of *Rad50* and *Mre11* expression levels in the wheat species. The analysis was carried out for each species separately. In all cases the expression of *Mre11* was used as the calibrator

cloned sequences of the *Rad50* genes to their genomes, and confirmed the initial results in which the sequences isolated from diploid species were assumed to belong to the A and D genomes. In addition, the B sequence characterized in tetraploid species was clearly assigned to the B genome. This kind of approach has been widely used in the identification of wheat sequences (Wicker et al. 2003; Bottley et al. 2006; Bottley and Koebner 2008; Chalupska et al. 2008).

The *Rad50* genes characterized showed a high level of sequence homology and coded for proteins with a similarity of around 99–98%. Similar results were found in the study of the *Mre11 genes* (De Bustos et al. 2007). In both cases RAD50B was the least related protein, reflecting its greater evolutionary distance (Quian Gu et al. 2004) and suggesting that the B genome diverged from the common ancestor before the separation of the A and D genomes. It has been established that genes with important functions are more conserved (Ciaffi et al. 2006). The present results clearly support this since almost no variation was found between the proteins belonging to the same genome or between genomes, despite the divergence of the diploid species having occurred 2–4 My ago and the divergence of the tetraploid species having taken place some 0.5 My ago.

Most of the functions of the MRN complex are influenced by its structure and intricate architectural arrangement (Kinoshita et al. 2009). It has been established that the core of the MRN complex is a heterotetramer formed by the aggregation of the MRE11 and RAD50 (MR complex) proteins (de Jager et al. 2001; Hopfner et al. 2001). The RAD50 proteins of wheat showed similar characteristics to those previously characterized in other species, as also shown for their counterpart MRE11 proteins (De Bustos et al. 2007). This indicates that all the components necessary for the formation of the 'MR complex' are present in wheat. The complex has the capacity to bind to DNA via the globular domain formed by the Walker A and B motifs of each RAD50 monomer together with an MRE11 dimer in such a manner that the DNA binding sites of the latter lie close to the two RAD50 ATPase domains (For a deeper explanation see online resource 6).In this tetrameric structure the RAD50 proteins are held together only by their interaction with the MRE11 dimer, which is stable in itself (Hopfner et al. 2001). The present interaction analysis results showed that the RAD50 wheat fusion proteins used in the yeast study cannot form dimers themselves, but only with MRE11 proteins. Dimers formed between the MRE11 wheat proteins alone, however, were found to exist.

Expression analysis

One of the most used techniques in the analysis of gene expression is quantitative QPCR. However, accurate quantification using this technique requires the standardization of several critical variables, including the RNA and cDNA quality, the efficiency of primer amplification, and the selection of an appropriate endogenous control. In this study, the quantification of the expression of each gene was undertaken using a pair of specific primers plus a specific probe composed of locked nucleic acid (LNA), a high affinity DNA analogue that allows experiments to be performed under highly stringent annealing temperatures (60°C), ensuring adequate duplex stability and high specificity (You et al. 2006). The strict recommendations suggested by Paolacci et al. (2009) were also followed. For each gene, pairs of primers were designed; only those whose efficiency and R^2 values were close to 100% and 1 respectively were used.

The choice of the endogenous control for normalization is an important step in QPCR analysis since gene expression results can change depending on the control used. The use of more than one endogenous control was considered in order to improve the accuracy and reliability of the expression analysis (Paolacci et al. 2009). Following the indications of the latter authors, initial tests were made using three of the most stable reference genes (ADP-ribosylation factor, cell division control protein, and RNase L inhibitor-like protein), the latter two finally being selected for use. The results obtained correlated with the ploidy of the wheat species, showing the diploid species to have a low expression of *Rad50*, followed in ascending order by the tetraploid and the hexaploid species (Fig 3). It should be remembered, however, that our work was not designed to relate the expression of *Rad50* genes to the ploidy level of the wheat species analysed. The *Rad50* expression of the four wheat species was similar to that observed for the *Mre11* gene (De Bustos et al. 2007). In both studies, the species *Ae. tauschii* and *T. aestivum* showed the lowest and highest expression values respectively. However, a slight difference was observed in the other two species: *Mre11* expression was higher in *T. monococcum* than in *T. turgidum*, whereas the expression of *Rad50* in *T. turgidum* was slightly higher than in *T. monococcum.* Both results are equally possible since, although the expression of most genes increases with ploidy, some genes show an inverse relationship with ploidy or an unexpected deviation (Comai 2005). In the present case, higher expression in polyploid species ought to be expected given the role of the MRN complex. It has been proposed that the expression of genes required for the repair of DNA damage by homologous recombination is higher in polyploid than diploid species (Storchova et al. 2006). The latter authors related the increased requirement of homologous recombination genes in polyploids to the increase in spontaneous DNA damage associated with DNA replication involving extra chromosome sets.

It is well documented that the components of the complex exist in equimolar amounts (Trujillo and Sung 2001;

Anderson et al. 2001; De Jager et al. 2002). We therefore compared the levels of expression of the *Rad50* and *Mre11* genes for each wheat species. The expectedly similar level of expression of these genes was observed in the species *T. monococcum* and *T. turgidum*. However, the values observed in *Ae. tauschii* and in *T. aestivum* were different, indicating the absence of a relationship at the level of transcripts in these species. Post-transcriptional modifications could act to balance the level of proteins present in the cell.

The expression of *Rad50* homoeologues was also assessed in the two polyploid species *T. turgidum* and *T. aestivum*. In both cases an overexpression of *Rad50B* was detected. In the tetraploid the expression of *Rad50B* reached 2.6 times the level observed for its homoeologous gene *Rad50A*, whereas in the hexaploid the expression of *Rad50B* was 2.3 and 3.9 times higher than that of *Rad50A* and *Rad50D* respectively. The biased expression of the counterpart homoeologous *Mre11* genes was also seen in earlier work (De Bustos et al. 2007), but in the opposite direction. Thus, the *Mre11B* homoeologue was always underexpressed in both polyploid species with respect to the other two *Mre11* homoeologues. Expression divergence between duplicate genes has long been a subject of great interest since it is an important step in the emergence of new gene functions (Li et al. 2005). Several possible fates for duplicated genes have been proposed (for a review see Lawton-Rauh 2003). The most common fate for genes that have been recently duplicated by polyploidization, as is the case in wheat, is gene maintenance (Cronn et al. 1999 and references therein), s. However, other duplicated genes can accumulate mutations such that both genes are required for the original function (subfunctionalization, see e.g., Hartung et al. 2007). Another possible fate of duplicated genes is neofunctionalization, where one copy retains the original function and the second accumulates mutations leading to a gain of novel function. Finally, some duplicated copies may lose their function due to the accumulation of mutations and the formation of pseudogenes. The *Rad50* homoeologous genes investigated here, and the previously analysed *Mre11* homoeologues (De Bustos et al. 2007), are expressed in both polyploid species and retain the capability of interaction between themselves, indicating the maintenance of function. These results contradict the idea that duplicated genes involved in DNA repair are preferentially lost (Blanc and Wolfe 2004), and support the idea that genes that code for the subunits of protein complexes, as do *Rad50* and *Mre11*, maintain their original functions, thus avoiding changes that could lead to a nonfunctional complex (Adams and Wendel 2005b). However, in both cases the biased expression of homoeologues was detected. In the active, ongoing process of polyploidization in wheat, this might lead to the silencing of one copy. This has been well documented in several polyploid plant species, in which variable expression levels and silencing have been detected in different organs and tissues (Adams et al. 2003; Adams 2008; Buggs et al. 2010), leading to the subfunctionalization of the copies. In wheat, the expression of these genes in more organs and tissues is required to determine whether a similar process is underway.

Although no studies have provided evidence to support the hypothesis that biased expression and silencing is directed towards one wheat genome (Bottley and Koebner 2008), different expressions of *Rad50B* and *Mre11B* were seen with respect to the other homoeologues. A similar tendency has been reported for other wheat genes (Ciaffi et al. 2001; Nemoto et al. 2003; Nomura et al. 2005; Kawaura et al. 2005; Shitsukawa et al. 2007; Bottley and Koebner 2008). This might be related to the idea of the extent of the changes in expression depending on the genetic distance between the parental genomes (Otto 2007). In this regard, it has been established that the B genome diverged from the common ancestor before the A and D genomes (Quian Gu et al. 2004), explaining its greater genetic diversity (Wendel 2000). The changes observed in the expression of *Rad50* and *Mre11* cannot be attributed to mutations accumulated during the process of polyploidization, as demonstrated by the high homology (98–99%) detected between the homoeologues of both genes, at least in the coding region (De Bustos et al. 2007; this work). Further, the recent origin of hexaploid wheat around 10,000 year ago (Huang et al. 2002) suggests there has been insufficient time to accumulate a significant quantity of point mutations (Hegarty and Hiscock 2008). Although the conservation of the biased expression pattern in the tetraploid *T. turgidum* and hexaploid *T. aestivum* might indicate a stable epigenetic alteration of the regulatory expression elements of both genes. It is necessary to bear in mind that the changes observed in the non-coding regions of the genes could also affect expression levels and messenger stability.

Interaction analysis

Since the characterization of the MR genes, a number studies have examined the physical interaction between their components (Johzuka and Ogawa 1995; Dolganov et al. 1996; Petrini et al. 1997; Anderson et al. 2001). However, to our knowledge, no analysis of interaction between the proteins encoded by the *Mre11* and *Rad50* genes has been undertaken in polyploid species. We believe the present work is the first to examine the interactions of the homoeologues of both genes. One of the aims of this study was to determine how homoeologous genes interact with their counterparts, thus showing whether the protein binding required to form the MRN complex is restricted to the products of genes carried by the same genome or

whether proteins encoded by different genes, independent of the genome to which they belong, are involved. The results obtained indicate the inexistence of specificity in the protein interactions; all possible combinations between the proteins coded for by the different genomes of wheat were detected. Similar results have been also observed in wheat class E MADS box genes (Shitsukawa et al. 2007). From an evolutionary point of view this could represent an advantage since, if one of the homoeologues is inactivated by mutations, transposable elements, or for any other reason (for details see Adams 2008), the formation of this important complex would be guaranteed by the interaction between the duplicated products of the homoeologous genes. Although the regulatory region has not been analysed, it is unlikely to influence the direction of interactions since duplicate genes are likely to share *cis*-regulatory motifs (Li et al. 2005). Also, if interaction between genes were constrained to counterparts belonging to the same genome, a coordinated level of expression between them would be expected. This was not observed for *Rad50B* and *Mre11B*, the levels of expression of which were opposing, supporting the idea that no specificity of interactions operates in MRN wheat genes.

The first analysis of the function of the MRN complex in a polyploid species, the archaeon *Haloferax volcanii*, has recently been reported (Delmas et al. 2009). The results obtained showed great differences in the function of this complex with respect to that observed in diploid species. *mre11* and *rad50* mutants were more resistant to DNA damage than the wild-type, but wild-type cells recovered faster from DNA damage. According to the latter authors, the MRN complex would prevent the repair of DSBs by homologous recombination (HR), directing repair via the non-homologous end joining (NHEJ) pathway. This change in the type of repair in *H. volcanii* might be related to the difficulty of repair via HR, a consequence of the multiple partners presents in a polyploid species for each DNA end originated at the break. These surprising findings indicate a novel role for the MRN complex, at least in *Archaea*. It would be interesting to determine whether the same is true in eukaryotic polyploid organisms such as wheat.

Acknowledgments This study was funded by grants from the Spanish Ministry of Science and Innovation (AGL2006-090018-c02 and AGL 2009-10373). The authors thank Adrian Burton for linguistic assistance.

References

Acharya SN, Many AM, Schroeder AP, Kennedy FM, Savytskyy OP, Grubb JT, Vincent JA, Friedle EA, Celerin M, Maillet DS, Palmerini JH, Greischar MA, Moncalian G, Williams RS, Tainer JA, Zolan ME (2008) Coprinus cinereus rad50 mutants reveal an essential structural role for Rad50 in axial element and synaptonemal complex formation, homolog pairing and meiotic recombination. Genetics 180:1889–1907

Adams KL (2008) Insights into the evolution of duplicate gene expression in polyploids from Gossypium. Botany 86:827–834

Adams KL, Wendel JF (2005a) Novel patterns of gene expression in polyploid plants. Trends Genet 21(10):539–543

Adams KL, Wendel JF (2005b) Polyploidy and genome evolution in plants. Curr Opin Plant Biol 8:135–141

Adams KL, Cronn R, PerciWeld R, Wendel JF (2003) Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-speciWc reciprocal silencing. Proc Natl Acad Sci USA 100:4649–4654

Anderson DE, Trujillo KM, Sung P, Erickson HP (2001) Structure of the Rad50_Mre11 DNA repair complex from *Saccharomyces cerevisiae* by electron microscopy. J Biol Chem 276(40):37027–37033

Assenmacher N, Hopfner KP (2004) MRE11/RAD50/NBS1: complex activities. Chromosoma 113:157–166

Berkovich E, Monnat RJ, Kastan MB (2007) Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. Nat Cell Biol 9(6):683–690

Blanc G, Wolfe KH (2004) Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. Plant Cell 16:1679–1691

Bleuyard JY, Gallego ME, White CI (2004) Meiotic defects in the Arabidopsis rad50 mutant point to conservation of the MRX complex function in early stages of meiotic recombination. Chromosoma 113:197–203

Borde V, Cobb J (2009) Double functions for the Mre11 complex during DNA double-strand break repair and replication. Int J of Biochem Cell Biol 41:1249–1253

Bottley A, Koebner RMD (2008) Variation for homoeologous gene silencing in hexaploid wheat. Plant J 56:297–302

Bottley A, Xia GM, Koebner RMD (2006) Homoeologous gene silencing in hexaploid wheat. Plant J 47:897–906

Buggs RJA, Elliott NM, Zhang LZ, Koh J, Viccin LF, Soltis DE, Soltis PS (2010) Tissue-specific silencing of homoeologs in natural populations of the recent allopolyploid *Tragopogon mirus*. New Phytol 1(1):175–183

Chalupska D, Lee HY, Faris JD, Evrard A, Chalhoub B, Haselkorn R, Gornicki P (2008) Acc homoeoloci and the evolution of wheat genomes. Proc Natl Acad Sci USA 105(28):9691–9696

Ciaffi M, Paolacci AR, Dominici L, Tanzarella OA, Porceddu E (2001) Molecular characterization of gene sequences coding for protein disulfide isomerase (PDI) in durum wheat (*Triticum turgidum* ssp. durum). Gene 265:147–156

Ciaffi M, Paolacci AR, D'Aloisio E, Tanzarella OA, Porceddu E (2006) Cloning and characterization of wheat PDI (protein disulfide isomerase) homoeologous genes and promoter sequences. Gene 366:209–218

Comai L (2005) The advantages and disadvantages of being polyploid. Nat Rev Genet 6:836–846

Cronn RC, Small RL, Wendel JF (1999) Duplicated genes evolve independently after polyploid formation in cotton. Proc Natl Acad Sci USA 96:14406–14411

D'Amours D, Jackson SP (2002) The Mre11 complex: at the crossroads of DNA repair and checkpoint signalling. Nat Rev Mol Cell Biol 3:317–327

Daoudal-Cotterell S, Gallego ME, White CI (2002) The plant Rad50-Mre11 protein complex. FEBS Lett 516:164–166

De Bustos A, Jouve N (2003) Characterization and analysis of new HMW-glutenin alleles encoded by *Glu-R1* locus of *Secale cereale*. Theor Appl Genet 107:74–83

De Bustos A, Jouve N (2006) Characterisation and phylogenetic analysis of the genes coding for high molecular weight glutenin

subunits in three diploid species of *Aegilops*. Int J Plant Sci 167(2):359–366

De Bustos A, Rubio P, Jouve N (2000) Molecular characterization of the inactive allele of the gene Glu-A1 and the development of a set of AS-PCR markers for HMW glutenin subunits of wheat. Theor Appl Genet 100:1085–1094

De Bustos A, Rubio P, Jouve N (2001) Characterisation of two gene subunits on the 1R chromosome of rye as orthologs of each the *Glu*-1 genes of hexaploid wheat. Theor Appl Genet 103:733–742

De Bustos A, Pérez R, Jouve N (2007) Characterization of the gene *Mre11* and evidence of silencing after polyploidization in *Triticum*. Theor Appl Genet 114:985–999

De Jager M, van Noort J, van Gent DC, Dekker C, Kanaar R, Wyman C (2001) Human Rad50/Mre11 is a flexible complex that can tether DNA ends. Mol Cell 8:1129–1135

De Jager M, Wyman C, van Gent DC, Kanaar R (2002) DNA end-binding specificity of human Rad50/Mre11 is influenced by ATP. Nucleic Acids Res 30(20):4425–4431

De Jager M, Trujillo KM, Sung P, Hopfner KP, Carney JP, Tainer JA, Connelly JC, Leach DRF, Kanaar R, Wyman C (2004) Differential arrangements of conserved building blocks among homologs of the Rad50/Mre11 DNA repair protein complex. J Mol Biol 339:937–949

Delmas S, Shunburne L, Ngo HP, Allers T (2009) Mre11-Rad50 promotes rapid repair of DNA damage in the polyploid Archaeon Haloferax volcanii by restraining homologous recombination. PLoS Genet 5(7):e1000552–e1000552

Dolganov GM, Maser RS, Novikov A, Tosto L, Chong S, Bressan DA, Petrini JHJ (1996) Human Rad50 is physically associated with hMre11: identification of a conserved multiprotein complex implicated in recombinational DNA repair. Mol Cell Biol 16:4832–4841

Gallego ME, Jeanneau M, Granier F, Bouchez D, Bechtold N, White CI (2001) Disruption of the Arabidopsis RAD50 gene leads to plant sterility and MMS sensitivity. Plant J. 25:31–41

Gherbi H, Gallego ME, Jalut N, Lucht JM, Hohn B, White CI (2001) Homologous recombination in plants is stimulated in the absence of Rad50. EMBO Rep 2:287–291

Hartung F, Puchta H (2004) What comparative genomics tell us about the evolution of eukaryotic genes involved in recombination. Curr Genomics 5:109–121

Hartung F, Suer S, Puchta H (2007) Two closely related RecQ-helicases have antagonistic rolesin homologous recombination and DNA repair in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104:18836–18841

Hegarty MJ, Hiscock SJ (2008) Genomic clues to the evolutionary success of polyploid plants. Curr Biol 18:R435–R444

Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP, Tainer JA (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell 101:789–800

Hopfner KP, Karcher A, Craig L, Woo TT, Carney JP, Tainer JA (2001) Structural biochemistry and interaction architecture of the DNA double strand break repair Mre11 nuclease and Rad50-ATPase. Cell 105:473–485

Hopfner KP, Craig L, Moncalian G, Zinkel RA, Usui T, Owen BA et al (2002) The Rad50 zinchook is a structure joining Mre11 complexes in DNA recombination and repair. Nature 418:562–566

Huang S, Sirikhachornkit A, Su XJ, Faris J, Gill B, Haselkorn R, Gornicki P (2002) Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum/Aegilops* complex and the evolutionary history of polyploid wheat. Proc Natl Acad Sci USA 99:8133–8138

Johnson JC, Bhave M (2004) Molecular characterisation of the protein disulphide isomerase genes of wheat. Plant Sci 167:397–410

Johzuka K, Ogawa H (1995) Interaction of Well and Rad50: two proteins required for DNA repair and meiosis-specific double-strand break formation in *Saccharomyces cereukiae*. Genetics 139:1521–1532

Kawaura K, Mochida K, Ogihara Y (2005) Expression profile of two storage-protein gene families in hexaploid wheat revealed by large-scale analysis of expressed sequence tags. Plant Physiol 139:1870–1880

Kinoshita E, Linden EVD, Sanchez H, Wyman C (2009) RAD50, an SMC family member with multiple roles in DNA break repair: how does ATP affect function? Chromosome Res 17:277–288

Lawton-Rauh A (2003) Evolutionary dynamics of duplicated genes in plants. Mol Phyl Evol 29:396–409

Li WH, Yang J, Gu X (2005) Expression divergence between duplicate genes. Trends Genet 21(11):602–607

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402–408

Moncalian G, Lengsfeld B, Bhaskara V, Hopfner KP, Karcher A, Alden E, Tainer JA, Paull TT (2004) The Rad50 signature motif: essential to ATP binding and biological function. J Mol Biol 335:937–951

Nemoto Y, Kisaka M, Fuse T, Yano M, Ogihara Y (2003) Characterization and functional analysis of three wheat genes with homology to the CONSTANS flowering time gene in transgenic rice. Plant J 36:82–93

Nomura T, Ishihara A, Yanagita RC, Endo TR, Iwamura H (2005) Three genomes differentially contribute to the biosynthesis of benzoxazinones in hexaploid wheat. Proc Natl Acad Sci USA 102(45):16490–16495

Otto SP (2007) The evolutionary consequences of polyploidy. Cell 131:452–462

Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10:11

Pérez R, De Bustos A, Jouve N, Cuadrado A (2009) Localization of *Rad50,* a single-copy gene, on group 5 chromosomes of wheat, using a FISH protocol employing tyramide for signal amplification (Tyr-FISH). Cytogenet Genome Res 125:321–328

Petrini JH, Bressan DA, Yao MS (1997) The *RAD52* epistasis group in mammalian double strand break repair. Semin Immunol 9:181–188

Puchta H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J Ex Bot 56(409):1–14

Quian Gu Y, Coleman-Derr D, Kong X, Anderson OD (2004) Rapid genome evolution revealed by comparative sequence analysis of orthologous regions from four Triticeae genomes. Plant Physiol 135:459–470

Rupnik A, Grenon M, Lowndes N (2008) The MRN complex. Curr Biol 18(11):455–457

Shitsukawa N, Tahira C, Kassai KI, Hirabayashi C, Shimizu T, Takumi S, Mochida K, Kawaura K, Ogihara Y, Murai K (2007) Genetic and epigenetic alteration among three homoeologous genes of a class E MADS box gene in hexaploid wheat. Plant Cell 19:1723–1737

Stein N, Feuillet C, Wicker T, Schlagenhauf E, Keller B (2000) Subgenome chromosome walking in wheat: a 450-kb physical contig in *Triticum monococcum* L. spans the *Lr10* resistance locus in hexaploid wheat (*Triticum aestivum* L.). Proc Natl Acad Sci USA 97:13436–13441

Steininger S, Gomez-Paramio I, Braselmann H, Fellerhoff B, Dittberner D, Eckardt-Schupp F, Moertl S (2008) Xrs2 facilitates crossovers during DNA double-strand gap repair in yeast. Gene Repair 7:1563–1577

Storchova Z, Breneman A, Cande J, Dunn J, Burbank K, O'Toole E, Pellman D (2006) Genome-wide genetic analysis of polyploidy in yeast. Nature 443:541–547

Stracker TH, Theunissen JW, Morales M, Petrini JH (2004) The Mre11 complex and the metabolism of chromosome breaks: the importance of communicating and holding things together. DNA Repair 3:845–854

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting matrix choice. Nucleic Acids Res 22:4673–4680

Trujillo KM, Sung P (2001) DNA structure-specific nuclease activities in the Saccharomyces cerevisiae Rad50.Mre11 complex. J Biol Chem 276:35458–35464

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7): research0034.1–0034.11

Waltes R, Kalb R, Gatei M, Kijas AW, Stumm M, Sobeck A, Wieland B, Varon R, Lerenthal Y, Lavin MF, Schindler D, Dörk T (2009) Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. Am J of Hum Genet 84:605–616

Waterworth WM, Altun C, Armstrong SJ, Roberts N, Dean PJ, Young K, Wei CF, Bray CM, West CE (2007) NBS1 is involved in DNA repair and plays a synergistic role with ATM in mediating meiotic homologous recombination in plants. The Plant J 52:41–52

Wendel JF (2000) Genome evolution in polyploids. Plant Mol Biol 42:225–249

Wicker T, Yahiaoui N, Guyot R, Schlagenhauf E, Liu ZD, Dubcovsky J, Keller B (2003) Rapid genome divergence at orthologous low molecular weight glutenin loci of the A and A_m genomes of wheat. Plant Cell 15:1186–1197

Williams RS, Williams JS, Tainer JA (2007) Mre11-RAa50-NBS1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. Biochem Cell Biol 85:509–520

You Y, Moreira BG, Behlke MA, Owczarzy R (2006) Design of LNA probes that improve mismatch discrimination. Nucleic Acids Res 34(8):e60