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ORIGINAL PAPER

Characterization of the gene *Mre11* and evidence of silencing after polyploidization in *Triticum*

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Abstract The MRE11 protein is a component of the highly conserved MRN complex, along with RAD50 and NBS1. This complex is crucial in the repair of breaks in double stranded DNA, and is involved in many other cell processes. The present paper reports the molecular characterization of *Mre11* gene in all three genomes of wheat, making use of the diploid species *Triticum monococcum* (genome A) and *Aegilops Tauschii* (genome D), the tetraploid *T. turgidum* (genomes A and B), and the hexaploid *T. aestivum* (genomes A, B and D). The genomic sequences characterized ranged from 4,662 to 4,766 bp in length; the cDNA corresponding to the processed mRNA was 2,440–2,510 bp long. In all cases, *Mre11* coded for a highly conserved protein of 699 amino acids with a structure involving 22 exons. *Mre11* expression was determined by real-time PCR in all the species analysed. The tetraploid species showed an expression similar to that of the diploid *Ae. tauschii* and lower than that of *T. monococcum*. Stronger expression was detected in the hexaploid *T. aestivum*. The SSCP technique was modified by introducing fluorescent labelling to the procedure in order to analyse the expression of the different *Mre11* genes (i.e., those belonging to the different genomes) in the polyploid species. In both polyploids, the *Mre11* gene belonging to the B genome was the least expressed. This probably reflects a first step in the process of silencing duplicate genes after polyploidization.

Abbreviations

DSB	Double stranded breaking
HR	Homologous recombination
MRN	Protein complex formed by the *M*re11, *R*AD50 and *N*BS1 protein
SSCP	Single stranded conformational polymorphism

Introduction

The MRE11 protein is a key player in the repair of double stranded DNA breakages (DSBs). *RAD50* and *NBS1* are also essential in this process (D'Amours and Jackson 2002). The proteins encoded by these genes form a protein complex known as MRN, which is involved in virtually all aspects of DNA metabolism, including DSB detection, DSB processing, homologous recombination, meiosis, and telomere maintenance (Hopfner et al. 2001; Van den Bosch et al. 2003; Assenmacher and Hopfner 2004). Mutations in these genes lead to hypersensitivity to methyl methane sulphonate (MMS) and ionizing radiation, and impede homologous and illegitimate recombination (Furuse et al. 1998; Uziel et al. 2003) as well as the DSB-activated cell cycle checkpoint response (Assenmacher and Hopfner 2004). Functional studies have shown that this complex interacts with the DNA break points in sister chromatids to maintain the original structure and favour repair (Van den Bosch et al. 2003). The protein encoded by *Mre11* is the core protein of the complex (D'Amours and Jackson 2002) and possesses binding

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sites for both RAD50 and NBS1 (Assenmacher and Hopfner 2004). It is also able to form dimers with itself. The protein consists of two separable functional domains with nuclease activity at the N terminal region and a double stranded (ds) DNA binding site in the C terminal domain (Furuse et al. 1998). Nuclease activity is due to the phosphoesterase motifs; these form a nuclease domain with 3′–5′ dsDNA exonuclease activity and single stranded (ss) DNA endonuclease activity (D'Amours and Jackson 2002).

Mre11 is highly conserved (Matthew et al. 2004; Hartung and Puchta 2004). It has been characterized in many organisms ranging from unicellular fungi to vertebrates (D'Amours and Jackson 2002). In plants, information on this gene is mostly available in the context of sequenced whole genomes; its molecular characterization has only been undertaken in *Arabidopsis thaliana* (Hartung and Puchta 1999). Despite the great importance of this gene, no studies have analysed its expression in plants.

The study of duplicate genes and their relationship with allopolyploidy is now a matter of much discussion. In recent years, the rapidly increasing number of complete genomes and incomplete genomic sequences available has permitted the study of evolutionary biology on the basis of comparative genomics (Hartung and Puchta 2004). One of the main revelations of this kind of study is that genome doubling (polyploidy) has been an important force in evolution, especially in plants (for a review, see Adams and Wendel 2005). All higher plants appear to have undergone several rounds of polyploidy during their evolution (Bowers et al. 2003; Paterson et al. 2004; Blanc and Wolf 2004a). For example, the genomes of *Arabidopsis thaliana* and rice showed up to 90 and 62% of duplicated genes (respectively) and it has been estimated that duplication of genes has occurred in 70–80% of angiosperm species (Simillion et al. 2002; Blanc et al. 2003; Bowers et al. 2003; Paterson et al. 2004). In some cases one of the duplicate genes has been lost after doubling (pseudogenization), while the other has maintained their original function or even acquired new roles (neofunctionalization). Hughes (1994) suggested that the change in the functions of duplicated genes could be due to complementary mutations that alter one or more subfunctions (subfunctionalization model). Theoretical and experimental studies in the past few years have let to a rethinking of the paradigm of duplicate genes that provided the conceptual foundations of studies on duplicate gene evolution (Moore and Purugganan 2003, 2005; Blanc and Wolf 2004a, b).

Triticum is a genus in which polyploid species arose from the merging of diploids and also newly formed polyploid (to get to the hexaploid for example), most of which still coexist. These species belong also to the *Aegilops* genus as the D genome progenitor *Ae. tauschii* and the putative B donor *Ae. speltoides*. The speciation process of the wheat group has occurred recently (Huang et al. 2002) from a common ancestor that diverged into the different diploid species around 2.5–6 million years ago (Chantret et al. 2005 and cites herein). The tetraploid species *T. turgidum* appeared around 0.5–3 million of years ago, and the hexaploid species *T. aestivum* ssp. *spelta* and *T. aestivum* L just 7,000–9,000 years ago. Wheat provides an interesting model by which the first steps in the fate of duplicated genes can be examined. The aim of this work was to study the structure of the *Mre11* genes in polyploid wheats, and to analyse their expression in order to advance in our understanding of the evolutionary dynamics operating on these genes, This should provide information useful in directing gene targeting, and thus improve plant transformation technology.

Materials and methods

Plant material

The molecular characterization of the *Mre11* genes of the diploid species *Triticum monococcum* L. (genome A) and *Aegilops Tauschii* Coss. (genome D), the tetraploid species *Triticum turgidum* L. cv. Vitron (genomes A and B), and the hexaploid wheat *Triticum aestivum* L cv. Chinese spring (genomes A, B and D) was undertaken. All the material proceeds from our own plant stocks.

DNA and RNA isolation

Genomic DNA was purified from young leaves following the method of Sharp et al. (1988). RNA was isolated from pollen mother cells. Immature spikelets were collected at the meiosis stage, using the morphological development of the flower organs as an indicator of the development of the microspores. Anthers were collected at the microspore mid to late mononucleate stage (confirmed microscopically). Total RNA was purified using Tripure reagent (Roche) following the manufacturer's recommendations. To eliminate contaminating DNA, the RNA was purified using the Turbo DNA-free Kit (Ambion).

Amplification and cloning of sequences

Two pairs of primers were used to amplify the genomic sequence: Mre11-L/Mre11-R2 (5′ TGCCATCTG

GGCTACATGGA 3′ and 5′AACAAGAGGCCAT GA AATGAG 3′ respectively), and Mre11-L2/Mre11-R (5′ CGATTTGGTCAGAAGTATG 3′ and 5′ GAGAATCATCTCCTCTTGAC 3′). The Mre11-L primer included the start codon and the Mre11-R included the stop codon. The primers Mre11-R2 and Mre11-L2 were designed over the 12th intron and the 12th exon, respectively. PCR reactions were performed in a volume of 50 µl containing 100 ng of genomic DNA, 0.2 µM of each primer, 400 µM of each dNTP, 2.5 U of PfuTurbo DNA polymerase (Stratagene) and 1× PfuTurbo DNA polymerase buffer. All reactions were performed in a T3 thermocycler (Biometra). The reaction conditions were one cycle of 5 min at 95°C, 35 cycles of 45 s at 94°C, 45 s at 52–50°C (for the first and second pair of primers, respectively), 3 min at 72°C, and a final extension cycle of 7 min at 72°C. The amplified fragments were cloned into the *Eco*RV site of the BlueScript plasmid (Stratagene) and sequenced using an ABI Prism 377 sequencer (Applied Biosystems).

First strand cDNA synthesis was performed using 5 µl of DNA-free RNA (~2 µg input RNA) using the Transcriptor Reverse Transcriptase and Oligo $(dT)_{15}$ primer (Roche) following the protocol provided by the manufacturer. To obtain the cDNA *Mre11* sequences, 2 µl of first strand cDNA was subjected to PCR with the Mre11-L and Mre11-R primers. The PCR reaction, cloning and sequence procedures were as indicated above. Complete cDNA sequences were obtained using the RACE kit (Roche) following the manufacturer's recommendations. For 5′ RACE, the primers SP1 (5′ CCAAATGTTCAAGAACAGA 3′), SP2 (5′ CCATGGATAGTGAACACAGG 3′) and SP3 (5′ CACTGACAACCTGGAACTTCAC 3′) were used. SP5 primer (5′ ATTCGCAGTGAGGAGGTGGAC 3′) was used for 3′ RACE.

Southern blotting

This experiment was carried out to address the number of copies of these genes. Genomic DNA (20 µg) from the diploid, tetraploid and hexaploid wheat species was digested with *Hin*dIII and *Eco*RI restriction endonucleases (that cut only once in the genomic sequence of these genes) and size-fractionated in 1% agarose gel. Fragments were blotted onto nylon membranes (Roche). A fragment of the *Mre11* gene obtained by PCR was labelled with digoxigenin (Roche) and used as a probe. Hybridization was performed at 65°C; the detection of hybridization was performed according to the manufacturer's recommendations (Roche).

Alignment and sequence analyses

The analysis of the DNA sequences obtained was performed using the Sequence Navigator programme (Applied Biosystem). DNA and deduced protein sequences were aligned using the Clustal W 1.5 program (Thompson et al. 1994). The alignment of MRE11 included sequences obtained in this study and others previously published of rice (XP474032), *A. thaliana* (T52564), humans (AAC78721) and *S. cerevisiae* (NP013951). Phylogenetic trees were constructed using the distance method of the PHYLIP (Phylogeny Inference Package) Version 3.6 (Felsenstein 2004). Distance matrices were computed using the two-parameter method of Kimura (1980) with the PROTDIST program. Distance trees were constructed by the neighbour-joining method Saitou and Nei (1987).

Real-time quantitative PCR

Real-time PCR was used to analyse the expression of the *Mre11* genes. Two pairs of primers were designed using the Primer Express program (Applied Biosystem). The pair QPCR-L2/QPCR-R2 (5′ CCCACAA AGTGGACGGAAGA 3′ and 5′ GCTTGGCATGG AACCAGTGT 3′) amplified a fragment of about 100 bp in the cDNA of *Mre11*. The pair 18S RNA-L/ 18S RNA-R (5′ AATTGTTGGTCTTCAACGAGG AA 3′ and 5′AAAGGGCAGGGACGTAGTCAA 3′) amplified a fragment of similar size in the cDNA of the *18S rRNA* gene that was used as an endogenous control in PCR reactions. All reactions were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystem). For the *Mre11* gene, reactions were performed in a volume of 30 µl containing 3 µl of cDNA obtained as described above (about 300 ng of input RNA), 900 nM of each primer, and 1X SYBR Green mix (Applied Biosystem). For *18S rRNA* amplification, the primer concentration used was 166 nM. The reaction conditions were 10 min at 95°C, 40 cycles of 95°C for 15s, and 60°C for 1 min. The specificity of the amplifications was analysed by gel electrophoresis using the same PCR conditions described above. This specificity was further analysed in each real-time PCR reaction using ABI Prism Dissociation Curve Analysis Software. Duplicate samples were used in all reactions and each experiment was performed in triplicate. For this purpose, from the same RNA, three independent RT reactions were made and the cDNA obtained was used to perform real-time PCR following the same procedure described above.

The amplification efficiency of *Mre11* and the *18S rRNA* genes was determined as described by Li et al.

(2004). The cDNA of both genes was serially diluted twofold to obtain a standard curve. The relative changes in the expression of *Mre11* were calculated using the $2^{-\Delta\Delta Ct}$ method where Ct indicates the threshold cycle that is the fractional cycle number at which the amount of amplified target reaches a fixed threshold (Livak and Schmittigen 2001). ΔCt indicates the difference in threshold cycles for target and control genes. This is carried out to normalize the amount of target gene related to an endogenous gene (housekeeping). Finally, ΔΔCt indicates the amount of target, normalized to an endogenous reference, and relative to a calibrator.

SSCP analysis

A fluorescence SSCP technique was developed based on the radioactive technique described by Cronn and Adams (2003). The modifications made consisted mainly of amplification with fluorescence-labelled primers and the use of an automatic sequencer. First, amplification of *Mre11* cDNA was performed using primers SSCP-L (5′ AGAGGTAAATCTGTAGCA GC 3′) and SSCP-R2 (5′ GGATCTGAATTTTCAAC AACTTC 3′) without labelling. PCR was performed in a reaction volume of 30 µl, including 2 U of Taq polymerase and 1× buffer (Sigma), 100 µM dNTPs, 0.3 µM of each primer, and 1 µl of cDNA. The PCR cycling conditions consisted of an initial incubation for 2 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, plus a final extension step at 72°C for 5 min. The amplified products were loaded onto agarose gels and purified using the High Pure PCR Product Purification Kit (Roche). After purification, the products were internally fluorescently labelled with 6 FAM by PCR using the same primers as above. The main difference with the above PCR conditions was that a single primer was used in each reaction. These were performed using 0.5 µM of the single labelled primer in a volume of 10 µl containing 0.6 U and 1× Taq Buffer (Sigma), 175 µM dNTPs, and 5–8 ng of purified DNA fragment. The PCR cycle was as above. The reaction volumes were then diluted (1:10) and 5 µl of each were mixed with 20 µl of formamide and 0.5 µl of standard Rox500 (Applied Biosystems). Fluorescence analysis was performed in an ABI 3130 Avant Genetic Analyzer using a 50 cm capillary array and a performance-optimized polymer (POP-7) (Applied Biosystems). The manufacturer's default settings were used except for temperature (18°C) and time (3,500 s). Analyses were performed in triplicate and cDNA was synthesized. The proportion of each transcript was related to the fluorescence area of each peak obtained.

Results

Molecular characterization

The genomic characterization of the *Mre11* gene was performed in the diploid species *T. monoccocum* (genome A) and *Ae. tauschii* (genome D), in the tetraploid *T. turgidum* (genomes A and B) and in the hexaploid *T. aestivum* (genomes A, B and D). Two sets of primers were designed to amplify two fragments covering the entire genomic sequence. These were designed based on previously published sequences of the *Mre11* genes of *Arabidopsis thaliana* and rice. Information of sequences from the EST databank on wheat and closely related species was also used. All the above mentioned sequences were used to perform an alignment. Primers were designed in the EST sequences of wheat or in the most conserved regions of *Arabidopsis* and rice sequences. The fragments obtained were cloned, and the clones of each species sequenced. In the case of diploid species, no variation was observed between the clones. However, in the tetraploid *T. turgidum*, two types of sequences were found, and it was assumed that each belonged to a different genome. The assembly of the partial sequences of the two fragments amplified for each species provided the whole genomic sequence. The sizes obtained ranged from 4,662 for *T. turgidum* (genome A) to 4,766 bp for *Ae. tauschii* (genome D) (Table 1). A more detailed description of the gene sequences is available in the data bank (the accession number of each sequence is presented in Table 1)

Southern blotting was performed to determine the *Mre11* copy numbers. The genomic DNA of *T. monococcum, T. turgidum*, *Ae. tauschii* and *T. aestivum* was digested with two restriction enzymes (see Materials and methods) and probed with an *Mre11* fragment (Fig. 1). In all cases, Southern blotting revealed strong hybridization signals according to the number of genomes of each species, indicating that there is only one copy of the gene per genome in the wheat species analysed. In agreement with the nomenclature rules for the homoeologous genes of the three genomes of the allohexaploid wheat, the denominations *Mre11A, Mre11B* and *Mre11D* are proposed to distinguish the *Mre11* gene of each. This nomenclature is used from here on.

To characterize the different *Mre11* genes, their mRNAs were isolated when the microspores were at the meiosis stage. cDNA was obtained as described in Materials and methods. Amplification reactions were performed using cDNA and the specific *Mre11* primers (Mre11-L and Mre11-R) to obtain the complete *Mre11*

Table 1 Sizes of the DNA sequences of the wheat *Mre11* genes characterized

Specie	Genome	Size of genomic DNA (bp) (accession number)	Size of cDNA+ UTR (bp) (accession number)	Size of 5′ UTR (bp)	Size of 3′ UTR (bp)
T. monococcum	A	4,678 (AM049169)	2,457 (AM049175)	87	289
T. turgidum cv. Vitron	A	4,662 (AM049171)	2,510 (AM049174)	174	234
T. turgidum cv. Vitron	B	4,719 (AM049172)	2,440 (AM049173)	145	199
Ae. tauschii	D	4,766 (AM049170)	2,456 (AM049176)	73	283

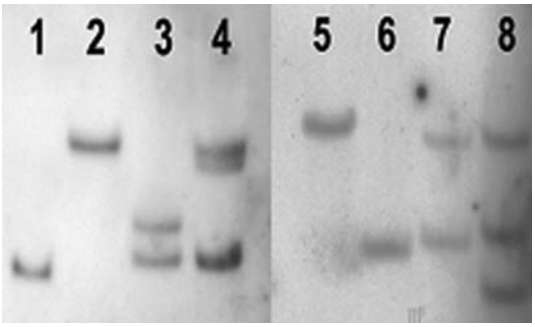

Fig. 1 Southern blot analysis of *Mre11* copy numbers. DNA from *T. monococcum* (genome A), *Ae. tauschii* (genome D), *T. turgidum* cv. Vitron (genomes A and B) and *T. aestivum* cv. Chinese spring (genomes A, B and D) was digested with *Eco*RI and *Hin*dIII and hybridized with a *Mre11* probe. *Lanes 1–4*, DNA digested with *Eco*RI (in the same order as above); *lanes 5–8*, DNA digested with *Hin*dIII

cDNA sequences of the diploid and tetraploid wheat species. A single fragment of around 2,100 bp was amplified in all species analysed. After the fragments were cloned, several clones of each species were sequenced once more. No differences were found between clones of the same species, except for the tetraploid species that showed two types of sequence. One of these was related to the sequence found in *T. monoccocum* (genome A); it was assumed that the second (which had more differences) belonged to the B genome. RACE experiments were also performed to obtain the sequence at the 3′ and 5′ cDNA ends. The size of the total cDNA sequences characterized ranged from 2,440 to 2,510 bp (genomes B and A, respectively) (Table 1).

The differences found between the coding regions of the cDNA sequences were all due to single nucleotide substitutions; no insertions or deletions were detected. These differences show the great similarity between the *Mre11* genes of the different wheat genomes: 98% of identity for *Mre11A* and *Mre11D*, and 97% of identity for both genes with respect to *Mre11B*. The comparison between genomic and cDNA sequences confirmed the presence of 22 exons in all the genomes. A characteristic of these genes appears to be the finishing of the first exon at the ATG start codon. The size of each exon was the same in all the sequences characterized, with exon 19 the largest at 74 codons. The size of the introns showed slight variation between the sequences analysed due to single nucleotide substitutions and insertions/deletions. Thus, the shortest intron was intron 5 with 71 bp in all the sequences characterized, while intron 16 was the largest, with a size varying between 466 bp in *Mre11A* to 477 bp in *Mre11D*. The open reading frame of the *Mre11* genes was the same size for all the sequences, with 2,097 bp coding for a 699 amino acid protein (estimated molecular weight around 78 kDa, theoretical pI 5.9).

Protein alignment

Protein alignment was performed to compare the deduced amino acid sequences for the different wheat species analysed, and to compare them with others previously published (Fig. 2). The alignment showed the very high conservation of the proteins encoded by the *Mre11* genes; a percentage of identity of 99% was seen between the species of wheat analysed. Conservation was also strong in the other species included in the alignment analysis, especially in the N-terminal region where the phosphodiesterase motifs are located. Overall, the MRE11 protein of rice showed 92% of identity with those of wheat, which in turn showed 84% of identity with the corresponding protein of *A. thaliana.* Homology with human and yeast *Mre11* was lower (60 and 57%, respectively).

The alignment results were used to build a phylogenetic tree (Fig. 3) showing the relationship between all the proteins analysed. The strongest similarity was found between proteins belonging to the A genome of *T. monoccocum* and *T. turgidum*. These proteins were closely related to their genome D counterparts in *Ae. squarrosa* and their genome B counterparts in the tetraploid *T. turgidum*. As expected, the evolutionary proximity between rice and wheat led to the rice protein showing more similarity with wheat proteins than with the protein of *A. thaliana*. The most distant proteins were those from yeasts and humans.

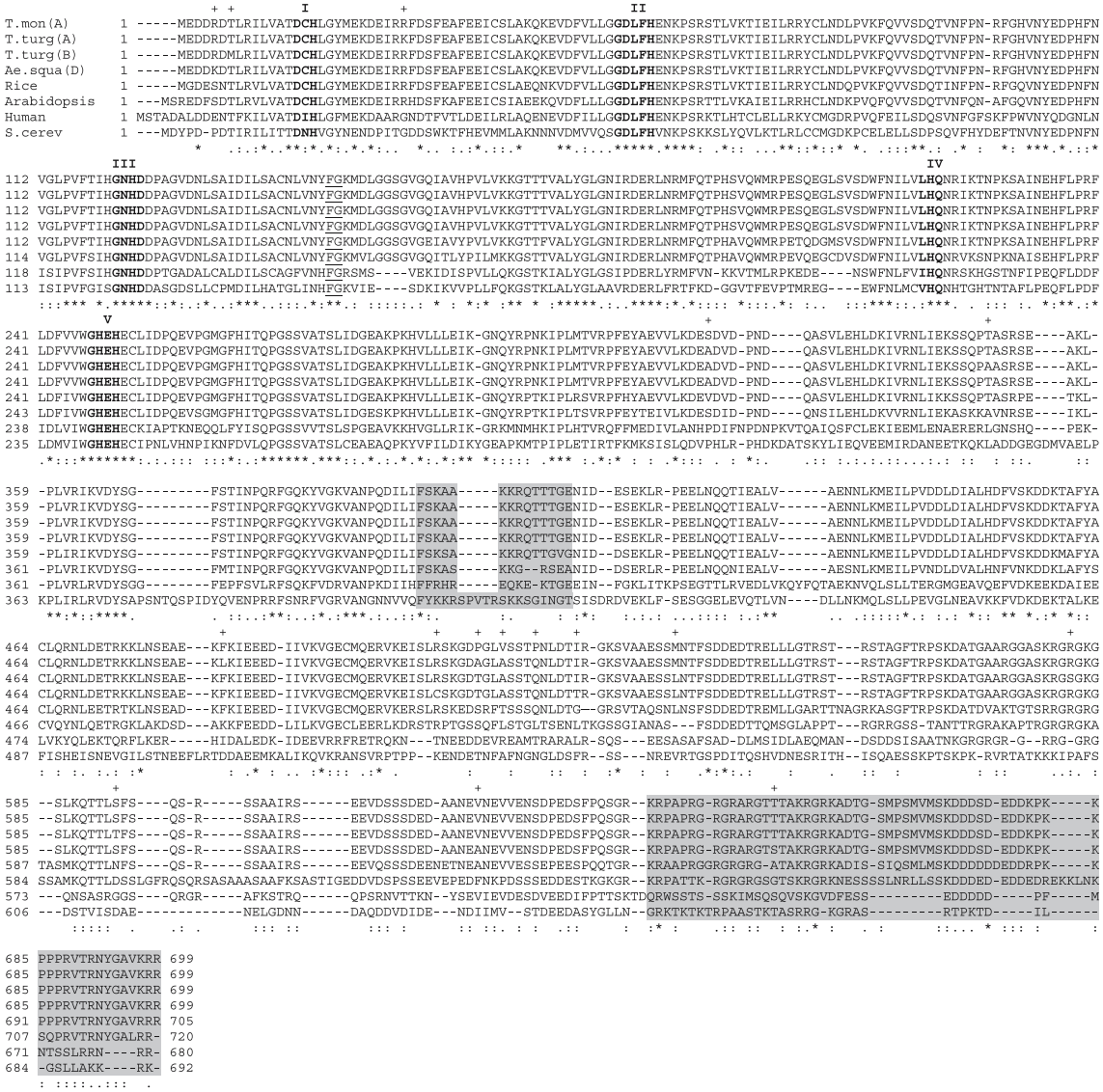

Fig. 2 Alignment of proteins encoded by the *Mre11* genes of the wheat species analysed and of previously published MRE11 of rice (XP474032), *A. thaliana* (T52564), humans (AAC78721) and *S. cerevisiae* (NP013951). The differences between the wheat proteins are denoted by the *plus* sign in the upper part of the alignment. In the lower part, *asterisk* indicates positions that have a single, fully conserved residue, and *dot* and *colon* indicate positions that have a single or two different residues, respectively. *Bold letters* indicate the five highly conserved phosphodiesterase motifs. *Underlined letters* denote the hydrophobic cluster. *Shaded regions* indicate the two DNA binding sites of MRE11

Analysis of the expression of the *Mre11* genes by real-time PCR

In a preliminary study, several genes were evaluated as internal controls. Thus, primers for alcohol dehydrogenase, polyubiquitin, puroindoline-b and the *18S rRNA* genes were designed as described in Materials and methods and their expression tested in the different species of wheat examined (data not shown). The 18S ribosomal gene showed the smallest variation in expression and was selected as a housekeeping gene in all real-time PCR experiments.

The efficiency of amplification was determined by making serial dilutions of Mre11 cDNA as described in Materials and methods. In a first test, the designed Mre11 primers showed different amplification efficiency to that of the primers for the *18S rRNA* gene. A second pair of Mre11 primers was then designed and the amplification efficiency obtained was very similar to that of 18S rRNA primers ($P = 0.058$, $R^2 = 0.1970$).

To ensure that the differences in expression were not due to differences in developmental stage, a pool of mRNA from different plants was obtained for each species analysed. Checks were performed by microscopy

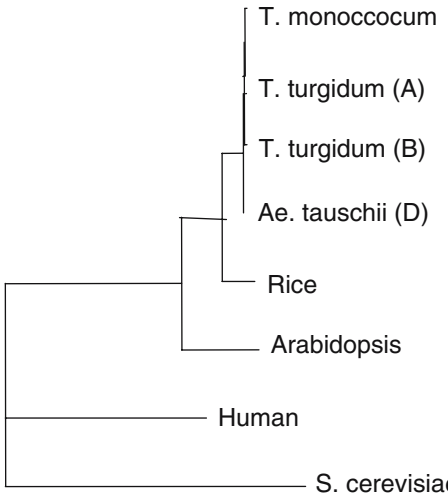

Fig. 3 Phylogenetic tree obtained from the deduced amino acid sequences of *Mre11* in the wheat species studied and in others (previously published data)

to confirm the microspore stage of the plant tissues from which this mRNA came. Table 2 shows the expressions obtained. The average expression of the two samples of *Mre11* and *18S* used in each real-time PCR reaction was calculated, and the ΔCt determined. This was performed for three replicates. The highest ΔCt obtained was for the diploid species *Ae. tauschii*, the hexaploid *T. aestivum* had the lowest. The other diploid species, *T. monoccocum*, had a higher ΔCt value than the hexaploid wheat, but a lower value than that shown by the tetraploid *T. turgidum*; the latter had a ΔCt value similar to that of the diploid *Ae. tauschii*. Figure 4a shows the differences in the expression of the wheat species. The species with the lowest expression was *Ae. tauschii*, which was used as a calibrator. The hexaploid species showed an *Mre11* expression six times that of the diploid *Ae. Tauschi*. The diploid *T. monoccocum* also showed a higher expression value than the calibrator (2.08-fold greater), whereas the expression of the tetraploid species was slightly higher (1.67-fold greater).

To analyse the intraspecific expression of the *Mre11* genes, three plants of each species were studied in the same way as described above (Table 3). The plants of *T. monoccocum* analysed showed variation in ΔCt. In plant 1, expression was similar to that seen in the hexaploid plants assayed, while plant 3 had a ΔCt value similar to the plants of *Ae. tauschii* and *T. turgidum*. The highest ΔCt value was that of plant 3 of *Ae. tauschii* (16.60). The other plants of this species showed levels of expression similar to that shown by the individual samples of *T. turgidum.* The plants of the tetraploid species showed the smallest range of variation in expression of the gene. The hexaploid plants analysed showed the smallest ΔCt values, with a minimum of 13.49 for plant 1. Figure 4b shows the variation of expression for all the individual plants analysed. Plant 3 of *Ae. tauschii* showed the weakest expression and was used as a calibrator. The maximum level of expression was found in the plants of *T. aestivum* (4.5- to 8.6-fold greater than the calibrator), with expression in the *Ae. tauschii* and *T. turgidum* plants very similar. The expression values for the *T. monoccocum* plants were intermediate.

SSCP analysis

The SSCP technique was used to analyse the relative expression of homoeologous genes in each polyploid wheat species. To evaluate the accuracy of the modified SSCP fluorescence technique, known amounts of the transcripts of *Mre11A* and *Mre11B* of *T. turgidum* were analysed in the same way as reported by Cronn and Adams (2003), who assumed that the level of expression observed with this technique agrees with the quantity of transcript used. The unlabelled SSCP-L and SSCP-R2 primers described in Materials and methods annealed within the cDNA clones of the *Mre11* genes to amplify a fragment of 237 bp. The A and B genome fragments were identical in size but differed in nine substitutions, yielding a pairwise nucleotide identity of 96.2%. Mixtures of purified A and B

Table 2 Results of real-time PCR expression analysis performed using pooled RNA

Specie	Replicate 1			Replicate 2			Replicate 3			Avg. ΔCt ± SD
	Avg. Ct Mre11	Avg. Ct 18S	ΔCt	Avg. Ct Mre11	Avg. Ct 18S	ΔCt	Avg. Ct Mre11	Avg. Ct 18S	ΔCt	
T. monoc	29.88	14.55	15.33	30.58	14.99	15.59	30.64	14.76	15.88	15.6 ± 0.27
Ae. Tausch	30.97	14.34	16.63	31.34	14.4	16.94	31.08	14.65	16.43	16.66 ± 0.25
T. turgid	29.98	14.37	15.61	30.79	14.68	16.11	30.28	14.24	16.04	15.92 ± 0.27
T. aestiv	28.57	14.88	13.69	29.14	15.18	13.96	28.43	14.44	13.99	13.88 ± 0.16

The Ct values are indicated for the *Mre11* and *18S rRNA* genes in three replicates. The normalized Ct (ΔCt) was calculated for each experiment and the average determined for the three experiments

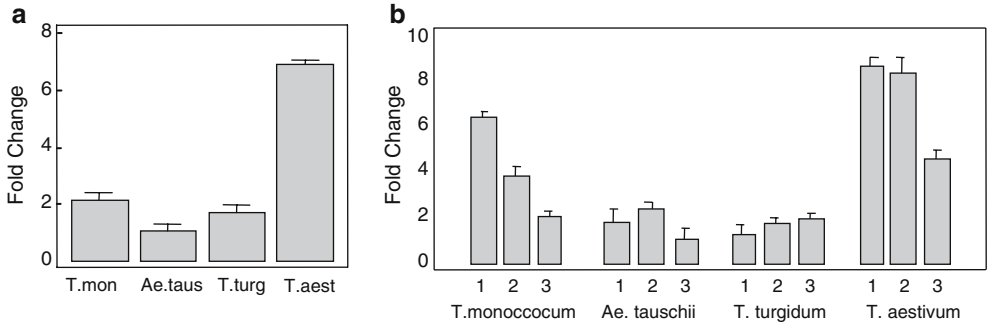

Fig. 4 **a** Fold change in gene expression of the pool of RNA obtained for the four wheat species used. The calculation of fold change was performed according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) as indicated in Materials and methods. The lower ΔCt value obtained (from *Ae. tauschii*) was used as calibrator. **b** Changes in the expression of *Mre11* gene in the individual plants studied. To perform the calculation of the fold change, the lower value of ΔCt of plant 3 belonging to *Ae. tauschii* specie was used as calibrator

Table 3 Expression levels in individual plants

Species	Replicate 1			Replicate 2			Replicate 3			Avg. ΔCt ± SD
	Avg. Ct Mre11	Avg. Ct 18S	ΔCt	Avg. Ct Mre11	Avg. Ct 18S	ΔCt	Avg. Ct Mre11	Avg. Ct 18S	ΔCt	
T. monoc										
1	28.65	14.67	13.98	28.20	15.05	14.15	28.32	14.67	13.65	13.92 ± 0.25
2	30.30	14.83	15.47	29.00	15.66	14.26	29.31	14.97	14.34	14.69 ± 0.67
3	30.56	14.67	15.98	31.09	15.66	15.43	30.20	14.78	15.42	15.58 ± 0.26
										14.73 ± 0.83
Ae. Tausch										
1	32.01	14.72	15.29	31.45	15.99	15.46	30.69	15.06	15.63	15.79 ± 0.60
2	31.89	14.40	15.49	30.89	15.87	15.02	31.20	15.63	15.57	15.36 ± 0.29
3	33.30	14.54	16.76	31.54	15.53	16.01	31.57	15.47	16.10	16.60 ± 0.53
										15.91 ± 0.62
T. turgidum										
1	31.97	14.43	15.54	31.54	15.66	15.86	29.63	14.66	14.97	15.45 ± 0.45
2	31.03	14.45	15.58	31.71	15.68	16.03	30.42	14.62	15.80	15.80 ± 0.22
3	31.02	14.74	15.37	30.04	15.55	15.89	30.41	14.92	15.49	15.58 ± 0.27
										15.61 ± 0.17
T. aestivum										
1	27.88	14.73	13.15	29.52	15.56	13.96	28.41	15.04	13.37	13.49 ± 0.41
2	28.91	14.54	14.37	28.07	14.87	13.20	27.67	14.62	13.05	13.54 ± 0.72
3	29.23	14.57	14.66	29.62	14.91	14.71	28.61	14.70	13.91	14.42 ± 0.44
										13.81 ± 0.52

Data are the results obtained for the three replicates performed. The average expressions of each individual plant and of the three plants of each species are indicated

PCR products were then made up for SSCP analysis in the following ratios (8 µl volume): 5 ng A + 0.05 ng B (100:1), 5 ng A + 0.25 ng B (20:1), 5 ng A + 0.71 ng B (7:1), 5 ng A + 1.66 ng B (3:1), 5 ng A + 5 ng B (1:1), 1.66 ng A + 5 ng B (1:3), 0.71 ng A + 5 ng B (1:7), 0.25 ng A + 5 ng B (1:20), and 0.05 ng A + 5 ng B (1:100). In addition, 5 ng of fragments A and B were used separately as controls. For each mixture, three independent labelling reactions were performed.

In this technique, gene expression is related to the area of the automatically obtained fluorescence peak. Expression values were obtained by calculating the average of the three replicates. Corrections for background were sometimes required for the 1:20 and 1:100 dilutions. Figure 5 shows the results for one of the replicates for each of the experiments performed. The technique clearly separates two peaks in each mixture, representing the transcripts of the *Mre11* genes of genomes A and B. The technique showed good reproducibility, as revealed by the very small standard deviations observed (Table 4). The 95% confidence intervals (CI) were calculated and the fractional expected ratios were well within the 95% CI calculated on observed signal ratio except for the 1:7 dilution. Fluorescence SSCP analysis of tetraploid *T. turgidum* genomic DNA was also performed to validate the method (data not

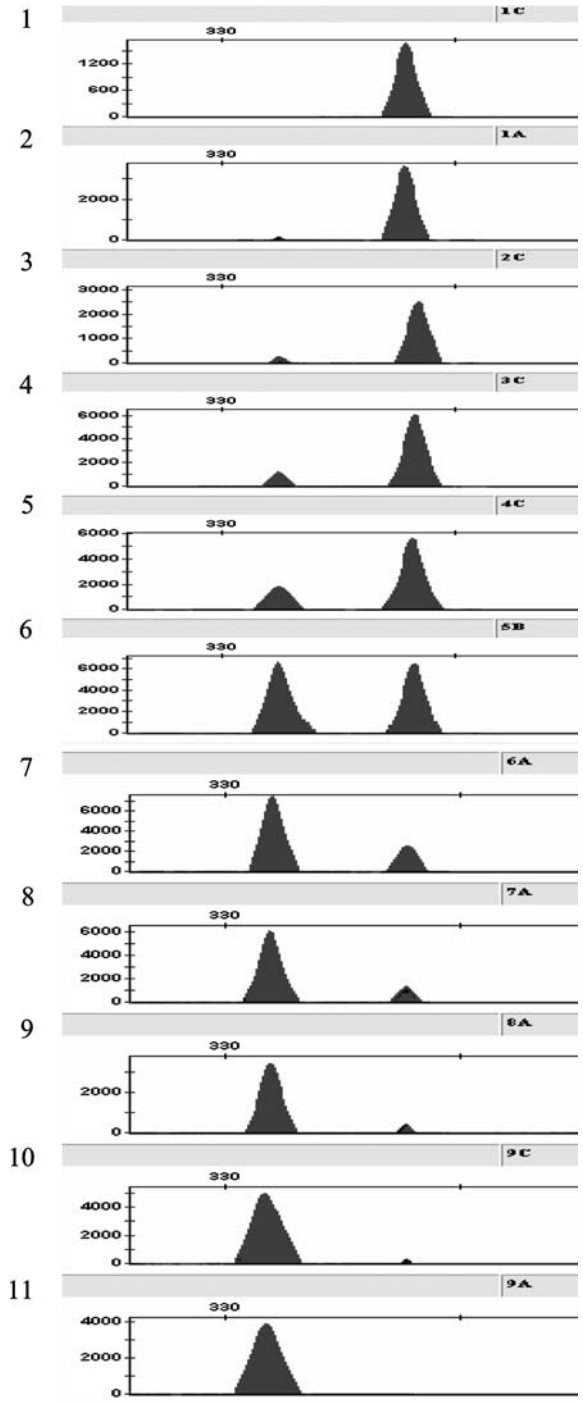

Fig. 5 Expression of *Mre11* as determined by automatic fluorescence analysis of one of the three replicates of each of the nine dilutions tested. Controls of the expression of the A and B genome genes were included (*1* and *11*, respectively): *2* = 1:100; *3* = 1:20; *4* = 1:7; *5* = 1:3; *6* = 1:1; *7* = 3:1; *8* = 7:1; *9* = 20:1; *10* = 100:1

shown). Identical levels of fluorescence would be expected for both genes. Values of 44.6 and 55.4%, close to those expected for the genes *Mre11A* and *Mre11B*, were obtained.

Table 4 Expression of *Mre11* belonging to the A and B genomes for the nine dilutions tested, and the confidence intervals obtained

Known dilution ratio	Fractional expected ratio	Observed signal ratio (±SD)	95% Confidence interval
1:100	0.010	0.016 ± 0.010	−0.010 to 0.042
1:20	0.048	0.030 ± 0.014	0.006–0.067
1:7	0.125	0.112 ± 0.004	0.101–0.124
1:3	0.250	0.237 ± 0.008	0.215–0.259
1:1	0.500	0.492 ± 0.008	0.471–0.514
3:1	0.750	0.755 ± 0.004	0.743–0.766
7:1	0.875	0.875 ± 0.002	0.869–0.881
20:1	0.952	0.952 ± 0.001	0.949–0.956
100:1	0.990	0.987 ± 0.003	0.979–0.996

The technique was then used to analyse the relative expression of homoeologous genes in the tetraploid species *T. turgidum* (AABB) using the diploid species *T. monococcum* (AA) and *Ae. tauschii* (DD) as controls. For this, pooled RNA was used to obtain cDNA. Three independent RT-PCR and labelling reactions were performed for each species. Figure 6 shows the results of one replicate. In this case, the fluorescence technique discriminated two peaks belonging to the A and B genomes. In agreement with Adams et al. (2003), the expression of the transcripts was considered biased when the transcript ratio for the two homoeologous genes was 60/40 or greater. As can be seen, biased expression of both genes was detected in the samples of *T. turgidum* analysed, the value for the *Mre11A* gene higher (69.4% ± 0.72) than that for *Mre11B* (30.5% ± 0.70). This technique was also able to distinguish between the transcripts of hexaploid *T. aestivum* (AABBDD) (Fig. 6). Thus, three different peaks were clearly resolved, corresponding to the three genomes of cultivated wheat. In this case, transcript expression was considered to be approximately equal if the transcript ratio for the three genes was around 33%. The expression of the *Mre11* genes of *T. aestivum* also showed differences between the three genomes, with higher values for the *Mre11A* and *Mre11D* genes (42.2 ± 0.62% and 38.4 ± 2.0%, respectively), again showing lower expression of the *Mre11B* gene (19.3 ± 1.8).

To assess the variation in expression within the polyploid species, three plants from *T. turgidum* and *T. aestivum* were studied. Table 5 shows the results obtained. Variation between plants was seen in the tetraploid species. Plant 1 showed expression values similar to those obtained in the analysis of the pooled RNA, with a great difference in the expression of the genes of the A and B genomes. The other two individuals analysed

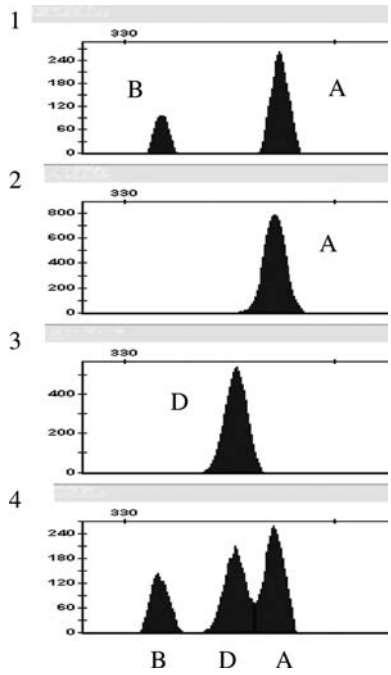

Fig. 6 Results of the fluorescence SSCP analysis of *Mre11* performed with *T. turgidum* (*1*), *T. monoccocum* (*2*), *Ae. tauschii* (*3*) *and T. aestivum* (*4*). Transcripts from the A, B and D genomes were identified

did not show clearly biased expression, although the expression of *Mre11A* was slightly higher than that of *Mre11B*. The expression of *Mre11* in individual plants of *T. aestivum* showed similar values. In all cases, *Mre11B* showed the weakest expression, with an average of 25.52 CT, slightly higher than the observed for the pooled RNA. The expressions of *Mre11A* and *Mre11D* differed slightly between plants but were similar to one another; in some cases that of *Mre11A* was stronger (plants 1 and 3), in others that of *Mre11D* was stronger (plant 2). These values were similar to that observed for the pooled RNA.

Discussion

The MRN complex is highly conserved, and genes orthologous to *Mre11* have been found in all taxonomic groups examined (Petrini et al. 1995; Hopfner et al. 2001; Van den Bosch et al. 2003). The MRE11 protein shows reasonable homology between organisms, indicating that the recombination machinery is an old complex that has been conserved during evolution (Hartung and Puchta 2004). This property allowed the use of combined comparative genomic strategies to isolate these genes in the wheat species analysed. Therefore, for the amplification of the *Mre11* genes, primers were designed using information on the orthologous genes of rice and *A. thaliana*, and from the EST database for wheat, barley and rye. This kind of strategy is currently being used in the characterization of genes in species for which no information on the sequence of the genome is available. The same method was previously used to characterize orthologous genes coding for the HMW glutenins in several species of *Triticum, Secale* and *Aegilops* (De Bustos et al. 2000, 2001; De Bustos and Jouve 2003, 2006).

Firstly, the hexaploid wheat 'Chinese Spring' (ABD genomes) was used to amplify the *Mre11* genes of the three genomes. However, the PCR experiments performed using different pairs of primers did not produce fragments of different size; only one fragment was obtained in all cases. This precluded the use of nulli-

Table 5 Results of SSCP analysis for the polyploid *T. turgidum* (A and B genomes) and *T. aestivum* (A, B and D genomes) plants

Individual plant	Genome	Replicate 1	Replicate 2	Replicate 3	Avg. expression (SD)
T. turgidum 1	A	68.60	71.08	74.49	71.93 (2.95)
	B	31.30	28.91	25.50	28.58 (2.91)
T. turgidum 2	A	56.13	58.00	57.72	57.28 (1.00)
	B	43.86	41.99	42.28	42.71 (1.00)
T. turgidum 3	A	55.35	54.49	50.08	53.30 (2.82)
	B	44.64	45.50	49.13	46.43 (2.38)
T. aestivum 1	A	38.69	36.09	41.08	38.61 (2.49)
	B	25..83	29.25	27.74	27.60 (2.93)
	D	35.47	34.64	31.16	33.75 (2.28)
T. aestivum 2	A	38.04	36.80	37.41	37.41 (0.62)
	B	21.74	21.87	24.04	22.55 (1.29)
	D	40.21	41.26	38.53	40.00 (1.37)
T. aestivum 3	A	37.67	41.89	37.70	39.08 (2.42)
	B	27.77	22.25	26.56	25.52 (2.90)
	D	34.55	35.84	35.73	35.37 (0.71)

Values are the percentage expression of the *Mre11* genes for the genomes of each sample (three replicates). The average *Mre11*expression for each genome is also indicated

tetrasomic lines to assign the gene a chromosome location. The diploid species *T. monoccocum* (genome A) and *Ae. tauschii* (genome D), and the tetraploid *T. turgidum* (genomes A and B), have all been used in the characterization of the orthologous genes of the three genomes of wheat (Johnson and Bhave 2004). *T. monoccocum* was chosen because it is thought to be very close to the A genome of cultivated wheat and *Ae. tauschii* is the donor of D genome (Zohary and Feldman 1962). However, the identity of the diploid donor of the B genome is still a matter of discussion, although some authors suggest that *Ae. speltoides* is the current diploid species with the most similar genome to the B genome of cultivated wheat (Petersen et al. 2006; De Bustos and Jouve 2006). In the absence of diploid species carrying the primitive B genome, we used tetraploid wheat, assuming that two different sequences would be characterized from this species, one corresponding to the *Mre11A* gene of *T. monoccocum* and the other matching *Mre11B*. The comparison of the two sequences obtained with that belonging to genome A of *T. monococcum* allowed the identification of the one belonging to the genome B.

The localization of these genes in the chromosomes of wheat was also attempted using fluorescence in situ hybridization (FISH). However, no results were obtained due to the technical difficulties in mapping low-copy number genes in large plant genomes such as that of wheat (Jiang and Gill 1994). Neither was any mapping information available on ESTs of this gene (GrainGenes wEST Resource, http://www.wheat.pw.usda.gov/wEST/). New strategies are therefore needed to map the loci of these genes.

Southern experiments were performed to determine the number of copies of this gene in each species. The results show that there was only a single copy of the *Mre11* gene per genome. This contrasts with that reported in humans, in which a pseudogene has also been detected (Petrini et al. 1995). The wheat results agree with the existence of the single *Mre11* sequence for the plant species available in the EST databank (Hartung and Puchta 2004).

The proteins encoded by the *Mre11* genes of the different wheat species showed characteristics similar to those of other species. The five phosphodiesterase motifs, plus the hydrophobic cluster typical of these proteins, have been found in the amino terminal region of MRE11 protein (Usui et al. 1998; Hopfner et al. 2001; Matthew et al. 2004). The nuclease activity of this protein is specified by the phosphodiesterase motifs. A 3′–5′ double stranded DNA with exonuclease activity and single and double stranded DNA endonuclease activity has been reported (D'Amours and Jackson 2002). The central and C-terminal regions contain additional DNA binding sites as well as a binding site for RAD50 and for MRE11-MRE11 dimerization (Assenmacher and Hopfner 2004).

The *Mre11* genes characterized showed strong conservation of coding sequences and exon/intron structures. This conservation was also found in the comparative analysis of this gene in plants and animals, where the intron positions were identical in more than 35% of cases (Hartung and Puchta 2002). The similarity between the sequences of the genes of the wheat studied here was 97–98%. Similarity reached 99% when comparing the proteins. This strong conservation of these genes indicates the important functional role of the proteins for which they code (Ciaffi et al. 2006). However, a clear difference in the degree of conservation is seen when comparing the conservation of these genes with that of other genes of the same genomes. Thus, the wheat genes coding for the *Glu1* endosperm proteins showed more variation than the *Mre11* genes (De Bustos et al. 2000; Kawaura et al. 2005). The sequences characterized from the A genome of *T. monococcum* and *T. turgidum* showed the greatest similarity, and were also very similar to those of genomes B and D. This close relationship supports the generally accepted hypothesis of a common ancestor for the three genomes of wheat (Bowden 1959; Löve 1984).

Mre11 expression

Real-time PCR is widely used for the quantification of mRNA and is a critical tool in basic molecular research, with applications in biomedicine and biotechnology (Bustin 2002). This technique has also been used in many plant studies (Gachon et al. 2004), including the analysis of gene expression in wheat (Yan et al. 2003; Li et al. 2004; Huang et al. 2004). An important feature in the use of this technique is the normalization of the RNA content between samples. This is often achieved using an endogenous control gene expressed at nearly constant levels in all the samples analysed (Brunner et al. 2004). The choice of the best candidate for this function is of great importance. In the present work, four genes amply used in this kind of study were analysed: those coding for alcohol dehydrogenase, puroindoline-b (Li et al. 2004), polyubiquitin, and 18S ribosomal RNA (Brunner et al. 2004). With the exception of the *18S rRNA* housekeeping gene, those tested showed variation, and in some wheat species the polyubiquitin gene could not be amplified. In the present study, only the *18S rRNA* gene was

expressed at the same level for all the plant species analysed, and was used as a control because the samples were not enriched in mRNA, and because the *Mre11* gene is weakly expressed in the meiosis stage (Bustin 2000).

The comparison of *Mre11* expression between wheat species was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittigen 2001). To apply this method, the amplification efficiency of the target and the endogenous control must be approximately equal. In the present case it was necessary to test different pairs of primers in order to amplify *Mre11* with efficiency similar to that seen for the amplification of the *18S rRNA* gene. The degree of similarity in amplification efficiency is obtained form the slope in linear regression analysis (Livak and Schmittgen 2001). If the value of the slope is close to zero, the efficiencies are similar. In the present work, the value $P = 0.058$ permitted the use of this method for analysing the relative expression of *Mre11*.

Mre11 expression was analysed using a pool of mRNA for each species. Although there are many studies comparing the level of expression between diploid species, to our knowledge this is the first time that the relative expression of a gene has been compared between species with different levels of ploidy. The hexaploid *T. aestivum* showed the strongest expression and the diploid *Ae. tauschii* the weakest. The other two species presented intermediate levels of expression showing the diploid *T. monoccocum* greater expression than the tetraploid *T. turgidum* (this species showed a degree of expression more similar to that of the other diploid species). The hexaploid species expressed *Mre11* over six times more than the diploid *Ae. tauschii*, and three times more than the tetraploid. In addition, the expression of the tetraploid was intermediate between the two diploid species. This could be attributed to differences in the development stage of the samples. However, the pooling of mRNA from different plants and the microscopically confirmed developmental stage of the material indicate this variation to have some other cause.

When the analysis was extended to individual plants, the variation in expression observed was even higher than that seen in the first experiment, although overall the variation of expression between species was similar. These results could suggest the existence of a complex mechanism of expression and silencing duplicate *Mre11* genes; this complexity probably related to the great importance of this gene. Alternatively, a lack of fine control of expression between duplicate genes is also possible.

SSCP analysis

Most higher eukaryotes have genomes with redundant genes, mainly arising from genome doubling or polyploidy (Adams et al. 2003). Differences in the expression levels of duplicate genes have long been a subject of great interest since new genes can arise from redundant duplicates (Li et al. 2005). The accurate assessment of the expression of each member of a homoeologous gene pair is therefore of great importance. Such analysis requires a technique that can discriminate between the expressions of two (or more) almost identical genes (homoeologues). Northern blotting, the most commonly used technique in gene expression analysis, has, however, frequently failed in this area. Recently, Cronn and Adams (2003) developed a useful PCR-SSCP technique for the characterization and quantification of the relative expression of homoeologous genes in polyploid cotton. We modified this technique to improve some of the negative aspects associated with the use of radioactive products.

The fluorescence SSCP technique described here conserves the advantages of radioactive labelling (Cronn and Adams 2003), but this was replaced by fluorescence. To determine the efficiency of the technique, known amounts of transcript were used to compare the expression level of the genes using the original technique (Cronn and Adams 2003). The results were highly reproducible, as shown by the non-significant differences found between the replicates. Another advantage of fluorescence SSCP is that the process is less time-consuming. Moreover, it eliminates the dangers associated with the use of radioactive compounds, as well as the use of acrylamide gels. In addition, the results are obtained automatically in less than an hour, thus avoiding lengthy exposure times (sometimes several days) and obviating the need to measure band intensities. When the modified technique was used to analyse the expression of homoeologous genes in polyploid species, it showed itself to be a powerful tool. This is the first time that the analysis of the expression of homoeologous genes has been achieved in hexaploid species. This technique should be valuable in the study of the expression of homoeologous genes in other species at least as complex as hexaploid wheat.

The expression of the *Mre11* genes in meiosis was not the same for all the genomes of the polyploid species, especially for the B genome. The results obtained clearly show a different expression level for *Mre11B* with respect to *Mre11A* and *Mre11D* in both the tetraploid and hexaploid species. The weaker expression of *Mre11B* would have a greater impact in the tetraploid than in the hexaploid species, in which the A and D

genomes are expressed at a similar level. This result shows that no relationship exists between the level of expression of the genes studied and the ploidy level of the species. However, it remains unexplained why the diploid species show differences in their expression of *Mre11* (in *T. monoccocum* [A] twice that seen in *Ae. tauschii* [D]). This could be explained by the action of other epigenetic factors (Lee and Chen 2001). This is suggested by the similar expression of the genes *Mre11A* and *Mre11D* in the hexaploid species, in which the maintenance of the differences in the expression of the genes observed in the diploids should be expected (Adams et al. 2003), especially when it is remembered that *T. aestivum* is of recent origin (estimated as appearing around 8,000 years ago) (Huang et al. 2002).

The individual plants analysed in both polyploid species showed a slight variation in *Mre11B* expression. This would indicate that the variation in the level of expression of these homologous genes at meiosis stage is an ongoing process that started after the recent polyploidization of wheat species (Blanc and Wolf 2004b; Chantret et al. 2005). The expression level might also be influenced by environmental or biological factors and also it can be variable between organs of the same plant (Adams et al. 2003, 2004). There is no clear reason why *Mre11B* is more weakly expressed in polyploids than the genes belonging to the other genomes, although similar results have been also reported for the expression of the α/β-gliadin genes of the B genome (Kawaura et al. 2005). It should be borne in mind that the B genome diverged from the common ancestor before the A and D genomes (Quian Gu et al. 2004); at present it is the genome with the greatest genetic diversity (Wendel 2000). This could be related to the differences observed in the expression of the analysed genes.

After polyploidization, a progressive process of adjustment may have occurred to compensate for the level of expression of the orthologous genes in the allopolyploid species. This may have taken place via the reduction or even silencing of some of the duplicated genes. Following genome duplication, changes in the expression of many of duplicate genes are produced, leading to different levels of expression, silencing or even the elimination of some genes (Adams et al. 2003; Blanc and Wolfe 2004a, b; Chantret et al. 2005). Hughes (1994) proposed a model, referred to as subfunctionalization, which suggests that duplicate genes acquire debilitating yet complementary mutations that alter one or more subfunctions of the original genes, or in the more frequently occurring loss-of-function mutations in regulatory regions (Force et al. 1999). Subsequent empirical studies on expression divergence between duplicate genes suggest that changes in expression regimen occur both frequently and rapidly, consistent with the predictions of the subfunctionalization model. This model might be especially convincing for the orthologous genes of the allopolyploid species. Functional divergence can occur rapidly after allopolyploidization. This phenomenon has been demonstrated in both naturally and in synthetic polyploids. For instance, among 40 homoeologous duplicate genes in natural tetraploid cotton, nine gene pairs exhibited biased expression, with the homeolog from one parental genome contributing more to the transcription than the other (Adams et al. 2003). Wang et al. (2004) studied the changes in duplicate gene expression between siblings of multiple selfed generations of synthetic *Arabidopsis* allopolyploids. They found that duplicate gene silencing is rapid and can vary stochastically between siblings. They also show that the silencing is potentially controlled by DNA methylation.

In the allopolyploid wheats these changes may have occurred soon after polyploidization or several generations after doubling, perhaps in connection with epigenetic factors (Adams and Wendel 2005). The observed expression of *Mre11B* would indicate the initiation of a process of silencing after polyploidization. This has been observed for some duplicated genes involved in DNA repair in *A. thaliana*, which have been selectively lost (Blanc and Wolfe 2004b). The analysis carried out has been performed at meiosis stage, in which the activity of the *Mre11* genes is critical. It would be interesting to extend the analysis to other tissues and different developmental stages to confirm if different copies of homoeologous genes issued from duplication have undergone a subfunctionalization process or are not regulated in the same way either in spatial or temporal dimensions in the allopolyploid wheats.

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