The role of *AtMSH2* in homologous recombination in *Arabidopsis thaliana*

Eyal Emmanuel, Elizabeth Yehuda, Cathy Melamed-Bessudo, Naomi Avivi-Ragolsky & Avraham A. Levy⁺

Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel

During homologous recombination (HR), a heteroduplex DNA is formed as a consequence of strand invasion. When the two homologous strands differ in sequence, a mismatch is generated. Earlier studies showed that mismatched heteroduplex often triggers abortion of recombination and that a pivotal component of this pathway is the mismatch repair Msh2 protein. In this study, we analysed the roles of *AtMSH2* in suppression of recombination in *Arabidopsis*. We report that *AtMSH2* has a broad range of anti-recombination effects: it suppresses recombination between divergent direct repeats in somatic cells or between homologues from different ecotypes during meiosis. This is the first example of a plant gene that affects HR as a function of sequence divergence and that has an anti-recombination meiotic effect. We discuss the implications of these results for plant improvement by gene transfer across species.

Keywords: mismatch repair; genome stability; somatic recombination; meiotic recombination; sequence divergence

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INTRODUCTION

Mismatch repair (MMR) systems have an important role in promoting genetic stability by repairing DNA replication errors (Kornberg & Bake, 1992; Modrich & Lahue, 1996), inhibiting recombination between divergent DNA sequences (Petit *et al*, 1991), maintaining barriers against massive genetic flow (Rayssiguier *et al*, 1989), preventing productive meiosis in interspecies hybrids (Hunter *et al*, 1996) and participating in responses to DNA damage (Stojic *et al*, 2004).

The best-characterized MMR pathway is the *Escherichia coli* methyl-directed MutHLS system that is involved in mismatch recognition and removal (Modrich & Lahue, 1996). MutS is an ATPase involved in mismatch recognition; MutL, another ATPase, couples mismatch recognition by MutS to downstream processing steps, and MutH is a methylation-sensitive endonuclease (Aravind *et al*, 1999). Both eubacteria and eukaryotes express highly

conserved MutS and MutL proteins. In eukaryotes, there are at least six MutS and five MutL homologues of the bacterial proteins (Modrich & Lahue, 1996; Flores-Rozas & Kolodner, 1998).

In *Arabidopsis*, several MutS homologues have been isolated. Four MutS homologues have been identified on the basis of their sequence conservation and through inspection of the *Arabidopsis* genomic sequence: *AtMSH2*, *AtMSH3*, *AtMSH6* and *AtMSH7* (Culligan & Hays, 1997; Eisen, 1998; Ade *et al*, 1999). *AtMSH1* was cloned through map-based cloning of a chlorophyll variegated mutant (Abdelnoor *et al*, 2003). *AtMSH4* was identified and cloned on the basis of sequence similarity with the human, mouse and yeast Msh4 amino-acid sequences (Higgins *et al*, 2004). Biochemical studies also point to conservation of protein functions (Culligan & Hays, 2000; Ade *et al*, 2001). The AtMsh2 protein was shown to form heterodimers with AtMsh3 and AtMsh6 *in vitro*, and the resulting complexes have mismatch recognition specificities similar to those shown by the corresponding yeast and mammalian complexes (Culligan & Hays, 2000). In addition, AtMsh2 forms heterodimers with AtMsh7, and this complex has a mismatch recognition spectrum distinct from that of the other two AtMsh2-containing complexes (Wu *et al*, 2003).

The plant *MSH*-like genes are involved in a broad range of functions, all of which are related to genome stability. AtMsh1 is involved in maintaining the stability of the mitochondrial genome (Abdelnoor *et al*, 2003). *AtMSH4* was shown to be expressed in floral tissues, and a mutant in this gene is defective in chromosome synapsis during meiosis, giving rise to a partially sterile phenotype (Higgins *et al*, 2004). The best-characterized plant gene so far is *AtMSH2*. Functional studies on the *Atmsh2* mutant in *Arabidopsis* showed microsatellite instability, indicating a direct involvement of *AtMSH2* in the repair of replication errors (Leonard *et al*, 2003; Depeiges *et al*, 2005). Moreover, the *Atmsh2* mutant shows a mutator effect when propagated for a number of generations, thus highlighting its importance in the control of genome stability (Hoffman *et al*, 2004).

In a previous study, we have shown that a minor sequence divergence can cause a drop-off in the rates of somatic recombination between direct repeats that differ by only 1 out of 618 identical base pairs (Opperman *et al*, 2004). Similarly, sequence divergence was shown to repress homologous recombination (HR) between inverted repeats (Li *et al*, 2004). The implication of *AtMSH2* or of any other plant MMR gene in such repression has not yet been tested.

Department of Plant Sciences, Weizmann Institute of Science, Rehovot 76100, Israel

⁺Corresponding author. Tel: +972 8 9342734; Fax: +972 8 9344181;

E-mail: avi.levy@weizmann.ac.il

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In this study, we tested the role of *AtMSH2* in two different types of HR: somatic and meiotic. The *Atmsh2-1* mutant we used is similar to that used in earlier studies (Alonso *et al*, 2003; Leonard *et al*, 2003; Hoffman *et al*, 2004). The somatic assay of recombination between divergent repeats has been described previously (Opperman *et al*, 2004). The effect of *AtMSH2* on the rates of meiotic recombination was determined using a novel seed-based assay in which linked markers (green fluorescent protein (GFP) or red fluorescent protein (RFP)) become separated following crossover (Melamed-Bessudo *et al*, 2005). We show that *AtMSH2* has a broad range of anti-recombination effects. We compare the roles of *MSH2* in different kingdoms and the implication of our results for plant improvement by broad gene transfer.

RESULTS

Somatic recombination between divergent repeats

To explore the role of *AtMSH2* on HR frequency in the presence of sequence divergence, we tested HR in the background of the *Atmsh2-1* mutant (Leonard *et al*, 2003; Hoffman *et al*, 2004) using the previously described assay (Opperman *et al*, 2004). The assay construct contains two overlapping parts of the β -glucuronidase (*uidA*) gene (GUS gene), namely the U repeat, as shown for the GU-NPT-US construct in Fig 1. The U repeat is the segment in which mismatches were inserted. HR between the two direct repeats (U sequences) leads to the formation of an intact *GUS* gene and results in a blue sector on histochemical staining with the enzyme substrate 5-bromo-4-chloro-3-indolylglucuronide (X-gluc). We used a construct with two identical repeats (A0) and a construct with ten mismatches between the repeats (A10), as described previously (Opperman *et al*, 2004).

The *Atmsh2-1* mutant, in the background of ecotype Columbia, was previously shown (Leonard *et al*, 2003) to be a true knockout in the *AtMSH2* gene (At3g18524). This was confirmed in this study by reverse transcription (RT)-PCR (data not shown). The *Atmsh2-1* homozygote and the wild-type plants were transformed with the assay constructs (Fig 1). About 40 transformants from each of the four combinations studied (2 genotypes $\times$ 2 mismatch categories) were grown, representing a broad range of position effects in the genome. The number of spots per seedling was determined in 120–150 individuals of each combination and served as an estimate of the recombination frequency (supplementary information online). In the absence of mismatches (A0), the *Atmsh2-1* mutant and wild type showed a statistically similar frequency of recombination (Fig 2). In the presence of mismatches, there was a significant drop-off in recombination frequency in the wild type, of the same magnitude as that previously reported (Opperman *et al*, 2004). However, in the *Atmsh2-1* background, this drop-off was not observed. Conversely, there was an increase in recombination frequency, although this increase was not statistically significant.

Meiotic recombination

The role of *AtMSH2* in meiotic recombination was determined using tester line Le5-11/22 (Melamed-Bessudo *et al*, 2005) that is in the Landsberg background. This line harbours two seed markers, RFP and GFP, driven by a seed-specific *Napine* promoter, linked in *cis*, ~ 6 cM apart. This tester enables one to estimate meiotic recombination rates by counting seeds expressing both parental markers (red and green) or recombinant seeds

Fig 1 | Assay for recombination between divergent repeats. A schematic representation of the assay construct is shown, before (top) and after (bottom) recombination. The GU-NPT-US construct (top) contains two overlapping halves of the β -glucuronidase GUS gene, GU and US, with the U (= 618 bp) overlapping region and the NPTII gene in-between the repeats. Mutations are introduced in the U part of the GU half. Homologous recombination (HR) between the U direct repeats gives rise to an active GUS reporter gene (bottom). A blue sector is obtained following X-gluc histological staining in the cell in which HR occurred and in its daughters. RB and LB represent the right and left borders of the pMLBART binary vector.

Fig 2 | Recombination frequency between identical and divergent sequences in wild-type or mutant background. The recombination frequency is expressed as the average number of blue spots per seedling. It was determined for the recombination between identical (A0) and divergent repeats (A10) in wild-type and *Atmsh2-1* backgrounds. Black circles represent *Atmsh2-1*; black squares represent wild type. Statistical analysis between the different groups was carried out with the Wilcoxon's test for non-parametric variables. n.s. indicates that the difference in the distribution of spots between the groups was not statistically significant ($P(\chi^2) > 0.05$). ** and *** indicate that the difference between the groups was statistically significant with $P(\chi^2) < 0.05$ or $P(\chi^2) < 0.01$, respectively.

expressing only one marker (red or green). To test meiotic recombination in the mutant background, the marker genes were introgressed in *Atmsh2-1*, as shown in Fig 3. The tester is in the Landsberg background, whereas the mutant is in the Columbia background. Therefore, in this plant material, crossover occurred between two non-identical homologues. Recombination in the mutant *Atmsh2-1* background was compared with a control in the *AtMSH2* background. In both mutant and control, recombination rates were tested in the same genetic background, namely the BC₁F₂ generation (see Methods; Fig 3).

In each genotype, we tested 4–6 plants and an average of 500 seeds per plant (Table 1). *Atmsh2-1* showed a significant 40% percent increase in crossover rates between the markers (8.8 versus 6.3 cM) compared with the *AtMSH2* wild type (Table 1). Note that each of the markers segregated in a mendelian 3:1 ratio,

Fig 3 | Scheme for introgression of the green fluorescent protein and red fluorescent protein meiotic markers in the *Atmsh2-1* background. (A) The tester line with the green fluorescent protein (GFP) and red fluorescent protein (RFP) markers, in the Landsberg background, is on the left side in the top row. The RFP and GFP markers are marked by a red and green square, respectively. The *AtMSH2* locus is indicated by a + sign on a different chromosomal pair. On the right side is the *Atmsh2-1* mutant in the Columbia background. The mutated *Atmsh2* locus is shown by a – sign. As a control, the same scheme of crosses was carried out, using the wild-type *AtMSH2* (Columbia background) for the first cross (F_1) as well as for the backcross (BC_1). (B) Crossover rates are determined by counting seeds of the parental types, seen as red and green (r&g) or non-fluorescent (n) versus seeds of the recombinant types seen as red only (r) or green only (g). This analysis was carried out in the self-pollinated seeds from plants that were heterozygote for the tester markers and homozygote for *Atmsh2-1* (or homozygote for *AtMSH2* in the control), namely in the BC_1F_2 generation.

and the number of ‘green-only’ was similar to the number of ‘red-only’ seeds, indicating that there was no silencing of the marker genes.

DISCUSSION

Across-kingdom comparison of *MSH2* genes functions

This study shows the first example of a plant gene, *AtMSH2*, involved in the repression of HR between divergent sequences.

Repression was found for somatic recombination between direct repeats and for meiotic recombination between homologous chromosomes derived from different ecotypes. *MSH2*-mediated suppression of recombination between divergent repeats has been previously reported in yeast (Chen & Jinks-Robertson, 1999) and in mammalian cells (Elliott & Jasin, 2001) and thus seems to be a conserved feature across kingdoms. The role of *MSH2* in meiotic recombination between homologous chromosomes is less well known. Studies on the role of the mouse *MSH2* homologue in meiotic recombination have not been conclusive (Qin *et al*, 2002). Studies on meiotic recombination in yeast have focused on *MSH2*-mediated repression of meiotic recombination between repeats (Chen & Jinks-Robertson, 1999) or between homeologous chromosomes that originated from different species, for example, *Saccharomyces cerevisiae* versus *S. paradoxus* (Chambers *et al*, 1996). The divergence in nucleotide sequences between *S. cerevisiae* and *S. paradoxus* is 10% on average (Kellis *et al*, 2003). This divergence is associated with a decrease in recombination rates in the *MSH2* wild-type background. In the *msh2* mutant, recombination between the homeologous chromosomes was enhanced 5.5-fold (Chambers *et al*, 1996). In this study, we show a 40% increase in meiotic recombination rates in the absence of *AtMsh2* compared with the wild-type background. This increase is lower than that reported in yeast; however, it was found for recombination between homologous, rather than homeologous, chromosomes that diverge by ~2% at the nucleotide level in the region between the two markers in tester Le5-11/22. To our knowledge, this is the first report on *MSH2*-mediated repression of meiotic recombination between homologous chromosomes. We assume that the increase in meiotic recombination reported here results from the lack of suppression caused by sequence divergence between the two ecotypes, Landsberg and Columbia. Indeed, we found, in a different set of experiments (Fig 4), that when recombination between the RFP and GFP markers in tester Le5-11/22 is measured in an isogenic background (Landsberg $\times$ Landsberg), it is higher than in a non-isogenic background (Columbia $\times$ Landsberg). The same, divergence-related repression of recombination was found for a different tester (data not shown).

The anti-recombination mechanism

There are several alternative pathways that can lead to recombination between repeats (Prado *et al*, 2003). How exactly it occurs in plants is not fully understood, although there is evidence for the involvement of single-strand annealing pathways (Gorbunova & Levy, 1999; Puchta, 2005). With these pathways, the mismatched heteroduplex is formed following the annealing of two single-strand DNA molecules. Heteroduplex rejection and abortion of the recombination process would thus occur after annealing, possibly, as proposed in yeast (Sugawara *et al*, 2004; Goldfarb & Alani, 2005), by Msh2-mediated recruiting of Sgs1p, a helicase that would unwind the annealed region. During meiotic recombination, Holliday junction formation is associated with annealing of single-stranded DNA from two homologues, forming a heteroduplex-containing Holliday junction. Statistically, there is more chance for forming a heteroduplex when recombination is initiated between homeologous chromosomes that diverge by ~10% at the nucleotide level (for example, *cerevisiae* versus *paradoxus*) than between homologous chromosomes that diverge

Table 1|Recombination rates between the green fluorescent protein and red fluorescent protein markers in tester line Le5-11/22 in the background of *Atmsh2-1* compared with wild type

Genotype	Total seeds	Seed phenotype*						Percentage of recombination	Standard error
		Total red	Total green	Red only	Green only	Red and green	Non-glowing		
<i>Atmsh2-1</i>	550	401	402	25	26	376	123		
	508	374	380	17	23	357	111		
	505	374	371	23	20	351	111		
	537	388	387	22	21	366	128		
Total	2,100	1,537	1,540	87	90	1,450	473	8.81	± 0.68
Percentage of total		73.2 [‡]	73.3 [‡]						
<i>AtMSH2</i>	543	404	406	10	12	394	127		
	456	319	325	11	17	308	120		
	493	336	328	22	14	314	143		
	517	384	379	24	19	360	114		
	532	392	394	14	16	378	124		
	546	407	411	12	16	395	123		
Total	3,087	2,242	2,243	93	94	2,149	751	6.31	± 0.56
Percentage of total		72.6 [‡]	72.7 [‡]						

*Seed phenotypes were determined in the BC₁F₂ generation for both *Atmsh2-1* and *AtMSH2* plants, as described in Methods and in Fig 3.
[‡]These ratios do not differ from a mendelian 3 (coloured):4 (total) segregation: $P(\chi^2 > 0.13)$.

Fig 4|Divergence-related repression of meiotic recombination frequencies. Meiotic recombination between the red fluorescent protein and green fluorescent protein markers in tester Le5-11/22 is measured in an isogenic background (Landsberg × Landsberg) and in a non-isogenic background (Columbia × Landsberg).

by ~2%, as reported here, thus increasing the chances for recombination abortion. The mechanism whereby Msh2 mediates the rejection of heteroduplex-containing Holliday junctions is still unknown.

Biological significance and applications

The repressing effect of sequence divergence on HR is well known in plants, as well as in other species. In plants, this repression is necessary to stabilize the repeat-rich genome, to maintain barriers between species and to prevent promiscuous recombination

between homeologous chromosomes in allopolyploid species. For example, meiotic recombination between chromosomes or chromosomal segments of the edible tomato and those of its wild relatives is strongly suppressed (Rick, 1969; Chetelat *et al*, 2000). In hexaploid wheat, there is virtually no recombination between the homeologous chromosomes in the wild-type *PH1* background (Sears, 1976). The findings reported here, that *AtMSH2* is involved in anti-recombination between divergent DNA fragments in plants, are therefore relevant to the understanding of plant genome stability and evolution and to the improvement of crops by sexual transfer of genes from distant wild relatives. *AtMSH2* is certainly not the only barrier for gene transfer between distant species; however, this work suggests that it has an important role. Crops mutated in *MSH2*, or in other MMR genes, might therefore promote distant gene transfer. It has been suggested that the *PH2* locus that represses pairing between homeologous chromosomes in wheat might be an MMR gene (Dong *et al*, 2002).

METHODS

Plant material and *Agrobacterium*-mediated transformation. *Arabidopsis thaliana* and homozygous mutant plants were grown and transformed by *Agrobacterium*-mediated transformation, using the dipping procedure (Clough & Bent, 1998). Plants were transformed with the A0 and the A10 constructs described previously (Opperman *et al*, 2004). Transformed seeds (T₀) were collected, planted and selected for BASTA resistance. The selected plants (T₁) were seed harvested as pools for intrachromosomal recombination experiments (30–40 plants per pool).

Intrachromosomal recombination experiments and GUS staining.

Histochemical staining for GUS activity was carried out in 3-week-old plants, using the following staining protocol. Plants were grown on agar plates with 1/2 MS (Murashige and Skoog) medium (Duchefa, Haarlem, The Netherlands), 2% sucrose and 25 mg/l glufosinate. Growth conditions were 16–18 h of light at 25–28 °C. Plants were harvested and incubated for 24–48 h at 37 °C in sterile staining buffer containing 1 mg/ml X-gluc substrate (Duchefa) in a final concentration of 50 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.1% dimethylsulphoxide and 5 mM potassium ferricyanide and potassium ferrocyanide trihydrate (Sigma-Aldrich, Rehovot, Israel). Bleaching was carried out at 25 °C using 70% ethanol.

DNA extraction, genotyping and reverse transcription-PCR. Total genomic DNA was extracted from 50–100 mg young leaves, as described previously (Melamed-Bessudo et al, 2005). All the plants were genotyped using PCR. Primers that were used for insertion verification and RT-PCR analysis are salk_0020708L 5'-AGCGCAATTTGGGCATGTCT-3' and salk_0020708R 5'-CCTCCCATGTTAGGCCCTGTT-3'. Multiplex PCR reactions were carried out using these primers in combination with the LBb1 primer (5'-GCGTGGACCGCTTGCTGCAACT-3') recommended by the Salk Institute. PCR conditions were 3 min at 95 °C followed by 35 cycles of 93 °C 30 s, 60 °C 45 s, 72 °C 120 s and a final step of 8 min at 72 °C. Confirmation of the null phenotype was carried out using RT-PCR-based analysis (data not shown).

Meiotic recombination. The *Atmsh2-1* mutant previously described (Leonard et al, 2003) was crossed with the previously described tester line Le5-11/22 in the Landsberg ecotype background (Melamed-Bessudo et al, 2005). The GFP and RFP markers of this tester line are located on chromosome 5, 6 cm apart. To exclude hybrid seeds that might result from self-pollination, the tester line was used as male. Seeds of the F₁ generation expressing the GFP and RFP markers were sown and backcrossed with the homozygous mutant. The resulting F₁ plants were backcrossed with the mutant (BC₁ generation). BC₁ seeds were selected for expressing both RFP and GFP markers and for being homozygote for the *Atmsh2-1* mutation. The recombination rate was monitored in self-pollinated seeds of this *Atmsh2-1* homozygote BC₁ plants (BC₁F₂ generation), as described (Melamed-Bessudo et al, 2005). As a control for the effect of the mutation, the same scheme of crosses and backcrosses was carried out with wild-type (*AtMSH2*) Columbia plants. The recombination rate in the *AtMSH2* background was also checked in BC₁F₂ seeds.

Statistical analysis. The mitotic recombination data were analysed using the Wilcoxon's non-parametric test. With this test, the number of blue sectors of the respective mutant and the wild type are converted to rank order data. The ranks from each group are compared. The meiotic recombination data were analysed by two-way ANOVA. All statistical analyses were carried out using the JMP program 5.0.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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