Regulation of plant MSH2 and MSH6 genes in the UV-B-induced DNA damage response

Luciana D. Lario1, Elena Ramirez-Parra2,3, Crisanto Gutierrez2, Paula Casati1,* and Claudia P. Spampinato1,*

1 Centro de Estudios Fotosintéticos y Bioquímicos (CEFEB), Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina
2 Centro de Biología Molecular ‘Severo Ochoa’, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain
3 Present address: Centro de Biotecnología y Genómica de Plantas, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Universidad Politécnica de Madrid, Campus de Montegancedo, 28223 Pozuelo de Alarcón, Madrid, Spain

* To whom correspondence should be addressed. E-mail: casati@cefobi-conicet.gov.ar or spampinato@cefobi-conicet.gov.ar

Received 7 October 2010; Revised 27 December 2010; Accepted 7 January 2011

Abstract

Deleterious effects of UV-B radiation on DNA include the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6–4PPs). These lesions must be repaired to maintain the integrity of DNA and provide genetic stability. Of the several repair systems involved in the recognition and removal of UV-B-induced lesions in DNA, the focus in the present study was on the mismatch repair system (MMR). The contribution of MutSα (MSH2–MSH6) to UV-induced DNA lesion repair and cell cycle regulation was investigated. MSH2 and MSH6 genes in Arabidopsis and maize are up-regulated by UV-B, indicating that MMR may have a role in UV-B-induced DNA damage responses. Analysis of promoter sequences identified MSH6 as a target of the E2F transcription factors. Using electrophoretic mobility shift assays, MSH6 was experimentally validated as an E2F target gene, suggesting an interaction between MMR genes and the cell cycle control. Mutations in MSH2 or MSH6 caused an increased accumulation of CPDs relative to wild-type plants. In addition, msh2 mutant plants showed a different expression pattern of cell cycle marker genes after the UV-B treatment when compared with wild-type plants. Taken together, these data provide evidence that plant MutSα is involved in a UV-B-induced DNA damage response pathway.

Key words: Cyclobutane pyrimidine dimers, mismatch repair, MutS homologues, ultraviolet-B radiation.

Introduction

Absorption of ultraviolet-B (UV-B) radiation by DNA induces the formation of covalent bonds between adjacent pyrimidines, giving rise to cyclobutane pyrimidine dimers (CPDs) and, to a lesser extent, pyrimidine (6–4) pyrimidone photoproducts (6–4PPs) (Friedberg et al., 1995). These lesions have been shown to disrupt base pairing and block DNA replication and transcription if photoproducts persist, or result in mutations if photoproducts are bypassed by error-prone DNA polymerases (Britt, 1996). Thus, accumulation of such lesions must be prevented to maintain genome integrity, plant growth, and seed viability. Since plants constantly require sunlight for photosynthesis, and the amount of solar UV-B radiation reaching the biosphere is increasing due to the reduction of the stratospheric ozone layer, UV-B-induced DNA damage will become more important in the future. Even worse, plants lack a reserved germline, and mutations occurring in somatic cells could be transmitted to the progeny. Therefore, plants not only have evolved mechanisms that filter or absorb UV-B to protect against DNA damage (Mazza et al., 2000; Bieza and Lois, 2001), but also have different DNA repair systems to remove or tolerate DNA lesions (reviewed by Hays, 2002; Bray and West, 2005; Kimura and Sakaguchi, 2006). Here, the role of the mismatch repair (MMR) system in the response pathway to UV-B-induced DNA lesions was analysed.

The MMR system is best known for its role in the recognition and correction of mispaired or unpaired bases...
(reviewed by Modrich and Lahue, 1996; Kunkel and Erie, 2005; Schofield and Hsieh, 2003; Iyer et al., 2006). These mismatches result from replication errors, deamination of 5-methylcytosine, and recombination between divergent sequences. In addition, the MMR system is also involved in the recognition of damaged DNA bases induced by oxidation, alkylation, or UV irradiation in bacteria, yeast, and animals (reviewed by Kunkel and Erie, 2005; Iyer et al., 2006; Jiricny, 2006; Hsieh and Yamane, 2008; Li, 2008). Moreover, Culligan et al. (2000) have reported that the MMR system is able to recognize pyrimidine dimers in DNA.

In eukaryotes, the MMR system functions through a complex interaction among multiple proteins (Kunkel and Erie, 2005; Iyer et al., 2006; Hsieh and Yamane, 2008; Jiricny, 2006; Boland et al., 2008; Spampinato et al., 2009). The initial recognition of DNA errors is carried out by heterodimer complexes known as MutSα (MSH2–MSH6) and MutSβ (MSH2–MSH3). MutSα recognizes base–base mismatches and single insertion/deletion loops, while MutSβ mainly binds insertion/deletion loops of 2–12 bases (Acharya et al., 1996; Genschel et al., 1998). A third protein complex known as MutSγ (MSH2–MSH7) has been exclusively described in plants (Ade et al., 1999; Horwath et al., 2002; Tam et al., 2009). MutSγ recognizes some base–base mismatches (Culligan and Hays, 2000; Wu et al., 2003) and it was also reported to play a role in meiotic recombination (Lloyd et al., 2007). Subsequent to DNA lesion recognition, MutS complexes, in the presence of ATP, interact with MutLα to direct DNA repair (Prolla et al., 1994; Li and Modrich, 1995). MutLα is a heterodimer of MLH1–PMS2 in humans and MLH1–PMS1 in yeast and plants.

Previous studies performed in mammals indicated a rapid increase of MSH2 in epidermal cells after exposure to UV-B (Lu et al., 1999) and have shown that MSH2 or MSH6 deficiencies increase the susceptibility to UV-B-induced skin cancer (Meira et al., 2002; Young et al., 2004) and reduce the ability to undergo UV-B-induced apoptosis (Peters et al., 2003; Young et al., 2004; Narine et al., 2007; Seifert et al., 2008), suggesting that MSH proteins are implicated in cell cycle regulation. However, information on the role of the MMR system in post-UV responses in higher plants is still limited. An up-regulation of a transcript encoding a putative MSH protein was described in maize following UV-B exposure by microarray analysis (Casati et al., 2006). In this work, the in vivo contribution of MMR genes, MSH2 and MSH6, to the UV-B-induced DNA damage response in two plant species, Arabidopsis thaliana (MSH2, At3g18524; and MSH6, At4g02070), and maize was first investigated. By quantitative reverse transcription-PCR (RT-qPCR), it was shown that MSH2 and MSH6 transcripts are increased following a UV-B treatment. It was then demonstrated that msh2 and msh6 mutant plants accumulated more CPDs relative to wild-type (WT) plants. Finally, MSH6 was experimentally validated as an E2F target gene and it was demonstrated that msh2 mutant plants showed an altered expression pattern of cell cycle marker genes after the UV-B treatment in comparison with WT plants. Together, the data provide evidence that deficiencies in the MMR system affect UV-B-induced DNA damage repair and cell cycle regulation.

**Materials and methods**

**Plant material and growth conditions**

*Arabidopsis thaliana* ecotype Columbia (Col-0) and the T-DNA insertion lines were sown directly on soil and placed at 4 °C in the dark. After 3 d, pots were transferred to a greenhouse and plants were grown at 22 °C under a 16 h/8 h light/dark regime. For *in vitro* growth of plants, seeds were sterilized and incubated at 4 °C for 72 h before plating on MS (Murashige and Skoog salt) medium supplemented with 3% (w/v) sucrose and 0.8% agar. Seedlings were allowed to grow for 9 d followed by exposure to UV-B irradiation when indicated.

The maize (*Zea mays*) W23 line was grown in a greenhouse with supplemental visible light (1000 mE m⁻² s⁻¹) under a 15 h/9 h light/dark regime without UV-B for 28 d. Alternatively, plants were grown at the field.

The insertional msh2 (SALK_002708 and SALK_083844) and msh6 (SALK_089638) Arabidopsis lines were obtained from the Salk-T-DNA mutant collections (Alonso et al. 2003) while the CS90359 mutant was originated in the Arabidopsis TILLING Project. All these lines were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA).

**UV-B irradiation of plants**

*Arabidopsis* plants were exposed for 4 h to UV-B radiation from UV-B bulbs (Bio-Rad, Hercules, CA, USA) in a growth chamber, while maize plants were irradiated for 8 h with UV-B bulbs (F40UVB 40 W and TL 20 W/12; Phillips, Eindhoven, The Netherlands) in a greenhouse. UV-B lamps were covered with cellulose acetate filters (100 mm extra clear cellulose acetate plastic; Tap Plastics, Mountain View, CA, USA) and placed 30 cm above the plants. The cellulose acetate sheeting excludes wavelengths <280 nm but does not remove any UV-B radiation from the spectrum. The UV radiation intensities measured with a UV-B/UV-A radiometer (UV203 AB radiometer; Macam Photometrics, Scotland, UK) were 2 W m⁻² and 0.65 W m⁻² for UV-B and UV-A, respectively.

Control plants without supplemental UV-B radiation were exposed for the same period of time to light sources described above covered with polyester filters (100 μm clear polyester plastic; Tap Plastics). This polyester filter absorbs both UV-B (0.04 W m⁻²) and wavelengths <280 nm (UV-A radiation intensity was 0.4 W m⁻²).

Immediately after irradiation, samples from at least three independent biological replicates were collected, frozen in liquid nitrogen, and stored at –80°C.

**Quantitative RT-PCR**

Total RNA was isolated from ~100 mg of tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer’s protocol. The RNA was incubated with RNase-free DNase I (1 U ml⁻¹) following the protocol provided by the manufacturer to remove possible genomic DNA. Then, RNA was reverse-transcribed into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) as a primer. The resultant cDNA was used as a template for qPCR amplification in a MiniOPTICON2 apparatus (Bio-Rad), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter and Platinum Taq Polymerase (Invitrogen). Primers for each of the genes under study were designed using the PRIMER3 software.
Identification of insertional T-DNA mutants

The genotypes of plants with T-DNA constructs were determined using a PCR-based approach. Initial screening was performed using genomic DNA isolated from leaves by a modified cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russell, 2001) and three combinations of primers. Two primers hybridize specifically to CPDs (TDM-2) were from Cosmo Bio Co., Ltd (Japan). After treatment, plant samples (0.1 g) were collected, immediately immersed in liquid nitrogen, and stored at –80 °C. The induction of CPDs was determined using an assay described in detail previously (Stapleton et al., 1993).

DNA damage analysis

The induction of CPDs was determined using an assay described in detail previously (Stapleton et al., 1993). Monoclonal antibodies specific to CPDs (TDM-2) were from Cosmo Bio Co., Ltd (Japan). After treatment, plant samples (0.1 g) were collected, immediately immersed in liquid nitrogen, and stored at –80 °C. The expression of both genes has been previously reported to remain unchanged by UV-B treatment (Casati and Walbot, 2004; Ulm et al., 2004).

Table 1. Primers used for RT-qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-At-MSH2</td>
<td>TGGTAAACGTCGTTAATCCAG</td>
</tr>
<tr>
<td>RP-At-MSH2</td>
<td>TTCTTTCCCACTCTTTCGCA</td>
</tr>
<tr>
<td>RP-At-MSH6</td>
<td>ACTACCTGGCAGCAAGCTTAC</td>
</tr>
<tr>
<td>RP-At-MSH6</td>
<td>TACAGGCTTCCGTGATAGCC</td>
</tr>
<tr>
<td>RP-At-CPK3</td>
<td>AGGCTGCGGAAATGTCAAAATC</td>
</tr>
<tr>
<td>RP-At-CPK3</td>
<td>CGGTGTTAAGCTTCCAG</td>
</tr>
<tr>
<td>RP-Zm-MSH2</td>
<td>TACAGTGGAGAATGGAGAGT</td>
</tr>
<tr>
<td>RP-Zm-MSH6</td>
<td>GCCAGTCGATGGACTG</td>
</tr>
<tr>
<td>RP-Zm-MSH6</td>
<td>GGAAGCGACAACAGATGACT</td>
</tr>
<tr>
<td>RP-Zm-Trx like</td>
<td>CCATGTAACATGGCCTTCCAC</td>
</tr>
<tr>
<td>RP-Zm-Trx like</td>
<td>AGCAGACGAGCTGCAAG</td>
</tr>
<tr>
<td>LP-UVR2</td>
<td>GACCGAGCTGATATGGTGG</td>
</tr>
<tr>
<td>LP-UVR2</td>
<td>GAGCTGTTCCTGACGTTCCC</td>
</tr>
<tr>
<td>LP-UVR7</td>
<td>TACATTGGCGGCCTTGCTG</td>
</tr>
<tr>
<td>LP-UVR7</td>
<td>TCTCGTGTCTTCCAAAGG</td>
</tr>
<tr>
<td>LP-At-CYC1B1</td>
<td>AGTCCATGGCAAGAAG</td>
</tr>
<tr>
<td>LP-At-CYC1B1</td>
<td>CTGTTGGCTTACAGTGG</td>
</tr>
<tr>
<td>LP-At-H4</td>
<td>CAAGCTGCGCATGGAGAG</td>
</tr>
<tr>
<td>LP-At-H4</td>
<td>TCCATGGGCTGACAG</td>
</tr>
<tr>
<td>LP-At-Knolle</td>
<td>CGTCTTCAAGCTTACATGCT</td>
</tr>
<tr>
<td>LP-At-Knolle</td>
<td>AGACATATTTCTCAGAG</td>
</tr>
<tr>
<td>RP-At-EIF4</td>
<td>GAGCAAGGCTGCAAG</td>
</tr>
<tr>
<td>RP-At-EIF4</td>
<td>AGACGACGCGTCAAAATC</td>
</tr>
</tbody>
</table>

Table 2. Primers used for identification of homozygous mutant lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-M2-708</td>
<td>GCAAGACGCGAAGTGAAGTTC</td>
</tr>
<tr>
<td>RP-M2-708</td>
<td>GCCTTTCAGATATGCGTGGG</td>
</tr>
<tr>
<td>LP-M2-844</td>
<td>AAACCTGGATGCGTGGT</td>
</tr>
<tr>
<td>RP-M2-844</td>
<td>GGTGTTAGCGGGTGGG</td>
</tr>
<tr>
<td>LP-M6-688</td>
<td>GCTTTGTCAGCATGCTGTTG</td>
</tr>
<tr>
<td>RP-M6-3Psal</td>
<td>CGGCTCGATGGTAGTGGTTACGCC</td>
</tr>
<tr>
<td>LP-M6-BpE1</td>
<td>TGAGCGCTGGTTATCTGAGG</td>
</tr>
<tr>
<td>Rp-M6-BpE1</td>
<td>AGCTTCTGCGCTTTCTAGTGGTGGT</td>
</tr>
<tr>
<td>LB-Salk</td>
<td>ATATATGGCGGCTAAACGCTAG</td>
</tr>
</tbody>
</table>

Table 3. Nucleotide sequences of the probes used for EMSA analysis

Probe E2F consensus-wt contains a canonical binding site (Ramirez-Parra and Gutierrez, 2007) while the E2F consensus-mut contains two point mutations (underlined) within the core binding site.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSH6-E2F1-F</td>
<td>GTGGTTTCCCGCGCAATTAAGC</td>
</tr>
<tr>
<td>MSH6-E2F1-R</td>
<td>GTGGTTTCCCGCGCAATTAAGC</td>
</tr>
<tr>
<td>MSH6-E2F2-F</td>
<td>GACACCTTGCAGCGCTTGGT</td>
</tr>
<tr>
<td>MSH6-E2F2-R</td>
<td>GAAATAACGCCGCCGGAAGAGG</td>
</tr>
<tr>
<td>MSH6-E2F3-F</td>
<td>GAATAATCCCGGGGGCATCTT</td>
</tr>
<tr>
<td>MSH6-E2F3-R</td>
<td>AAAGATGTTGGGGGATGAGG</td>
</tr>
<tr>
<td>E2F consensus-wt-F</td>
<td>ATTAAGTTTGGGCGCCCTTCCCA</td>
</tr>
<tr>
<td>E2F consensus-wt-R</td>
<td>TTGAGAAGGCGCGCAAGCTTAAAT</td>
</tr>
<tr>
<td>E2F consensus-mut-F</td>
<td>ATTAAGTTTGGGCGCCCTTCCCA</td>
</tr>
<tr>
<td>E2F consensus-mut-R</td>
<td>TTGAGAAGGCGCGCAAGCTTAAAT</td>
</tr>
</tbody>
</table>
described (Ramirez-Parra and Gutierrez, 2000). For the competition experiments, 100-fold annealed unlabelled probe or unlabelled mutated probe (Table 3) was included in the reactions. After incubation at 4°C for 20 min, DNA–protein complexes were loaded onto 4% native polyacrylamide gels and electrophoresed in 0.5× TBE buffer. Labelled DNA–protein complexes were visualized by autoradiography.

Results

UV-B regulation of MMR transcripts in Z. mays and A. thaliana

To investigate the role of the MMR system in the UV-B-induced DNA damage response pathway in plants, the MMR transcriptional regulation was studied by RT-qPCR analysis. Two plant species were chosen for the analysis. *MSH* transcripts were first analysed in maize plants, as previous reports showed UV-B up-regulation of a transcript encoding a putative MSH protein by microarray analysis (Casati *et al.*, 2006). Experiments with this species were carried out under two different conditions. In the first set of experiments, plants were grown in a greenhouse without UV-B during 4 weeks and then irradiated with UV-B lamps in the absence (treated plants) or presence of a plastic that absorbs UV-B (control plants) (Fig. 1A). In a second experiment, plants were field grown for 21 d under either a polyester plastic that absorbs UV-B but transmits UV-A and white light (no UV-B treatment) or a control plastic that transmits all solar radiation, including UV-B (solar UV-B, Fig. 1B). Total RNA was extracted from leaf samples after each treatment and used for RT-qPCR analysis. A non-UV-B-regulated gene, encoding a putative thioredoxin protein, was included as a control. As shown in Fig. 1A, *MSH2* and *MSH6* transcript levels increased 5- and 2-fold, respectively, after 8 h of UV-B treatment in the greenhouse. However, *MSH2* and *MSH6* transcript levels were not significantly increased in field-grown maize plants after natural UV-B exposure (Fig. 1B). These results suggest that plants in the field are already acclimated to this radiation and show a different response to plants grown in the greenhouse that never received any UV-B.

UV-B regulation of *MSH* transcripts was also studied in *Arabidopsis* plants. Plants were exposed under UV-B lamps for 4 h (2 W m⁻²) in growth chamber conditions. After the treatment, leaf tissue from 4-week-old plants was collected for RNA extraction. In addition, plants were irradiated after flowering, and RNA was extracted from flowers to determine transcript levels of MMR genes after UV-B irradiation in reproductive tissues. Figure 1C shows that *MSH2* and *MSH6* transcript levels were significantly increased in leaves after UV-B irradiation. In flowers, a similar increase was observed for all *MSH* transcripts studied (Fig. 1D). Induction of *MSH2* and *MSH6* by UV-B was observed as early as 1 h after the UV-B treatment; levels were similar to those detected after a 4 h treatment (Supplementary Fig. S1 available at *JXB* online).

Fig. 1. Relative transcript levels of MMR genes from leaves of maize grown in a greenhouse (A), leaves of field-grown maize (B), and leaves (C) and flowers (D) of *Arabidopsis* measured by RT-qPCR. Plants were irradiated with UV-B light (UV-B) or kept under control conditions (control) as indicated in the Material and methods. Data show mean values ±SD of at least three independent experiments. Asterisks denote statistical differences applying the Student’s *t*-test (*P* <0.05).
Functional role of MSH proteins in repair of UV-B-damaged DNA

To investigate further the role of MSH proteins in the repair of UV-B-induced DNA lesions, several Arabidopsis mutants defective in MSH2 and MSH6 genes were identified in the SALK and Arabidopsis TILLING Project collections. For MSH2, two independent T-DNA insertional lines, SALK_002708 (msh2-1; Leonard et al., 2003) and SALK_083844 (msh2-2), with insertions in the seventh exon and the 5′-untranslated region (UTR), respectively, were identified (Supplementary Fig. S2 at JXB online) by a PCR screen using gene-specific primers and one specific primer for the T-DNA left border (Table 2). Insertional inactivation of MSH2 in the SALK_002708 line was confirmed by RT-PCR (Supplementary Fig. S2). The consequences of insertion of the T-DNA in the 5′ UTR from the SALK_083844 line was confirmed by RT-qPCR on plants homozygous for the mutant allele (Supplementary Fig. S2). The results indicate that the MSH2 transcript abundance in SALK_083844 lines was five times lower than in WT plants.

For the MSH6 gene, a T-DNA insertion in the 17th exon was obtained (SALK_089638, msh6-1). Supplementary Fig. S2 at JXB online shows that homozygous plants had undetectable levels of MSH6 transcripts when analysed by RT-PCR. The expected product (181 bp) was observed in the WT and in the msh2 homozygous lines, but not in the msh6 homozygote mutant (Supplementary Fig. S2). The CPK3 (At1g23650) gene was used as a control, and products of the correct size (183 bp) were present in all the genotypes analysed. A second mutant named the SALK_090359 line was obtained from the Arabidopsis TILLING Project collections (msh6-2). A point mutation in the MSH6 gene replaces a tryptophan codon (TGG) with a stop codon (TGA). As a result, a truncated protein of 226 amino acids is produced, instead of the 1324 amino acid WT protein (Supplementary Fig. S2). The genotype was identified by PCR using a pair of primers flanking the point mutation followed by digestion of the amplified product with the BpuEI restriction enzyme.

Excision products were separated by gel electrophoresis to confirm the presence of the point mutation (Supplementary Fig. S2).

None of the described mutants showed any visible phenotype under standard growth chamber conditions in the first and second generations. It is important to mention that the Atmsh2-1 insertion mutant showed a wide variety of abnormalities but only after the fifth generation (Hoffman et al., 2004).

To test the hypothesis that the MMR system can contribute to UV-B-induced DNA repair in vivo, Arabidopsis WT plants and the msh mutants were grown in the growth chamber in the absence of UV-B for 4 weeks, and plants were then exposed for 4 h to UV-B radiation (2 W m⁻²). As a control, different sets of plants were irradiated with the same lamps covered with a polyester plastic that absorbs UV-B (see Material and methods). Leaf samples from control and treated plants were collected immediately or 24 h after the end of the UV-B treatment. These plants were either kept under dark conditions or maintained under a 16 h photoperiod to allow photoreactivation. DNA was extracted and the CPD abundance was compared in each mutant relative to that in WT plants by an immunological sensitive assay; this assay detects CPDs by a monoclonal antibodies specifically raised against them. A calibration curve was generated by plotting signal values of UV-B-treated samples versus the corresponding amounts of DNA loaded. As shown in Supplementary Fig. S3 at JXB online, a linear relationship was observed up to 2 μg of DNA; thus, 1.5 μg of DNA was used for each sample in the analysis. Comparison of the CPD accumulation in samples from WT and mutant plants after the UV-B treatment and in control conditions in the absence of UV-B is shown in Fig. 2. In the absence of UV-B, the steady-state levels of CPDs in WT and msh mutants were similar (~200 optical density (IOD) in all samples). However, after 4 h of exposure to UV-B radiation, a significant accumulation of CPDs in msh2-1 and msh2-2 mutants than in the WT plants; this was true both in light (Fig. 2A, C) and in dark conditions (Supplementary Fig. S4 at JXB online). In addition, both msh6 mutants also accumulated more CPDs (65% in msh6-1 and 85% msh6-2) than WT plants (Fig. 2B, C). After 1 d of recovery in the absence of UV-B, CPD levels in the msh2 and msh6 mutants were still higher than in WT plants (Fig. 2), demonstrating that the repair of UV-B-induced DNA lesions is less efficient in these two lines of MMR mutants, under both dark and light conditions that allow photoreactivation. To discard the possibility that the mutations in both MSH genes may be affecting the expression of DNA repair enzymes of other repair systems, transcript levels of UVR2 (encoding a CPD photolyase, At1g12370) and UVR7 [encoding ERCC1, a DNA excision repair protein of the nucleotide excision repair (NER) system, At3g05210] were similar in WT and MMR-deficient plants, both under control conditions and after the 4 h UV-B treatment (Fig. 3). These results suggest that major CPD removal mechanisms are unaffected in mutant plants and that both MSH2 and MSH6 indeed participate in UV-B-induced DNA repair.

E2F-dependent transcriptional regulation of MSH6

Taking into consideration the finding that MSH genes are up-regulated after UV-B exposure and that MutSα is also required for cell cycle arrest and apoptotic responses in mammalian cells, a search for putative E2F-binding sites in the MSH promoter sequences was conducted. The family of E2F/dimerization partner (DP) transcription factors are key components of the cyclin/retinoblastoma/E2F pathway that participate in controlling cell cycle transitions in multicellular organisms, in both animals and plants (Gutierrez et al., 2002). In plants, E2F factors contribute to the transcriptional induction of genes upon DNA damage (Shen, 2002; Mariconti et al., 2002; Ramirez-Parras et al., 2007; Lincker et al., 2008). Arabidopsis contains six functional E2F genes that, according to their structural and functional features, can be divided into two distinct groups. The first group...
includes three members, AtE2Fa (At2g36010), AtE2Fb (At5g22220), and AtE2Fc (At1g47870), which possess a conserved DNA-binding site, a heterodimerization domain, and a transactivation domain embracing a retinoblastoma-related (RBR)-binding site. These AtE2Fs associate with an AtDP protein (AtDPα or AtDPβ) to form a functional heterodimer which can specifically recognize E2F cis-elements, transactivate E2F-responsive reporter genes, and be negatively regulated by RBR protein (Mariconti et al., 2002; Shen, 2002).

The second group (E2F/DEL) includes novel members which contain two conserved DNA-binding domains and lack a dimerization domain (Mariconti et al., 2002; Shen, 2002).

To assess if MSH2 and MSH6 are targets of the E2F transcription factors, the presence of putative consensus E2F-binding sites in the promoter regions of the corresponding genes was first bioinformatically analysed. This analysis showed that the promoter sequence of MSH2 lacks any consensus E2F-binding sites but the MSH6 promoter region contains three consensus E2F-binding domains.

Fig. 2. CPD levels in the DNA of msh2-1 (A), msh6-1 (B), msh2-2 and msh6-2 (C), mutants relative to WT Arabidopsis plants after a 4 h UV-B treatment (UV-B) or under control conditions without UV-B (control). Experiments were performed under light conditions to allow photorepair. CPD levels were quantified immediately after the end of a 4 h UV-B treatment (A–C), and 1 d after the end of the light treatments (A and B). DNA was extracted immediately following the treatment or after 24 h. A 1.5 μg aliquot of DNA was loaded in each well. Results represent the average ±SD of three independent biological replicates. Asterisks denote statistical differences applying the Student’s t-test (\( P < 0.05 \)).
TTTCCGCCCAAT (at –161 bp), TTCGCCGGCG (at –141 bp), and ATTCCGCGCAA (at –114 bp), where the first and the third sites contain two consensus motifs (Fig. 4A). Specifically, the first binding site includes the forward sequence TTTCCCGC at –161 bp and the reverse sequence ATTGGCGG at –157 bp, whereas the third site contains the forward sequence ATTCCCGC at –110 bp and the reverse sequence TTGCGCGG at –114 bp. These results are consistent with previous reports which identified MSH6 as a target of E2Fa-DPa transcription factors by comparing the transcriptomes of WT plants with plants overexpressing E2Fa-DPa by Affymetrix ATH1 microarray (Vandepoele et al., 2005) or Affymetrix Tilling 1.0R arrays (Naouar et al., 2009).

To determine experimentally whether the above-described E2F sites in the MSH6 promoter mediate E2F binding, an EMSA was carried out using labelled double-stranded oligonucleotides corresponding to each E2F site (Table 3, Fig. 4A). Recombinant Arabidopsis E2Fa, E2Fe/DEL1, and E2Ff/DEL3 proteins bound to all these probes in a specific and E2F site-dependent manner (Fig. 4B). Addition of a competitor oligonucleotide containing a general E2F consensus site (TTTCGGCGC, Table 3) greatly reduced the specific complex, but the yield of pre-formed complexes was unaffected when the challenge was performed with a mutated version (TTTCGATC, Table 3) of the consensus sequence (Fig. 4B). Note that the specific complex could be competed out with an oligonucleotide containing the canonical binding site, thus reflecting that the MSH6 promoter is indeed involved in the binding to E2F consensus sequences. Thus, E2F is able to bind in vitro to any of the three sites in the MSH6 promoter, suggesting that it could regulate the in vivo expression of this gene.

To validate this hypothesis, a set of transgenic Arabidopsis seedlings with altered E2F expression (Ramirez-Parra and Gutierrez, 2007) were used and MSH6 mRNA levels in these plants were analysed by RT-qPCR. Cell division cycle 6 (CDC6, At2g29680), a well-characterized E2F target gene (Castellano et al., 2001), was used as a positive control. Figure 4C shows a significant increase in MSH6 expression in plants which overexpress the E2Fa.

---

**Fig. 3.** Relative expression of UVR2 (A) and UVR7 (B) transcripts by RT-qPCR. MMR-deficient msh2-1, msh2-2, msh6-1, and msh6-2 and proficient MMR (WT) Arabidopsis plants were irradiated with UV-B for 4 h (UV-B) or were kept under control conditions without UV-B (control). Data show mean values ±SD of at least three independent experiments. For each transcript analysed, different letters indicate significant statistical difference (P <0.05).
and E2Fb transcriptional activators. The overexpression of the transcriptional repressor E2Fc contributed to repress the expression of MSH6. In addition, MSH6 expression also increased in plants that overexpress the atypical E2Fs [E2Fd/DEL2 (At5g14960), E2Fe/DEL1 (At3g48160), and E2Ff/DEL3 (At3g01330)] (Fig. 4C). Finally, MSH6 transcript levels were evaluated in plants overexpressing RepA, a viral protein which increases endogenous E2F activity by inactivating the RBR protein (At3g12280) through physical interaction, and in plants overexpressing a mutated version of RepA protein (RepA E198K), in which RBR interaction is abolished (Desvoyes et al., 2006). The results indicate that MSH6 transcript levels increased in the RepA-expressing plants relative to the RepA mutant plants (Fig. 4D). It should be noted that the interaction between E2F and RBR can result in either activation or inactivation of the expression of gene targets (Shen, 2002; Vandepoele et al., 2005). The differential regulation depends on the complex activator or repressor functions of the E2F-binding sites (Egelkroot et al., 2001; Ramirez-Parra and Gutierrez, 2007). Taken together, these results demonstrate that E2F family members can bind to the MSH6 promoter and regulate the expression of the gene.

Contribution of the Arabidopsis MMR system in the cell cycle response induced by DNA damage

A good indication of alterations in cell cycle progression is derived from measurements of transcript levels of genes that change during the cell cycle (Menges et al., 2005). Thus, to assess the role of MSH2 and MSH6 in cell cycle regulation after UV-B irradiation, the mRNA levels of HISTONE H4 (At2g28740, an S-phase-specific associated gene), CYCB1;1 (At4g37490, a G2 phase-specific gene), and CYCB1;4 (At2g26760, a mitotic cyclin) and KNOLLE (At1g08560, a G2/M transcription encoding a protein required for
cytokinesis) were measured in WT, msh2, and msh6 seedlings by RT-qPCR after UV-B irradiation. As shown in Fig. 5, the expression of HISTONE H4 was not increased by the UV-B treatment in seedlings of either WT, msh2, or msh6 mutant plants. In contrast, CYCB1;1 was up-regulated in seedlings from WT plants or msh6 mutants after the treatment (Fig. 5). This increase may represent an additional role for CYCB1;1 in the DNA damage response, as previously reported (Culligan et al., 2006). However, this CYCB1;1 transcript accumulation by UV-B was not observed in the msh2 mutants. On the other hand, CYCB1;4 as well as KNOLLE transcript levels were repressed in WT plants and msh6 mutants but remained unchanged in msh2 mutants (Fig. 5). Eukaryotic translation initiation factor 4A-1 (EIF4A1, At3g13920), a non-cell cycle-regulated gene, was used as a control. Taken together, these data indicate that msh2 plants had strikingly different effects on the expression of cell cycle marker genes compared with WT and msh6 plants.

The transcriptional regulation of MSH genes from maize and Arabidopsis after UV-B exposure was first studied. Using RT-qPCR, it was demonstrated that MSH2 and MSH6 transcript levels increase in maize leaves (Fig. 1A, B) after UV-B irradiation, although to different extents depending on the growth conditions. This difference may reflect an adaptive response of field-grown plants to UV-B, suggesting that the DNA repair systems are already induced in these plants to protect them from continuous DNA damage. MSH2 and MSH6 transcript levels were also significantly induced in Arabidopsis leaves (Fig. 1C) and flowers (Fig. 1D) by UV-B radiation. Increased MSH2 levels were also reported in mice epidermal cells after exposure to UV-B (Lu et al., 1999). Thus, the present results suggest that MutSα may be involved in the repair of UV-B-induced DNA damage. To investigate further if MSH2 and MSH6 genes are regulated by other conditions, the expression of both genes was checked in the eFP-browser under other abiotic stress conditions, such as cold, salt, drought, heat, wounding, and osmotic and genotoxic stress. All these stress conditions induce a down-regulation of MSH2, except the genotoxic stress, while MSH6 is only down-regulated by drought, heat, and genotoxic stress (Supplementary Table S1 at JXB online). Thus, abiotic stress conditions mostly down-regulate the expression of MSH2 and MSH6, in contrast to the up-regulation measured by UV-B in the present experiments. However, some hormone treatments (1-aminocyclopropane-1-carboxylate, zeatin, indole acetic acid, abscisic acid, methyl jasmonate, and brassinolide) significantly increased MSH6 transcripts levels, while MSH2 was only induced by brassinolides (Supplementary Table S1). Together, MSH2 and MSH6 are regulated by different abiotic stress and hormone treatments besides UV-B conditions.

To gain further insights into the response of the MMR system in UV-B-induced DNA repair in plants, the consequences of MSH2 and MSH6 disruptions in UV-B-induced DNA damage accumulation were evaluated. The
study was restricted to the analysis of CPD levels by an immunological approach. The results show that msh2 and msh6 plants were less efficient at removing UV-B-induced CPDs relative to WT plants, either immediately or 1 d after irradiation (Fig. 2, Supplementary Fig. S4 at JXB online). It is worth mentioning that DNA damage under control conditions was comparable in all plant genotypes studied. Thus, CPDs were more efficiently repaired in MMR-proficient than MMR-deficient Arabidopsis plants. The msh mutant plants showed similar levels of transcripts for UV R2 and UV R7, both under control conditions and after UV-B treatment, than WT plants (Fig. 3), thus indicating that major CPD removal pathways are unaffected in msh-deficient plants. The data correlate with several reports indicating an increased incidence of UV-B-induced skin tumorigenesis in MMR-defective mice (Meira et al., 2002; Yoshino et al., 2002; Young et al., 2004). In particular, the present results demonstrate that MutSα activity contributes to the removal of CPDs from Arabidopsis leaf tissues.

The functional relevance of MutSα in cell cycle progression in response to UV-B irradiation was further investigated. The major regulators controlling the expression of cell cycle genes are members of the E2F family of transcription factors. Interestingly, MSH6 has been identified as a target of the E2F transcription factors (Vandepoele et al., 2005). The MSH6 promoter contains three E2F-binding sequences, with two of them comprising double E2F-binding sites (Fig. 4A). All the sites were shown to be bound in vitro by E2Fa-DPa, E2Fe/DEL1, and E2F/DEL3 (Fig. 4B). Further investigation of the regulation of MSH6 expression showed an increased level of MSH6 transcripts in plants that overexpress the activators E2Fa and E2Fb (Fig. 4C). Previous results have shown that the Arabidopsis E2Fa gene is maximally expressed shortly before the S-phase peak while E2Fb transcripts accumulate maximally at the G1/S transition (Mariconti et al., 2002). On the other hand, plants that overexpress the E2Fc repressor showed lower MSH6 mRNA levels than WT plants (Fig. 4C). MSH6 transcripts also increased in plants overexpressing E2Fd/DEL2, E2Fe/DEL1, and E2Fi/DEL3. Both E2Fc and E2Fd/DEL2 increase during the progression into S-phase and peak after the passage into G2, while E2Fe/DEL1 and E2Fi/DEL3 are expressed at both the G1/S and S/G2 transitions (Mariconti et al., 2002). These results suggest that E2F transcription factors regulate the expression of MSH6, and consequently MMR activity, through cell cycle progression.

Finally, the transcriptional expression of cell cycle marker genes in msh2 or msh6 mutants after UV-B-induced DNA damage was studied. The expression of HISTONE H4 is linked to the S-phase whereas the expression of CYCB1;1 reaches a maximum at the G2/M transition and the expression of the CYCB1;4 and KNOLLE proteins show a characteristic and sharp peak during mitosis. RT-qPCR results showed that the transcript levels of the CYCB1;1 gene increased 2-fold in WT seedlings and the msh6 mutant, following UV-B irradiation, whereas that of HISTONE H4 was unaltered (Fig. 5). Up-regulation of CYCB1;1 appears to be related to DNA-damaging treatment (Culligan et al., 2006; Ramirez-Parra and Gutierrez, 2007). In addition, a significant decrease of CYCB1;4 and KNOLLE transcript levels was observed in WT seedlings and msh6 mutants. However, all these changes in the cell cycle marker genes were not evident in the msh2 mutants (Fig. 5). Taken together, these findings indicate a functional link of the MMR system to the cascade induced by UV-B damage, as the transcript levels of CYCB1;4 and KNOLLE genes remained unchanged in msh2 seedlings but were down-regulated in WT and msh6-deficient plants. It is important to note that MSH2 is an essential component of the MutS complexes, while the function of MSH6 could be replaced by MSH3 or MSH7. Additional evidence that shows a functional interaction between MMR and cell cycle genes has been demonstrated in mammalian cells. Reports indicate that MSH2 or MSH6 deficiencies were associated with reduced levels of apoptosis in mice (Young et al., 2004) and human cells (Narine et al., 2007; Seifert et al., 2008) in response to UV-B radiation.

In conclusion, in this work it was first demonstrated that both MSH2 and MSH6 transcripts are increased by UV-B irradiation in maize and Arabidopsis. Then, it was shown that UV-B induced the accumulation of CPDs in msh2 and msh6 mutants relative to WT plants. Further analysis validated MSH6 as a target of the E2F transcription factors, thus suggesting an interaction between MMR genes and the cell cycle control. Finally, it was demonstrated that msh2 mutant plants showed a different expression pattern of cell cycle marker genes after the UV-B treatment relative to WT plants. Taken together, the data provide evidence that plant MutSα is associated with the repair of CPDs and may be involved in the control of cell cycle progression following UV-B irradiation.

Supplementary data
Supplementary data are available at JXB online.

Figure S1. Time course analysis of MSH2 and MSH6 UV-B regulation in WT Arabidopsis leaves. Data show mean values ± SD of at least three independent experiments.

Figure S2. Location of the T-DNA insertions and polymorphisms in the MSH2 and MSH6 genes and analysis of their transcript levels. Exons are represented by black boxes, introns by thin black lines, and the UTRs by a white box. T-DNA insertions are indicated by triangles. Transcript levels were evaluated either by PCR followed by agarose gel electrophoresis or by RT-qPCR. Amplifications were performed using the primers shown in Table 3: LP- and RP-At-CPK3 for CPK3 (lane 1), LP- and RP-At-MSH2 for MSH2 (lane 2), and LP- and RP-At-MSH6 for MSH6 transcripts (lane 3) on RNA extracted from homozygous mutants or WT lines. Punctual mutation was detected by PCR amplification using LP-At-M6BpuE1 and RP-At-M6BpuE1 primers and genomic DNA from msh6-2 homozygous and WT lines followed by cleavage with BpuE1
restriction enzyme (New England Biolabs) and analysis of the excision products on agarose gels. Two migrating fragments (150 bp and 50 bp, line 5) were obtained for the mutant due to the enzymatic cleavage of the PCR product, whereas only one species (200 bp) was observed for the mutant in the absence of enzyme (line 4) and for the WT (line 6) due to resistance to enzymatic cleavage. The MW line shows the 50 bp molecular weight markers.

Figure S3. Calibration curve for the quantification analysis of dot-blot assays for CPDs accumulated in DNA of WT Arabidopsis leaves.

Figure S4. CPD levels in the DNA of msh2-1 relative to WT Arabidopsis plants after a 4 h UV-B treatment (UV-B) or under control conditions without UV-B (control). Experiments were performed under dark conditions to decrease photoreactivation. CPD levels were quantified before and immediately after the end of a 4 h UV-B treatment. DNA was extracted immediately following the treatment. A 1.5 μg aliquot of DNA was loaded in each well. Results represent the average ±SD of three independent biological replicates. Asterisks denote statistical differences applying the Student’s t test (P <0.05).

Table S1. Transcriptional regulation of MSH2 and MSH6 by different stress conditions (A) or hormonal regulation (B). Data were obtained from eFP-browser (Winter et al., 2007). Up-regulated transcripts are in red, while down-regulated transcripts are in green.

Acknowledgements

We thank the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA) for providing seed stocks. This research was supported by FONCyT grants PICT 2007-00711 and 2006-00957 to PC, Fundación Antorchas and PIP-5388 to CPS, grants BU2009-9783 and CSD2007-00057-B (Ministry of Science and Innovation) and P2006-GEN0191 (Comunidad de Madrid) to CG, and by an institutional grant from Fundación Ramón Areces to CBM. CPS and PC are members of the Researcher Career of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and LDL is a fellow of this Institution. We also thank to the European Molecular Biology Organization (EMBO) for a Short term fellowship to LDL to visit the Centro de Biología Molecular ‘Severo Ochoa’.

References


Culligan K, Hays J. 2000. Arabidopsis MutS homologs—AtMSH2, AtMSH3, AtMSH6, and a novel AtMSH7—form three distinct protein heterodimers with different specificities for mismatched DNA. The Plant Cell 12, 991–1002.


