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Rapid Communication

DNA Mismatch Repair in Plants[1]

An *Arabidopsis thaliana* Gene That Predicts a Protein Belonging to the MSH2 Subfamily of Eukaryotic MutS Homologs

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Sets of degenerate oligomers corresponding to highly conserved domains of MutS-homolog (MSH) mismatch-repair proteins primed polymerase chain reaction amplification of two *Arabidopsis thaliana* DNA fragments that are homologous to eukaryotic *MSH*-like genes. Phylogenetic analysis places one complete gene, designated *atMSH2*, in the evolutionarily distinct *MSH2* subfamily.

In contrast to multicellular animals, plants lack a reserved germ line; their gametes are formed late in their growth cycles by differentiation of somatic meristematic cells. Typically, the somatic precursors of gametophytes have divided many times, potentially subjecting their genomes to multiple rounds of spontaneous or environmentally induced mutagenesis (Walbot, 1985). However, plants do not seem to show extraordinarily high mutation rates. For example, long-lived trees presumably produce gametes from somatic cells that are themselves the products of many annual cycles of mitotic growth. Nevertheless, their mutation rates per zygote-to-meiosis generation, averaged over long reproductive lives, are only one order of magnitude or so higher than those of annuals (Klekowski, 1997).

How do plants combat the threats to genomic stability posed by somatic mutation? Mechanisms of selection against less-fit somatic cells during growth and development have been reviewed extensively by Klekowski (1988). Diploid meristematic cells that acquire even partially dominant deleterious mutations may drop out of the actively dividing pool, which is termed "diplontic selection." Additional sieving out of recessive mutations may occur when haploid cells compete to form sperm or eggs. However, Klekowski has suggested that these mechanisms alone are not powerful enough to protect plants against rapid accumulation of extraordinary mutational loads (Klekowski, 1988, 1997). Genomic-fidelity functions must therefore be at least as efficient in plants as those of microbes and animals, if not more so.

Estimates of spontaneous mutation frequencies suggest that DNA is replicated both in the bacterium *Escherichia coli* (Radman et al., 1981) and in human cells (Loeb, 1991) with error rates of approximately 10^{-11} to 10^{-10} per bp per generation. This appears to be typical of other organisms (Echols et al., 1991). To the extent that endogenous events such as depurination, deamination, and oxyradical attack contribute to mutant frequencies, actual replication error rates may be even lower. The limit of DNA polymerase accuracy mechanisms—primarily base selection, exonucleolytic proofreading, and inefficient extension of unpaired primer termini—appears to be about 10^{-7} to 10^{-8} (Radman et al., 1981), leaving a factor of 10^2 to 10^3 to be accounted for by postreplication error-correction processes.

Highly conserved long-patch mismatch-excision-repair activities that efficiently correct basemispairs and insertion/deletion "loopouts" have now been described in a wide variety of organisms (Modrich and Lahue, 1996). In bacteria MutS proteins bind mismatches and MutL proteins couple the recognition complexes to specific incisions of nascent DNA strands. *E. coli* achieves strand discrimination by delaying adenine-methylation at d(GATC) sites; MutH protein, when activated by a mismatch-MutS-MutL complex, incises the unmethylated strand at the nearest hemimethylated d(GATC) site, whether it is 5′ or 3′ to the mismatch. Excision of the incised strand then proceeds, respectively, 5′ to 3′ or 3′ to 5′ toward the mismatch. Bacteria naturally lacking both d(GATC) methylation and MutH proteins initiate repair from strand-specific nicks produced in some other way.

The MutS-MutL paradigm has been remarkably well conserved throughout evolution. Mismatch-repair systems in *Drosophila melanogaster* and human cells also show bidirectional excision capabilities (Fang et al., 1993); they efficiently repair in vitro substrates in which nicks have been placed either 5′ or 3′ to the mismatch in a particular strand. Genetic analyses have revealed multiple yeast and human homologs

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Abbreviations: RACE, 5′-rapid amplification of cDNA ends; RT-PCR, reverse-transcriptase PCR.

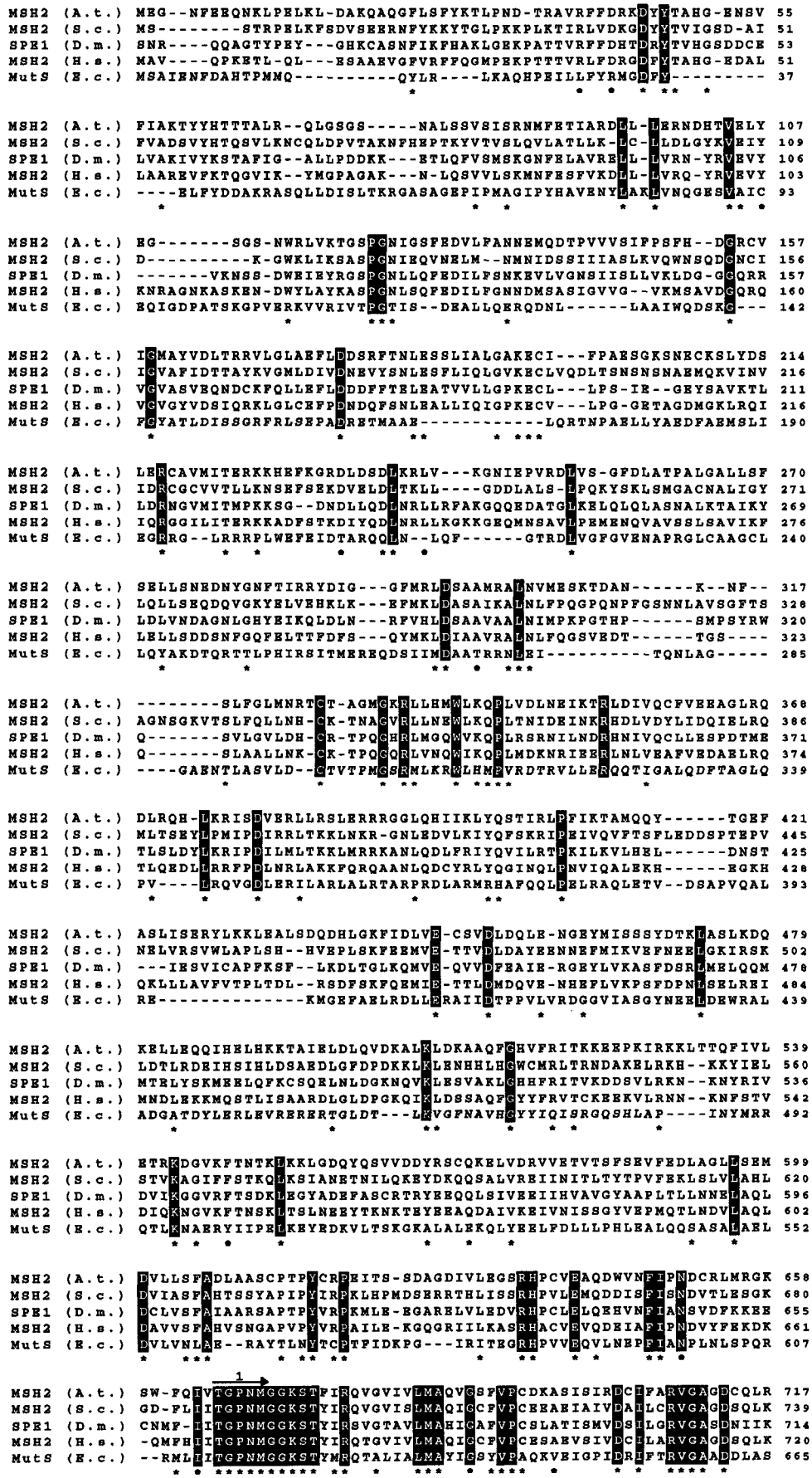

Figure 1. (Figure continues on facing page.)

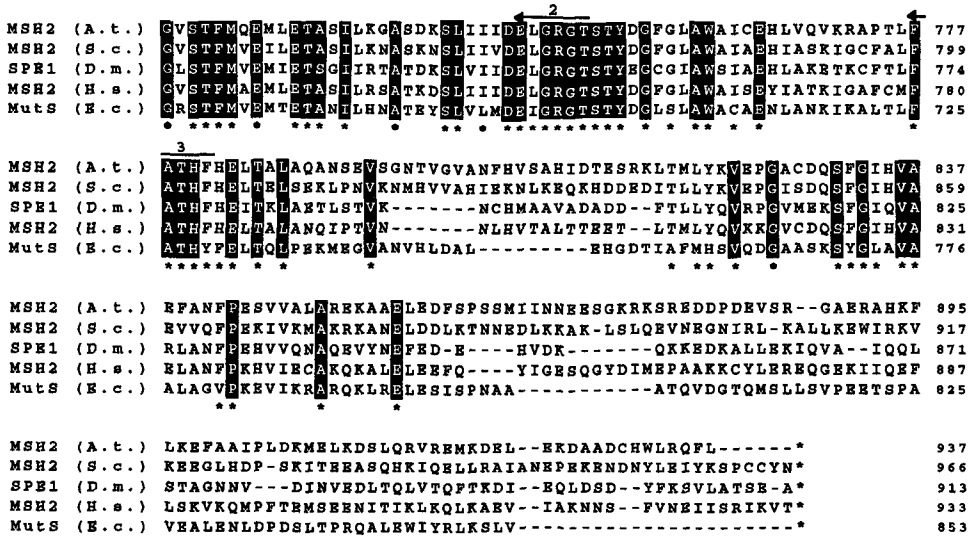

Figure 1. (Figure continued from facing page.) Alignment of MSH2-like proteins. Sequences were deduced from *MSH2* genes of *Arabidopsis thaliana* (A.t.), *Saccharomyces cerevisiae* (S.c.), *Drosophila melanogaster* (D.m.), and *Homo sapiens* (H.s.), and from *Escherichia coli* (E.c.) MutS. Black boxes indicate identical amino acids for all sequences listed; asterisks denote identical amino acids conserved in eukaryotic sequences; and dashes denote gaps. Degenerate primer locations are designated by arrows. Sequence comparisons (CLUSTAL method) were performed using Genetic Data Environment. All sequences were retrieved from GenBank. The complete *atMSH2* cDNA and protein sequences have been deposited as GenBank accession number AF002706.

of the single bacterial *mutS* and *mutL* genes (Marsischky et al., 1996; Modrich and Lahue, 1996); this multiplicity appears to be widespread among eukaryotes. Although the members of these broad homolog families show strong conservation of amino acids in certain critical regions, individual subfamilies show their own individual lines of evolutionary descent, apparently reflecting functional specialization. Thus, yeast *yMSH2* and human *hMSH2* appear more similar to one another than to *yMSH3* and *yMSH6* or *hMSH3* and *hMSH6*, respectively, and similarly for *MSH3* and *MSH6* homologs (Marsischky et al., 1996; Modrich et al., 1996). Biochemical studies and genetic studies in yeast and human cells suggest that mismatch recognition is carried out by hMSH2·hMSH6 or hMSH2·hMSH3 complexes, with different but overlapping substrate specificities (Acharya et al., 1996; Habraken et al., 1996; Johnson et al., 1996; Marsischky et al., 1996; Palombo et al., 1996).

As well as correcting replication errors, mismatch-repair systems also preserve genetic fidelity by antagonizing genetic recombination between imperfectly homologous DNA sequences on the same or different chromosomes (or on exogenous DNA fragments). The strong, positive effects of mismatch-repair deficiency on genetic exchange (Rayssiguier et al., 1989), on meiotic recombination involving partially diverged chromosomes (Hunter et al., 1996), and on recombination between imperfect repeats in the same genome (Petit et al., 1991) suggest that mismatch-repair systems help maintain interspecies barriers and protect chromosomes against rearrangements (Rayssiguier et al., 1989). This second role for mismatch repair has obvious implications for the fertility of hybrids between diverged plant species and for targeted alteration of plant genes by homologous recombination. Finally, both bacterial (Feng et al., 1991) and human mismatch-repair proteins (Mu et al., 1997) recognize some UV photoproducts and chemical adducts in DNA. To the extent that mismatch repair excises incorrect bases opposite UV photoproducts more efficiently than it excises canonical bases (Mu et al., 1997), this third function of mismatch repair may avert some mutagenic consequences of the inevitable exposure of plants to solar UV-B radiation.

Our long-range goal is to determine the extent to which MutSL-like mismatch-repair systems maintain the genetic integrity of plant genomes. Here we have addressed an initial question: do plants encode homologs of MutS proteins, and if so, do these homologs fall into the same distinct MutS-homolog subfamilies seen in other eukaryotes? We describe below the isolation of two *Arabidopsis thaliana* gene fragments encoding MutS-like proteins, using PCR amplification of DNA or RNA with degenerate primer sets based on evolutionarily conserved MutS amino acids. Phylogenetic analysis suggests that the complete sequence of one gene is an *MSH2* homolog.

MATERIALS AND METHODS

Growth Conditions

Arabidopsis seeds (ecotype Columbia) were sterilized in 50% commercial hypochlorite bleach, washed five times in sterile water, and aseptically grown in 250-mL flasks containing 100 mL of liquid Murashige-Skoog medium (Murashige and Skoog, 1962) with 0.5% Suc (pH 5.8). After 14 d seedlings were harvested for isolation of DNA (Murray et al., 1980) or mRNA (RNeasy RNA isolation kit [Qiagen,

Chatsworth, CA]; mRNA separator kit, [Clontech, Palo Alto, CA]).

PCR Techniques

We employed degenerate primer-oligonucleotide sets corresponding to highly conserved MutS/MSH2 protein domains (Fig. 1): primer 1, TGPNM (coding strand) 5′-AGI GGI CCI AA(T/C) ATG GG-3′; primer 2, ELGRGT (noncoding strand) 5′-GT ICC IC(T/G) ICC IA(A/G) (T/C)TC-3′; and primer 3, FATH(Y/F)H (noncoding strand) 5′-TG (G/A)(T/A)A (G/A)TG IGT IGC (A/G)AA. PCR amplification was performed in 100-μL reaction mixes containing 10 μL of 10× reaction buffer (Promega), 250 μmol of dNTPs, 2 units of *Taq* DNA polymerase (Promega), and 20 pmol each of degenerate primer pairs. A PCR optimization kit (Invitrogen, San Diego, CA) was used to optimize Mg^{2+} and pH conditions for each primer pair. Templates for initial PCR screening reactions using primers 1 and 3 were either 20 ng of genomic DNA or 100 ng of cDNA (produced from purified mRNA [see above] using an RT-PCR kit [Perkin-Elmer] in accordance with the instructions of the manufacturer). Amplification was carried out for 30 cycles, 30 s at 94°C, 30 s at 42°C, and 3 min at 72°C. We analyzed reaction products by electrophoresis on 2.5% agarose gels. Where cDNAs templated 0.35-kb products corresponding to the expected distance between primers 1 and 3 in *MSH2*-like sequences, these products were isolated and utilized as templates in PCR reactions, under similar conditions, using primers 1 and 2. These reactions yielded the expected 0.26-kb "nested" products. Among individual products initially templated by genomic DNA using primers 1 and 3, those (0.35 kb and larger) that were repeatedly obtained under a variety of reaction conditions were isolated and analyzed with primers 1 and 2 as above. PCR products templated by cDNA or genomic DNA using primers 1 and 3, and authenticated using primers 1 and 2, as above, were cloned in the T/A vector pCRII (Stratagene).

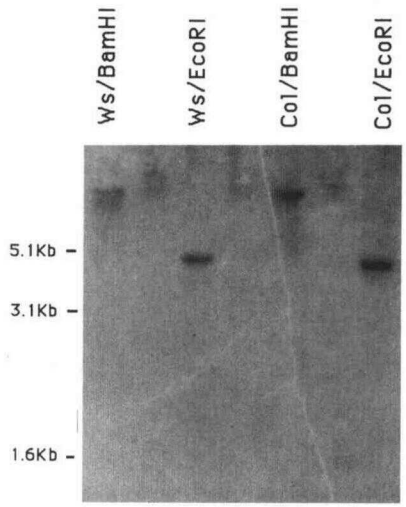

Figure 2. Southern analysis of genomic DNA from *A. thaliana* (ecotypes Columbia and Wassilewskija). A ^{32}P-labeled fragment (corresponding to genomic positions +2871 to +3518 of the DNA sequence deposited in GenBank) was used to probe genomic DNA digested with *Eco*RI or *Bam*HI restriction endonucleases.

Southern Hybridization

Arabidopsis genomic DNA was digested with *Eco*RI and *Bam*HI restriction enzymes, and 3 μg of the resulting fragments was separated by electrophoresis in 1% agarose. Transfer to nylon paper was as described (Maniatis et al., 1982). A ^{32}P-labeled probe was prepared by random priming of a 0.7-kb *atMSH2* clone using a DNA labeling kit (DECAprime II, Ambion, Austin, TX). Hybridization and subsequent washes were at 42 and 60°C, respectively. Final wash conditions were 2× SSC buffer (Maniatis et al., 1982) and 0.5% SDS, at 60°C for 30 min.

Isolation of cDNAs

We probed approximately 10^5 plaques of a phage/plasmid λ-YES vector Arabidopsis (ecotype Columbia) cDNA library with a 352-bp ^{32}P-labeled Arabidopsis *MSH2*-like DNA fragment (see above). Plaque purification, plasmid rescue, and cDNA isolation as described (Hoffman et al., 1996) yielded three partial cDNAs, 1 to 2 kb in length, encoding the same 3′-terminal *MSH2*-like sequences. The 2-kb partial cDNA clone was further used to probe a library of 3- to 6-kb Arabidopsis cDNAs inserted into phage λ ZAPII (Kieber et al., 1993; available from the Arabidopsis Biological Resource Center, Ohio State University, Columbus). Infection of XL1-Blue MRF′ cells with phage stocks prepared from five positive plaques, plus helper phage, yielded phagemids selectable by growth of transfected bacteria on ampicillin (100 μg/mL) plates (Bullock et al., 1987). In two cases, digestion with *Eco*RI endonuclease released 3.1-kb fragments, sufficient to encode a full-length *MSH2*-like cDNA.

Isolation of 5′-RACE Products

5′-RACE was performed using a Marathon cDNA Amplification Kit (Clontech) with purified mRNA (see above) as described by the manufacturer. The second-strand-synthesis cDNA products, ligated to blunt-ended Marathon adaptors, were used as templates in 50-μL PCR reactions containing 5 units of KlenTaq DNA polymerase mix (Clontech), 200 pmol of adaptor primer (AP1), 200 pmol of *atMSH2* gene-specific primer (see below), 5 μL of 10× reaction buffer, and 200 μM dNTPs. We employed a "touchdown" protocol to reduce background products: 1 cycle at 94°C for 1 min; 5 cycles at 94°C for 30 s and 72°C for 4 min; 5 cycles at 94°C for 30 s and 70°C for 4 min; 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 4 min. The gene-specific primer sequence (5′-ACC TCA GAG AAG CTG GTA ACA GTC-3′) corresponded to position +1763 relative to the first ATG of the longest open reading frame in the *MSH2*-like 3.1-kb cDNA (see above). The single product, a 1.9-kb fragment, was purified by agarose gel electrophoresis and inserted into the T/A cloning vector pCRII.

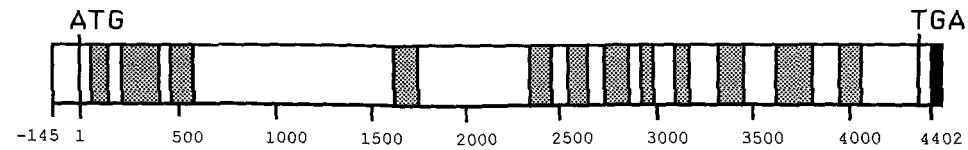

Figure 3. Structure of the *atMSH2* gene. Comparison of genomic and cDNA sequences revealed 12 introns (shaded boxes) throughout the longest reading frame of *atMSH2* (denoted as genomic position +1 [ATG] to +4259 [TGA] of genomic sequence). The black box 3′ to position +4402 denotes poly(A)$^+$ sequences observed in cDNAs. The genomic sequence has been deposited as GenBank accession number AF003005.

Genomic Clone Isolation

Genomic clones were identified by probing a λ ZAPII genomic library (Stratagene) with the 3.1-kb cDNA fragment, and phagemids were isolated as described above. Five genomic clones spanned the entire region of the full-length cDNA. Their coding-region sequences were identical with the cDNA sequences.

Analysis of DNA Sequences

Sequences were obtained through the Oregon State University Central Services Laboratory using a DNA sequencer and *Taq* Dye-Primer/Dye-Terminator cycle sequencing kit (Applied Biosystems), as described by the manufacturer, plus synthetic internal primers as needed. Sequence alignments and construction were performed using GCG (University of Wisconsin Genetics Computer Group, Madison), version 8.0. CLUSTAL alignments and phylogenetic analyses were performed using Genetic Data Environment.

RESULTS AND CONCLUSIONS

We utilized two sets of degenerate PCR primers, corresponding to all possible codons for three domains of five to six amino acids each that are highly conserved among eukaryotic and prokaryotic MutS-like proteins (Fig. 1) to amplify and clone segments of Arabidopsis cDNA and genomic DNA. Aseptically grown seedlings were used to reduce false positives from amplification of homologous sequences encoded by contaminating organisms. A 0.7-kb genomic DNA product that was consistently obtained under a variety of PCR reaction conditions using primers 1 and 3 was purified and reamplified using primers 1 and 2 (Fig. 1), yielding a 0.4-kb "nested" product. Similarly, RT-PCR consistently yielded a 0.35-kb fragment, which, when purified and reamplified with primers 1 and 2, yielded a 0.26-kb "nested" product, as predicted (Fig. 1). The 0.7- and 0.35-kb fragments proved to encode the same MutS-like protein; hybridization analysis demonstrated that they are fragments of an Arabidopsis gene (Fig. 2).

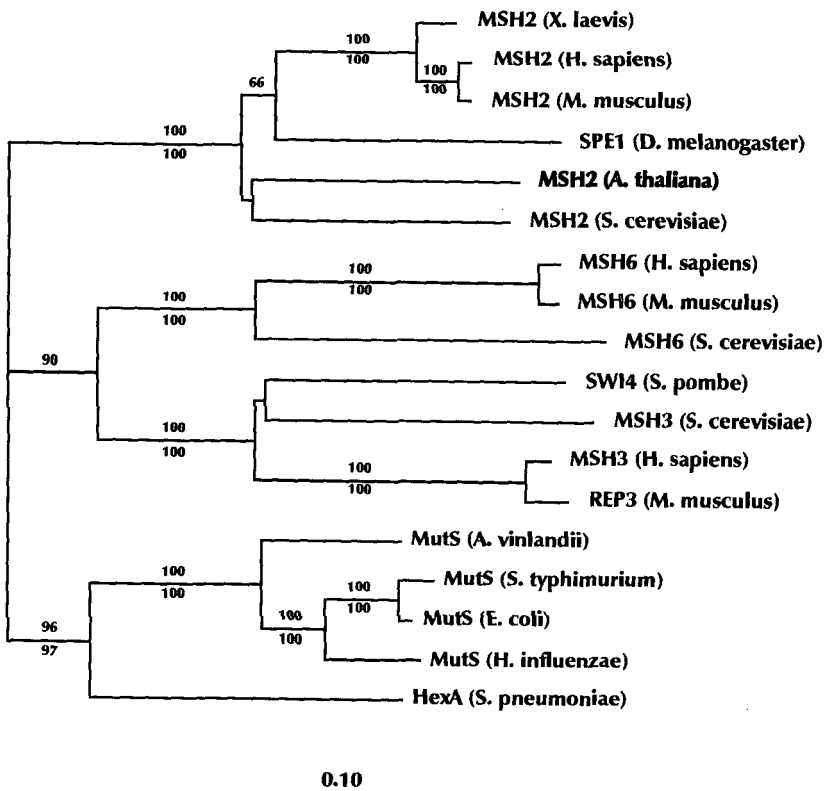

Figure 4. Phylogenetic analysis of MutS homologs. CLUSTAL sequence alignments were masked to exclude sequence gaps and analyzed with two phylogenetic methods to create two distinct trees using Phylip version 3.5. A neighbor-joining distance tree was constructed using PROTDIST (specifying the Dayhoff PAM matrix), and a protein parsimony tree was constructed using PROTPARS. A representative distance tree is shown; both trees showed very similar branching patterns. Bootstrap values (significant above 60) are shown above and below tree branches for distance and parsimony consensus trees, respectively. The distance branch-length scale (bottom) denotes 0.10 expected substitutions per sequence position. *S. cerevisiae* MSH1 (excluding the 21-amino-acid mitochondrial targeting sequence) was used as an outgroup (not shown). We believe MSH1 to be the most distant from the sequences used in this analysis, but it may be related to both prokaryotic and eukaryotic homologs. *S. cerevisiae* MSH4 and MSH5 were excluded because of low bootstrap confidence values for their phylogenetic relationships, and because these proteins appear to have significantly diverged, in both sequence and function, from other MutS homologs. Analyses were performed using Genetic Data Environment.

To clone the corresponding cDNA, we probed two Arabidopsis λ phage cDNA libraries with the 0.7-kb genomic DNA fragment. Five individual cDNAs were isolated, and two that were 2.0 and 3.1 kb in size were sequenced. To independently confirm that the 3.1-kb isolate encoded a complete cDNA, we used Arabidopsis 5′-RACE using a gene-specific "right-hand" primer site approximately 1.2 kb 5′ to the poly(A)$^+$ tails in the previously isolated cDNAs. The 5′-RACE product, corresponding to cDNA sequences 5′ to the primer site, was approximately 1.9 kb in length, indicating a full-length cDNA to be 3.1 kb.

The longest open reading frame identified, 2814 bp in length, would encode a protein of 937 amino acids, similar in size to other MutS homologs. The predicted amino acid sequence shows strong homologies with the MSH2 class of mismatch-repair proteins (Fig. 1); it is 37% identical to both yeast and human MSH2 proteins. We designate this gene *atMSH2*.

The fact that only a single Arabidopsis DNA restriction fragment hybridized to the 0.7-kb probe (Fig. 2) is an argument against a family of *atMSH2*-like genes with similar DNA sequences. However, the DNA sequences of genes encoding other Arabidopsis MutS-like proteins, for example, homologs of eukaryotic MSH3 or MSH6 proteins, might be expected to have diverged considerably. We randomly selected ten 0.35-kb clones and ten 0.26-kb clones, corresponding to RT-PCR products obtained using degenerate primer sets 1,3 and 1,2, respectively, and determined the DNA sequences. All 0.35-kb clones proved to be *atMSH2* fragments. Seven of the 10 0.26-kb clones were *atMSH2* fragments, but 1 encoded a protein fragment strongly resembling eukaryotic MSH6 proteins (deposited as GenBank accession no. AF009657). Two encoded an amino acid sequence more similar to yeast MSH2; the latter fragment did not hybridize to Arabidopsis DNA (data not shown), under conditions where an *atMSH2* fragment hybridized strongly (Fig. 2). In addition, the *MSH6*-like DNA fragment hybridized strongly to a single restriction fragment of Arabidopsis DNA under these conditions and the band pattern was distinct from that shown in Figure 2 for *atMSH2*, for each restriction enzyme tested (J. Leonard and J. Hays, unpublished results). These observations are consistent with the pattern of functionally specialized, evolutionary diverged MutS homologs observed in other eukaryotes (Modrich and Lahue, 1996).

Figure 3 schematically depicts the *atMSH2* gene structure deduced from the complete genomic sequence. The 12 introns in the coding sequence range in size from 80 to 230 bp, and all introns show consensus GT/AG splice signals. A plant consensus polyadenylation signal is present in the 3′ untranslated region (Mogen et al., 1990).

To determine the phylogenetic relationships of *atMSH2* to other *MSH*-like genes, we compared its predicted amino acid sequence with the sequences of all known eukaryotic MSH2, MSH3, and MSH6 proteins, and with those of bacterial MutS proteins. This also provided an opportunity to rigorously analyze the relationships of recently reported eukaryotic MutS-like protein sequences to one another. Figure 4 shows the resultant distance tree and the bootstrap values for the distance (top) and parsimony (bottom) consensus trees. Although *at*MSH2 branched with all other MSH2 sequences with a bootstrap confidence value of 100, it could not be unambiguously ordered within this subgroup. For example, there is a low bootstrap confidence value for the *Saccharomyces cerevisiae* MSH2 and *A. thaliana* MSH2 node. Preliminary analysis of the putative *at*MSH6 fragment places it in the MSH6 subgroup (data not shown). Furthermore, in both the distance and parsimony trees, the entire MSH3 and MSH6 subgroups branched together, with a bootstrap confidence value of 90 for the distance tree. This suggests that eukaryotic MSH3 and MSH6 proteins diverged from a common ancestral protein (itself distinct from MSH2), consistent with the functional overlap between MSH3 and MSH6 (Marsischky et al., 1996).

Recently, Cerovic and Radman demonstrated mismatch-specific excision repair of plasmid substrates in nuclear extracts prepared from tobacco callus and suspension-culture cells (G. Cerovic and M. Radman, unpublished data). These authors also reported the sequence of a PCR product, templated by Arabidopsis genomic DNA and cDNA. The cDNA sequence corresponds exactly to bp +1948 to +2300 of the *atMSH2* sequence deposited in GenBank. We suggest that plants employ mismatch-repair systems that are highly homologous to those found in other eukaryotes. In particular, we believe that their systems include at least two of the evolutionarily distinct MutS homologs described for yeast and mammals.

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