Rapid generation of plant traits via regulation of DNA mismatch repair

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Summary

The reversible inhibition of DNA repair is a novel approach to maximize genetic diversity within a plant’s genome in order to generate offspring exhibiting important de novo output traits. This process is based on the inhibition of the evolutionarily conserved mismatch repair (MMR) system. In this process, a human dominant negative MMR gene allele is introduced into the germline of a target plant, yielding progeny that can be screened to identify variants with commercially important agronomic output traits. Using this novel strategy, we generated MMR-deficient Arabidopsis thaliana plants that showed genome-wide instability of nucleotide repeats associated with chromosomal microsatellites, in addition to base substitution mutations. Functional screenings of the MMR-deficient Arabidopsis offspring identified variants expressing selectable traits (ethylene insensitivity and salt tolerance), as well as plants exhibiting altered morphologic traits (albinos and dwarfs). We determined by segregation analyses of variant plants that the de novo phenotypes were due to both recessive and dominant genetic mutations. Mutations caused by MMR deficiency showed a different spectrum compared with those derived using ethylmethane sulphonate (EMS) mutagenesis. Our finding demonstrates the feasibility of using reversible MMR deficiency via transient expression of a single human gene product to enhance genetic diversity in plants.

Introduction

Dramatic increases in crop yields in the 20th century have been attributed mainly to genetic improvements introduced through plant breeding (Phillips, 1993). However, this major accomplishment has been achieved through the genetic manipulation of only a few loci. It is widely believed that the demands of the expanding world population, climate changes, limited availability of arable lands, water shortages, soil salinization and erosion will strain the ability of the agricultural industry to meet the world’s increasing food needs. Therefore, strategies to generate new crop varieties with enhanced output traits are required (Miflin, 2000). Part of modern plant breeding relies on the genetic diversity obtained via insertional, chemical and radiation-induced mutagenesis. These methods may suffer from: (i) low frequencies of the mutational events; (ii) a limited spectrum of genomic changes attainable; (iii) high toxicity and difficult recovery of the mutated progeny when using chemical and radiation mutagenesis; and (iv) limitations to only loss-of-function or gain-of-function mutation in the case of insertional mutagenesis. Alternatively, genetic engineering can be employed to over-express previously characterized gene products to attain specific phenotypes; however, this method requires the understanding of the pathways controlling the specific phenotypes and the physical cloning of the genes involved in such pathways.

Mismatch repair (MMR) is a highly conserved process used by prokaryotes and eukaryotes to correct single base pair mismatches and slippage mutations within repetitive mono-, di- and trinucleotide repeats that commonly occur during DNA polymerization (Modrich, 1994; Kolodner and Marsischky, 1999). Independent studies have demonstrated that MMR-deficient cells or whole organisms exhibit a significant increase in frequency of point mutations and microsatellite instability (MSI) characterized by 1–4 base pair insertions/deletions within repetitive nucleotide tracts (Leach et al., 1993; Parsons...
et al., 1993). Although MMR-defective cells are hypermutable, they, as well as MMR-defective multicellular mammalian organisms, remain viable (Parsons et al., 1993; Baker et al., 1995). The human PMS2 (postmeiotic segregation increased 2) is a gene involved in MMR processes whose product has been found to dimerize with MLH1 (MutL homologue 1). The PMS2-MLH1 complex is believed to represent the human homologue, which couples the human mutS homologue (formed by the MSH2.MSH6 (MutS homologue 2/6) heterodimer) to the downstream DNA excision repair process (Kolodner and Marsischky, 1999). The PMS2-134 allele encodes a truncated form of PMS2 and was first identified in kindreds affected with hereditary non-polyposis colorectal cancer (Parsons et al., 1995). Subsequent studies demonstrated that the ectopic expression of the PMS2-134 gene product in eukaryotic cell lines exerted a dominant negative activity on the endogenous MMR apparatus in the presence of wild-type PMS2, leading to a ‘mutator’ phenotype (Nicolaides et al., 1998). Based on this principle, a reversible mutator phenotype strategy has been employed to create genetic diversity in mammalian production lines secreting therapeutic antibodies, with the goal of enhancing titres and binding affinity (Grasso et al., 2004).

The role and biology of MMR in plants are not as clearly defined as they are for mammalian and microbial organisms (Britt, 1999). Several components of the MMR machinery have been cloned by homology and low-stringency hybridization techniques employing MMR gene homologues from different species (Culligan and Hays, 1997; Ade et al., 1999; Jean et al., 1999). The analysis of the complete Arabidopsis genome sequences has also identified additional MMR gene homologues, including PMS2 (The Arabidopsis Genome Initiative, 2000). Recently, Hoffman et al. (2004) reported that MSH2 knockout in Arabidopsis resulted in a hypermutable phenotype similar to that observed in mammalian systems. Because of the high sequence homologies between mammalian and plant MMR genes, we hypothesized that the expression of the human PMS2-134 transgenic product in plants may result in a mutator phenotype. We reasoned that, if the PMS2-134 gene product can enhance genetic diversity in plants, offspring exhibiting important agronomic output traits could be generated using this approach.

In this article, we report the engineering of the transgenic PMS2-134 Arabidopsis thaliana. Genetic analyses of these plants showed that the activity of the human PMS2-134 gene was able to confer a dominant negative effect on MMR, leading to hypermutation within the plant genome. Importantly, the MMR-deficient plants yielded a wide range of variant offspring with de novo phenotypes, such as altered petal colour patterns, size, ethylene insensitivity and salt tolerance, while the PMS2-134 transgene was successfully crossed out after the desired phenotype, imparted by an endogenous plant gene, was selected. In addition, the PMS2-134-mediated MMR deficiency in Arabidopsis led to a broader spectrum of mutations and a higher mutation frequency compared with ethylmethane sulphonate (EMS)-mediated mutagenesis. This technology offers a novel approach to develop plants with genetically diverse germplasm, and complements other methods currently used in agriculture to develop elite strains of plants for commercial use.

Results

Increasing genetic diversity via induction of MMR deficiency

To test our hypothesis that the expression and activity of the PMS2-134 gene product in the germline of Arabidopsis plants may result in a mutator phenotype, we introduced a binary expression vector, containing the cDNA of the human PMS2-134 allele and a kanamycin (KAN)-resistant gene cassette, into A. thaliana (At) plants via Agrobacterium-mediated infection, and selected for KAN-resistant T1 seedlings. The expression analysis of these seedlings demonstrated that 79 of the 200 KAN-resistant T1 seedlings (hereafter referred to as At-PMS2-134 lines) expressed various levels of PMS2-134 RNA at the steady state (Figure 1). To distinguish between mutations induced by PMS2-134 activity and those potentially caused by the transformation procedure, we also generated A. thaliana plants that were transformed with a control vector (pBI121). We next assayed for the ability of the PMS2-134 gene product to cause genetic hypermutability in Arabidopsis by monitoring MSI in the genome of At-PMS2-134 and control plants. Microsatellite sequences consisting of mono- or dinucleotide repeats and representing simple sequence length polymorphisms (SSLPs) have been previously identified in the Arabidopsis genome and used for both genetic mapping (Bell and Ecker, 1994) and MSI monitoring. We arbitrarily chose and amplified the SSLP marker nga692 (consisting of GA dinucleotide repeats) from randomly sampled T2 plants derived from At-PMS2-134 or control lines, as described in ‘Experimental procedures’. We identified microsatellite contraction in two of 852 MMR-defective plants analysed (Figure 2). The altered alleles found in these two plants showed an apparent four-base deletion. To confirm the size of the deletion, we sequenced one of these mutated alleles and found that the contraction was due to the loss of two repeats, thereby changing the nga692 marker from (GA),13 in the wild-type to (GA),11. No alterations in the nga692 marker

were observed in more than 1000 transgenic control plants. These data indicate that the frequency of nga692 mutation is 0.12%, which is consistent with frequencies reported in MMR-defective mammalian cells (Parsons et al., 1995), and demonstrate the role of MMR in ensuring the integrity of the plant genome. We monitored the growth of three independently derived At-PMS2-134 lines for four generations and did not observe any differences in their germination and growth rate (see ‘Experimental procedures’), or in their reproduction and morphologic characteristics, compared with control plants, demonstrating that MMR deficiency is compatible with normal growth, reproduction and development. Noticeably, some of the mutants observed, such as albino and dwarf, showed a growth disadvantage, as expected.

MMR deficiency in plants leads to phenotypic variations

T3 seeds from three independent At-PMS2-134 lines (lines 111, 165 and 96) and control plants were screened for their ability to develop novel phenotypes (see ‘Experimental procedures’ for details on the propagation and screening schemes). Three thousand seeds per plate were plated in semisolid growth medium and scored for de novo phenotypes under non-selective conditions (Table 1). Albino mutant plants have been found to represent 0.4% of a single seed descendent family in heavily mutagenized populations using radiation or chemicals as mutagens (Feldmann et al., 1994). In addition, Jurgens (1992) observed albinos in 800 of 44 000 lines screened, corresponding to a frequency of 1.8%. During our screening, we observed albino mutants, including a chimeric albino plant (Figure 3A), with a frequency ranging from 1.8% to 5.3%
Mutations affecting plant hormone biosynthesis pathways have a higher preponderance of dwarf mutants via MMR deficiency. In 732 seeds screened, 19 dwarfs (19 dwarfs in 695 seeds screened) and seven dwarfs (seven dwarfs in 732 seeds screened), representing a 2.8-fold (2.73%) number of seeds screened) and 0.96% (seven dwarfs in 732 seeds screened) of dwarf mutants were noted, respectively, representing a 2.8-fold higher preponderance of dwarf mutants via MMR deficiency. In Arabidopsis, dwarfism has been previously demonstrated to reverse a subset of dwarf mutant phenotypes obtained through standard EMS mutagenesis. We tested 12 independent dwarf Arabidopsis plants derived from our three At-PMS2-134 lines. Our analysis found that one could be reversed to the wild-type phenotype via brassinosteroid and/or gibberellin supplementation, suggesting that the mutations had occurred in loci encoding for proteins or other factors of alternative pathways. In contrast, some of the EMS-derived dwarfs responded to the hormone supplementation treatments (data not shown).

Identification and characterization of agronomically important traits

Ethylene is a well-studied plant hormone that is involved in a variety of physiological processes, including fruit ripening, cell elongation, flower senescence, leaf abscission and sex determination. When germinated in the presence of ethylene in the dark, wild-type Arabidopsis plants exhibit the so-called ‘triple response’ consisting of exaggerated curvature of the apical hook, radial expansion of the hypocotyl and shortening of both hypocotyl and root (Guzman and Ecker, 1990). Ethylene-insensitive (Ein⁻) plants do not exhibit the triple response.

To explore whether our At-PMS2-134 plants could yield agronomically important output traits, such as ethylene resistance, a series of screenings were conducted under selective conditions. At-PMS2-134 lines 111, 165 and 96 were screened to identify ethylene-insensitive progenies by the absence of the triple response, as described previously (Guzman and Ecker, 1990). Screens were carried out by plateing 1000 seeds per plate, one plate for each of the 50 T2 plants per line (range, 40–57). Ein⁻ mutants were identified in each of the three lines with a frequency of 2% (three mutants in 150 T2 plants screened), which corresponds to at least a 20-fold increase over that obtained by chemical mutagenesis (range, 0.01%–0.1%) as reported previously by Guzman and Ecker (1990). In contrast, Ein⁻ plants were not observed in our control populations consisting of over 300 000 plants or in over one million wild-type Columbia (Col) A. thaliana plants (data not shown).

Table 1: Mutation frequencies of phenotypic mutants in PMS2-134-expressing single-seed descendant populations

<table>
<thead>
<tr>
<th>Independent transgenic line</th>
<th>Number of T2 descendants with phenotypic T3 mutants</th>
<th>Number of T2 descendants</th>
<th>dwf</th>
<th>albino</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>53</td>
<td>13 (24.5%)*</td>
<td>1 (1.8%)</td>
<td></td>
</tr>
<tr>
<td>165</td>
<td>57</td>
<td>30 (52.6%)</td>
<td>3 (5.2%)</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>40</td>
<td>10 (25%)</td>
<td>n/r</td>
<td></td>
</tr>
</tbody>
</table>

*Mutation frequency was calculated by dividing the number of descendants with mutants by the number of single-seed descendants screened, multiplied by 100.

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carried out on 100–200 seedlings from the Ein– plant as described in ‘Experimental procedures’. The Ein– mutant offspring were found to segregate at a ratio of three Ein– to one wild-type (data not shown, $\chi^2 = 0.04$), indicating that the defect in this mutant was most likely controlled by a single dominant mutant allele. The Ein– phenotype did not result from the genomic integration of the $\text{PMS2-134}$ gene, because the Ein– phenotype was found in only a subset of plants derived from the same At-$\text{PMS2-134}$ founder line (T1), and the Ein– phenotype was retained in the offspring whereby the $\text{PMS2-134}$ locus was lost through breeding, as discussed later. Finer genetic mapping was performed to localize the $\text{EIN}$ locus. An F2 population from a cross between a heterozygous Ein– mutant derived from a Col ecotype and a wild-type Landsberg erecta ecotype was segregated to map the linked allele, as described previously (Guzman and Ecker, 1990). The F1 progeny from this cross showed a clear segregation of the wild-type and Ein– plants at a ratio of 1 : 1, supporting the hypothesis that the phenotype is due to a single dominant allele. We employed a variety of validated polymorphic SSLP markers to perform linkage analysis on 1000 Ein+ plants from the F2 progeny, and analysed the data using Map Manager QTX software, as described in ‘Experimental procedures’. The mapping analyses suggested a linkage of Ein– with the AthCHIB marker and placed the linked allele between AthCHIB and nga172 markers on chromosome 3. Previous reports have identified a known ethylene receptor gene, termed $\text{EIN4}$, located in this region; the mutations in this gene have been shown to yield a dominant Ein– phenotype (Hua and Meyerowitz, 1998). Therefore, we considered $\text{EIN4}$ as a gene candidate and isolated it by polymerase chain reaction (PCR) from the mutant founder plant, the segregated offspring and the wild-type Col and Landsberg plants (see ‘Experimental procedures’). Sequence analysis revealed a G to A transition in the mutant at nucleotide position 1861 of the coding region of the $\text{EIN4}$ gene, which resulted in a non-conservative threonine to methionine amino acid change (Figure 4), thus confirming the ability of MMR-defective hosts to yield variant offspring containing single base pair mutations in functional genes. Interestingly, previous studies employing chemical mutagenesis found this same mutation, which was shown to be responsible for the Ein– phenotype via a dominant negative mechanism (Hua and Meyerowitz, 1998).

Salt tolerance is a highly desirable trait for farming because salinity stress is one of the factors limiting the viability and productivity of agricultural crops. In an attempt to generate salt-resistant mutants, we screened our seed library (generation T5, see ‘Experimental procedures’) and identified several mutant seedlings that could germinate and grow in medium containing a high concentration of sodium chloride (Werner and Finkelstein, 1995). One particular mutant (salt-tolerant germination, $\text{stg}$) was interesting in that it could germinate and grow normally for up to 3 weeks in medium containing 200 mM sodium chloride (Figure 5), while control seeds did not germinate under identical conditions. The nature of the mutation in this mutant was determined to be recessive by segregation analyses of F1 and F2 progenies in the cross with wild-type Landsberg ecotype, and not to be associated with the $\text{PMS2-134}$ insertion event. We observed an 80% penetrance for this trait, a phenomenon also observed by other researchers (Quesada et al., 2000). We mapped this trait in the region between marker R89998 (38.65 cm) and CDPK9 (44.55 cm) on chromosome 5. To our knowledge, none of the previously identified salt stress-related mutants have been mapped in this region (Werner and Finkelstein, 1995;
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Figure 5  Germination of salt-tolerant mutants (stg) in 200 mM sodium chloride medium. Seeds of Columbia (Col) wild-type and stg mutant were germinated under continuous light for 7 days in medium containing 200 mM sodium chloride, as described by Werner and Finkelstein (1995). The stg plants germinated and grew for up to 3 weeks despite the high concentration of salt.

Quesada et al., 2000, 2002). Further examination of this region of the Arabidopsis genome suggested the presence of several putative gene candidates, including cation:proton antiport open reading frames.

Restoration of genome stability and maintenance of trait expression

The major concerns about genetically modified plants relate to the possibility of harmful reactions that may affect consumers as a result of potential effects associated with the ingestion of recombinant proteins derived from the transgenes, as well as the risk of the transgene being introduced into wild plants. The phenotypes generated in this study are determined by mutations within endogenous plant genes. Once a desired phenotype is selected, two options are available: (i) breeding out the PMS2-134 transgene to restore the genetic stability and maintain the trait first selected; or (ii) maintaining the PMS2-134 transgene to select for additional traits. To demonstrate this, we selected F2 Ein- progeny from the cross between the Ein- mutant and wild-type (Col ecotype) plants, and assayed for the presence of the linked NPTII gene via KAN resistance. Of the 56 F2 Ein- seeds plated, we identified eight KAN-sensitive Ein- seedlings, indicating that the NPTII genes were segregated from the Ein- phenotype. RNA and DNA analysis of these NPTII null plants isolated from the Ein- phenotype confirmed the absence of the PMS2-134 gene locus (data not shown). These plants were genetically and phenotypically stable as demonstrated by: (i) a lack of MSI in the nga692 marker; (ii) absence of albino mutants over at least four generations; and (iii) maintenance of the ethylene insensitivity trait for at least four generations (data not shown). In addition, we were able to isolate albino mutants from Ein- plants that retained the PMS2-134 transgene (data not shown), providing the proof of principle for the use of our method to generate stacked traits.

Discussion

In this study, we demonstrated that the transient disruption of MMR processes in plants, mediated by a single human transgene product, PMS2-134, leads to genetically diverse offspring exhibiting a wide array of novel phenotypes identified under selective and non-selective conditions. This process is suitable for creating plant varieties with enhanced output traits but lacking foreign DNA fragments.

From a methodology standpoint, our transgenic-based approach, which relies on the negative dominant activity of PMS2-134 to produce reversible MMR deficiency, presents advantages over strategies involving MMR gene knockouts. In fact, in order to establish Arabidopsis knockout plants, large T-DNA insertion libraries need to be generated and screened by PCR amplification and DNA sequencing (Alonso et al., 2003). This undertaking may have a duration of up to 1 year, whereas a 3-month development time was required for the generation of our transgenic MMR-deficient Arabidopsis. Considering the fact that many crop plants are polyploid, such as soybean, potato, peanut, tobacco, wheat and oats, to name a few (Paterson et al., 2000), multiple copies of an essential MMR gene homologue (e.g. MSH2, PMS2) may need to be targeted in order to obtain an MMR-deficient plant. Unlike the transgenic approach described in this study, whereby a single copy of PMS2-134 is sufficient to inhibit DNA repair processes, the MMR gene knockout strategy may therefore not be practical for polyploid crop plants. Another viable approach for targeting MMR genes is RNA interference (Leonard et al., 2003).

Several components involved in MMR processes have been identified and cloned from A. thaliana based on homology (Culligan and Hays, 1997; Ade et al., 1999; Jean et al., 1999), including mutS and mutL homologues, whereas the genomic instability and increase in the mutational loading caused by MMR deficiency in Arabidopsis have been described elsewhere (Leonard et al., 2003; Hoffman et al., 2004). In this study, we showed that a protein encompassing 133 amino acids of the human PMS2 N-terminus possesses a dominant negative effect and is able to suppress the MMR apparatus in Arabidopsis. PMS2 dimerizes with MLH1 via its C-terminus (Nicolaides et al., 1998), whereas it is through MLH1 that PMS2 interacts with another critical MMR protein complex, the MSH2.MSH6 heterodimer (Plotz et al., 2003). Therefore, the negative
dominant activity of PMS2-134 could be exerted through its interaction with functionally conserved plant protein(s), different from MLH1, MSH2 or MSH6 homologues, which are essential for MMR processes. It is possible that such evolutionarily conserved proteins are the nucleases involved in the downstream DNA excision repair process.

Chemical mutagens, such as EMS, offer a robust method for generating genetic diversity in plants. When the dose is well balanced between desired and toxic effects, EMS-mediated mutagenesis almost exclusively results in G:C to A:T base substitution (Colbert et al., 2001). MMR deficiency obtained using our method produces both base substitution (Ein’ mutant) and deletion mutations (MSI), offering greater mutation diversity compared with an EMS-based approach. In addition, preliminary genetic analyses of our salt-resistant variants indicated the involvement of a locus never before reported to be associated with this phenotype, suggesting a different spectrum of mutations compared with other mutagenesis approaches.

Another advantage of our method over other mutagenesis techniques is the potential to generate plants exhibiting ‘stacked’ traits and to create maximal diversity as mutations gradually accumulate throughout the genome with each generation. This is supported by the fact that we were able to isolate albino mutants by simply propagating the Ein’ mutant plants before crossing out the PMS2-134 gene.

In summary, the process reported here offers some benefits over other mutagenesis strategies commonly used to produce maximum genetic diversity within selected crop plants. Some of these advantages include the wide spectrum of genetic alterations (base substitution and 1–4 base pair insertions/deletions), enhanced mutation frequencies and perpetual accumulation of mutation during propagation to produce multiple traits within the same line. Unlike genetic engineering strategies, this method does not require prior knowledge of the molecular mechanisms governing output traits, but instead is capable of randomly inducing in vivo genetic changes that affect endogenous plant genes. Because these attributes are important for maximizing genetic diversity, the method described here could potentially be used to efficiently generate plants exhibiting complex phenotypes.

**Experimental procedures**

**Plant materials and growth conditions**

Wild-type *A. thaliana* Col-0 and Landsberg erecta seeds were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH, USA). EMS-mutagenized *Arabidopsis* seeds were purchased from Lehle Seeds (Cat No. M2E-02-04 with a frequency of initial cells with albino embryo mutations of 0.38; Round Rock, TX, USA). *A. thaliana* plants were grown in Percival growth chambers under a 16 h photoperiod at 22 °C.

To screen Ein’ mutants, seeds were germinated on one-half-strength Murashige and Skoog (0.5 × MS) agar containing 1 μM ethylene precursor, 1-aminoacyclopropane-1-carboxylic acid, which mimics the effect of ethylene. For salt-tolerant mutant selection, 0.5 × MS agar containing 200 mM sodium chloride was used and germinated seeds were selected after 7 days, as described by Werner and Finkelstein (1995). To determine potential phenotypic reversion of the dwarf mutants in the presence of growth hormones, seeds were germinated and grown on 0.5 × MS agar containing 10 μM gibberellin (GA3) or 1 μM brassinosteroïds, and adult plants were phenotyped.

**PMS2-134 expression vectors and transformation of *A. thaliana***

A HindIII-EcoRI endonuclease restriction DNA fragment, isolated from the plasmid pBI121 (Cat. 18313-015, Invitrogen, Carlsbad, CA) and containing the cauliflower mosaic virus (CaMV) 35S promoter, the glucuronidase (GUS) cDNA and the NOS terminator, was inserted into the pGPTV-KAN plasmid (ATCC, Cat. 77388) digested with endonucleases HindIII and EcoRI, resulting in the CMV-GUS-KAN vector. The GUS gene was then replaced with the full-length *PMS2-134* cDNA by insertion of the *Pmel* endonuclease restriction fragment, isolated from the *pIND-PMS134* plasmid, into the CMV-GUS-KAN plasmid digested with endonucleases EcoCR and Smal, resulting in the CMV-Morpho-KAN vector. This vector, which contains the *PMS2-134* cDNA under the control of the CaMV 35S promoter and NOS terminator, was fully sequenced to verify the integrity of the promoter and the *PMS2-134* cDNA. After *Agrobacterium*-mediated transformation of *A. thaliana* Col-0 via the flower dipping method (Clough and Bent, 1998) with the CMV-Morpho-KAN vector, T1 seeds were pooled from several transformed plants. KAN-resistant T1 plants were selected by plating the T1 seeds on 0.5 × MS agar medium containing 50 μg/mL KAN. The selected T1 plants were allowed to self-pollinate to generate T2 generations. Adaptor-ligated PCR was used to confirm the insertion of T-DNA and to identify the position of the inserted T-DNA (Sperti et al., 1999).

**Propagation of transgenic lines**

In this paper, we refer to ‘line’ as a set of plants propagated through various generations from a single and independent
KAN-resistant T1 plant, having the transgene inserted in a distinctive genomic location. Three (At-PM52-134 lines 111, 165 and 96) of the 200 T1 KAN-resistant lines were selected based on expression levels of the PM52-134 transgene and uniqueness of the transgene insertion (as shown by adaptor-ligated PCR). Seeds were independently collected from each T1 plant per line and plated on 0.5 × MS agar to produce an average of 50 seedlings per line (range 40–57; see Table 1), representing generation T2. The remaining T2 seeds were appropriately stored. T2 plants were grown to maturity in soil and T3 seeds were harvested. T3 seeds from all of the T2 plants per line were germinated in separate plates, and phenotypic variants (albino and dwarfs), ethylene-insensitive mutants and germination rates were recorded. Approximately 600 T3 seedlings per line were randomly chosen, transferred to soil and grown to maturity. T4 seeds were combined from the three At-PM52-134 lines to generate a library of seeds that was propagated up to generation T8. This library was used: (i) to screen for salt-resistant variants (generation T5); and (ii) to compare mutation frequencies between MMR deficiency and EMS-mediated mutagenesis (generation T6).

Northern blot analyses

Total RNA was isolated from the rosette leaves of 2-week-old seedlings using the RNeasy kit (QIagen, Valencia, CA), and polyA+ RNA was isolated with oligo(dT) columns. For reverse transcriptase coupled PCR (RT-PCR), 1 µg of total RNA or 200 ng of polyA+ RNA was subjected to first-strand cDNA synthesis using oligo(dT) primers, and the synthesized products were then used as templates for PCR amplification using gene-specific primers (AA1–AA134), which do not amplify endogenous Arabidopsis MutL transcript. Arabidopsis tubulin gene-specific primers were used as an internal control. For Northern analysis, 20 µg of total RNA was separated on a 1.2% formaldehyde-agarose gel, transferred to Hybond-N+ (Amersham, Piscataway, NJ) membrane and probed with gene-specific probes labelled using South2North kit (Pierce, Rockford, IL).

Amplification and analysis of SSLPs

Genomic DNA was isolated from rosette leaves of 2-week-old seedlings using DNAZol (Invitrogen). A pair of primers was used: nga692-F with the sequence of TTTAGAGAGAGAGA-GAGCGCGG (TET-labelled) and nga692-R with the sequence of AGCGTTTAGCTCAACCCTAGG. PCR was used to amplify the SSLP marker nga692 using the genomic DNA as template (Bell and Ecker, 1994). The amplified products were loaded on to an ABI373 sequencer and historchromographs were generated using GeneScan software (Applied Biosystems, Foster City, CA). For sequence determination, the amplified products were separated on agarose gel and the specific fragments corresponding to the marker nga692 were isolated, cloned into PCR2.1 vector using a TA cloning kit (Invitrogen) and sequenced. At least 20 clones from TA cloning were sequenced to rule out PCR artefacts.

Genetic analyses

Crosses were performed as described by Guzman and Ecker (1990). To map ein or stg mutations, the homozygous Ein+ (an ein/EIN heterozygote) or STG- (an stg/stg homozygote) mutant was crossed with Landsberg erecta. In the case of ein mapping, Ein+ (EIN/ein) F1 progenies were allowed to self-pollinate. Wild-type (EIN/EIN) F2 progenies were selected for mapping. In the case of stg mapping, all F1 progenies (STG/stg) were allowed to self-pollinate. STG- (stg/stg) F2 progenies were selected for mapping. Various SSLP markers spanning the entire Arabidopsis genome were chosen. Genomic DNA was isolated from the rosette leaves of 2-week-old F2 seedlings and SSLP markers were amplified by PCR using standardized conditions (Bell and Ecker, 1994) employing genomic DNA as template. The amplified markers were separated on agarose gel for sizing. Scoring of alleles was performed and linkage maps were generated using Map Manager software (http://mapmgr.roswellpark.org/mmQTX.html).

Amplification and sequencing of EIN4 gene

To amplify EIN4, oligonucleotide primers were designed to cover the whole coding region by PCR with sufficient overlapping. The fragments were amplified from a wild-type Col plant, a heterozygous Ein+ mutant and a homozygous Ein- plant using genomic DNA prepared from these plants. Both strands of the resulting PCR products from several independent PCR amplifications were sequenced.

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in Arabidopsis, encodes a member of the raf family of protein kinases. Cell, 72, 427–441.


