A New System for Detecting Mutations in *Arabidopsis thaliana* and the Mutational Spectra Resulting from Ethylmethanesulfonate Treatment

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**Mutation spectrum/Plant/Arabidopsis/rpsL/EMS.**

A system was developed for the detection and analysis of mutations occurring on chromosomal DNA in plants. The plasmid pML4, carrying the *Escherichia coli rpsL* gene, a target gene for mutagenesis, was inserted into a shuttle vector, pCGN5138, to construct a plasmid which could be used for the transformation of plants. pML4 sequences were introduced into *Arabidopsis thaliana* mediated by *Agrobacterium*. The pML4 DNA was rescued from transgenic *Arabidopsis* plants exposed to mutagens, and the plasmids were introduced into *Escherichia coli* DH10B to isolate mutant clones. In this system, any form of inactivation mutation in the *rpsL* gene can be positively selected since it makes the *E. coli* cells resistant to streptomycin. Here we report that the system could detect the mutagenic effect of ethylmethanesulfonate (EMS). Further characterization of the mutants revealed that G:C to A:T transitions predominated among the EMS-induced mutations. This assay system is useful for the detection and analysis of mutations arising on chromosomal DNA in plants, and should be useful for evaluating analysis of the effects of environmental mutagens.

**INTRODUCTION**

All living organisms are exposed to a variety of genotoxic agents. Although some mutations arising in a genome can increase the genetic diversity, which is thought to be beneficial to the species for better adaptation to specific environmental conditions, many others are detrimental to the organisms; depending on the genes that were inactivated, various effects may result such as embryonal death, malformation, elevated frequency of malignancies, hereditary diseases etc. A variety of genotoxic substances in air, soil and water can affect the health of organisms. Also depletion of atmospheric ozone has led to exposure to elevated levels of ultraviolet (UV) radiation on the earth’s surface, and UV radiation is known to cause DNA damage. Investigation of induced mutations is necessary to estimate the mutagenicity of genotoxic, chemical or physical agents.

Several kinds of mutation assay based on microorganisms are available for mutagenicity tests. In eukaryotes, mice carrying the *E. coli rpsL*, *lacI*, *lacZ* or other genes, and zebrafish carrying the *rpsL* gene as a transgene have been developed to detect mutations occurring in genomic DNA.¹ ² These genes can be recovered from genomic DNA of the cells in the form of phage³ ⁴ ⁵ or plasmid.⁶ ⁷ However, there are only a limited number of systems available to analyze mutations in higher plants.⁸ ⁹ For example, Kovalchuk *et al.*³⁸ generated transgenic *Arabidopsis* lines by introducing the β-glucuronidase (*uidA*) gene with amber, opal or ochre nonsense codons at five different positions in the genome to detect somatic mutation. Reversion of any of the nonsense codons to original codons restores *uidA* activity, which can be monitored by visualization as a blue spot on plants following histochemical staining.¹⁰ This reversion system can detect A:T to G:C transitions, and A:T to C:G and A:T to T:A transversions directly on the plant but not base change of G:C pairs, which commonly takes place after exposure to alkylating agents. Another system for the detection of mutations in higher plants is TILLING (Targeting Induced Local Lesions In Genomes), a reverse genetic strategy,¹¹ which was used by Greene *et al.* to analyzed EMS-induced mutations in *Arabidopsis*.⁹ TILLING is one example of single-nucleotide polymorphism (SNP) detection technology applied to large-scale reverse genetics, and combines ran-
dom chemical mutagenesis with the screening of point mutations by PCR to detect mutations in target sequences.\(^{12}\)

Since bacterial \(rpsL\) gene is frequently used as a target gene for detection of forward mutation in microorganisms and animals, we thought it would be also applicable in higher plants because plants should be effective monitors for detecting genotoxic effects of environmental mutagenic substances. Furthermore, to make valid comparisons of the specificity of mutations between plants and other organisms, it is desirable to use the same reporter gene because there would be a bias in mutation spectra depending on the target gene. In addition, an efficient system for detecting mutations is a prerequisite for collecting a large number of mutant clones for the analysis of mutation spectra.

In the present study, we developed a transgenic system for detection of \textit{in vivo} mutations occurring in the genome of the \textit{Arabidopsis} plant, which can be used to determine mutational changes in the sequence of specific genes. The \(rpsL\) gene of \textit{E. coli} was used as a target gene, which is relatively small, comprising 375 base pairs, and confers a streptomycin-resistant phenotype in \textit{E. coli} cells when inactivated by mutations. Plasmid clones carrying a mutated \(rpsL\) gene can be positively selected in \textit{E. coli} and sequenced. We found that EMS-treated transgenic \textit{Arabidopsis} showed about 20 times higher mutant frequency compared to the frequency in the control group and that the observed mutations were predominantly G:C to A:T transitions.

**MATERIALS AND METHODS**

\textbf{Plant and Bacterial strains}

\textit{Arabidopsis thaliana} ecotype Columbia was used in the study for mutation analysis. Plants were grown under 14 h light and 10 h dark cycle. The light source was four fluorescent lamps (40W) with an illumination intensity of 6,800 lx. 

\textit{Agrobacterium tumefaciens} \textit{GV3101 (pMP90)} resistant to gentamycin\(^{13}\) was used for the transformation of \textit{Arabidopsis}. \textit{Escherichia coli} strain DH10B resistant to streptomycin was used for detection of mutations at \(rpsL\) gene contained in the plant DNA.

\textbf{Plasmids}

Plasmid pML4 carries \textit{E. coli} \(rpsL\) and kanamycin-resistance genes. pCGN5138 is a shuttle vector for the introduction of pML4 into plant cells. pML4 was inserted into the BamHI site of pCGN5138 to construct pPMA8.

\textbf{Transgenic Arabidopsis carrying \(rpsL\) gene}

\textit{Arabidopsis} plant was infected by \textit{Agrobacterium tumefaciens} \textit{GV3101 (pMP90)} carrying the binary vector pPMA8 using the floral dip method.\(^{14}\) For this purpose, \textit{Agrobacterium tumefaciens} cells carrying pPMA8 was cultured in 20 ml of LB medium containing 25 \(\mu\)g/ml of kanamycin and 5 \(\mu\)g/ml of gentamycin for two days at 28°C. A portion of the culture was diluted 50-fold into LB medium containing the same drugs and incubated at 28°C for overnight with shaking. \textit{Agrobacterium} cells were then collected and suspended in 500 ml of water containing 5% sucrose. \textit{Arabidopsis} was grown for a month and primary bolts were clipped. Just prior to dipping plants for infection, 12.5 \(\mu\)l of NUC-SILICONE L-77 (Nippon Unicar) was added to 250 ml of \textit{Agrobacterium} suspension. \textit{Arabidopsis} plants, six or seven days after clipping of the primary bolts, were dipped in \textit{Agrobacterium} suspension for several seconds in an upside down position. Treated \textit{Arabidopsis} was grown until seeds developed (T1 seeds). Seeds transformed with pML4 were screened on 1/2 B5 agar medium containing 50 \(\mu\)g/ml kanamycin, 1% sucrose, and 1/1000 Hyponex, since transformed seeds are kanamycin resistant. T3 seeds were obtained by repeated self-pollination, and homozygous strains regarding the transgenic \(rpsL\) gene were established and used in the present study.

Several lines of \textit{Arabidopsis} were produced which carry the \textit{E. coli} \(rpsL\) gene as a target. The transgenic lines appear to contain pML4 in a single locus of the chromosome judging from the segregation pattern of kanamycin resistant and sensitive plants of later generation (data not shown).

\textbf{EMS treatment}

Transgenic \textit{Arabidopsis} seeds were first treated with 0.05% Triton X-100 and washed with sterile water, and then immersed in a 0.33% EMS (Sigma) dissolved in water for overnight while gently shaking at room temperature. After the treatment, seeds were repeatedly washed with 70% ethanol and sterile water to remove EMS, mixed with 1 ml of 0.1% agar, and spread on 1/2 B5 agar medium containing 50 \(\mu\)g/ml kanamycin, 1% sucrose and 1/1000 Hyponex. After vernalization at 4°C for two days, seeds were allowed to germinate, and were grown at 25°C under 14 h light and 10 h dark cycle conditions. Two weeks later, kanamycin-resistant plants were transplanted to soil and grown under the same conditions for about 30 to 40 more days. DNA was isolated from 10 plants grown from 10 seeds treated with EMS.

\textbf{Detection of \textit{in vivo} mutations}

The aerial parts of 30- to 40-day old plants were harvested, and genomic DNA was extracted with the CTAB method from approximately 3 g of plants.\(^{15}\) Forty to 50 \(\mu\)g of DNA was digested with \textit{BamHI}, and the digests were treated with T4 DNA ligase (Takara Bio Inc.) to recover the plasmid pML4. The DNA was then dissolved in sterile water at a final concentration of 0.5 to 0.9 \(\mu\)g/ml, and introduced into \textit{E. coli} DH10B by electroporation. A small fraction of the treated \textit{E. coli} was plated on LB agar medium containing kanamycin to estimate the total number of cells carrying the pML4 plasmid. The remaining cells were spread on LB medium supplemented with streptomycin to screen for streptomycin-resistant mutant clones.\(^{7}\) Colonies were isolated and grown again on streptomycin-containing medium to confirm the
Table 1. Frequency of background and EMS-induced mutation

|                           | Total number of colonies | Number of colonies carrying mutated $rpsL$ | Mutant frequency$^3$ |
|---------------------------|--------------------------|-------------------------------------------|----------------------|
| Background mutation       | 524,682                  | 13                                        | $2.5 \times 10^{-3}$ |
| EMS treated plant         | 45,560                   | 26                                        | $5.7 \times 10^{-4}$ |

$E. coli$ DH10B transformed with DNA from Arabidopsis were grown on the medium supplemented with kanamycin or kanamycin and streptomycin
1. number of colonies formed on the medium supplemented with kanamycin.
2. number of colonies formed on the medium supplemented with kanamycin and streptomycin.
3. ratio of number of streptomycin resistant colonies to total kanamycin resistant colonies.

Table 2. Mutational spectra of transgenic Arabidopsis after EMS treatment

| Types of mutation | Number of mutations (% ratio to total mutation) |
|-------------------|--------------------------------------------------|
| Base substitution | Untreated control: 7 (53.8%) EMS: 25 (96.2%)   |
| Transition        | G:C to A:T: 2 (EMS: 24)                          |
| Transversion      | G:C to T:A: 3 (EMS: 1)                           |
|                   | A:T to C:G: 2 (EMS: 0)                          |
| Frameshift        | 3 (23.0%) (EMS: 1 (3.8%))                      |
| Deletion          | 1 (7.8%) (EMS: 0)                               |
| Sequence substitution | 2 (15.4%) (EMS: 0)                          |
| Total             | 13 (100%) (EMS: 26 (100%))                     |

![Fig. 1. DNA sequence changes in the $rpsL$ gene. Background mutations and EMS induced mutations are shown above and below the $rpsL$ sequence, respectively. The solid line above the sequence, from bases 44 to 206, indicates a deletion.](http://jrr.jstage.jst.go.jp)
drug resistance. The region of *rpsL* gene on the pML4 plasmid was sequenced with an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

## RESULTS

### Spontaneous mutation

When DH10B *E. coli* cells, transfected with DNA extracted from about 3 g of untreated plants, were plated on LB agar plates supplemented with kanamycin, a large number of colonies were observed. Among approximately $5.2 \times 10^5$ colonies, 13 mutants were found giving an estimated background mutant frequency of $2.5 \times 10^{-5}$ (Table 1). Among the 13 mutants found, 2 were transitions, 5 transversions, 3 frameshifts, and 1 deletion. Additional 2 were sequence substitutions (Table 2); e.g., TACC at base pairs 283 to 286 was changed to CTGACT in one clone, and ACCGTA in base pairs 326 to 332 to CGG in the second case (Fig. 1).

### EMS-induced mutagenesis

Chromosomal DNA isolated from plants grown from EMS-treated seeds was subjected to mutation analysis. It is mentioned that plant mature seeds are composed of various cellular tissues such as cotyledons, epicotyl, hypocotyl and radicle. Therefore, in a single *Arabidopsis* seed, a large number of somatic cells exist and are subjected to the EMS treatment. We found 26 mutations among 45,560 colonies and the mutant frequency was therefore estimated as $5.7 \times 10^{-4}$ (Table 1). This mutant frequency was about 20-fold higher than that of spontaneous mutations, i.e., $2.5 \times 10^{-5}$, as mentioned above. Among these mutations, 24 were G:C to A:T transitions, 1 G:C to T:A transversion, 3 frameshifts and 1 deletion. Additional 2 were sequence substitutions (Table 2); e.g., TACC at base pairs 283 to 286 was changed to CTGACT in one clone, and ACCGTA at base pairs 326 to 332 to CGG in the second case (Fig. 1).

### Discussion

Mutations occurring in the chromosomal DNA of *Arabidopsis* were readily detected with the system described here. It is now possible to compare the specificity of mutations in animals and plants because a common target gene, *rpsL*, is used.

In the present *Arabidopsis* system, the background mutant frequency was about $2.5 \times 10^{-5}$. In transgenic mice carrying the *rpsL* gene, the spontaneous mutation frequency in lung is reported as $3.5 \times 10^{-5}$ and similar frequency is reported in epidermal cells. Therefore, the background mutant frequency appears to be on the order of $10^{-5}$ in animal or plant systems using *rpsL* as a transgene.

Thirteen mutations were observed in the control group. The spectra of the spontaneous mutations were different from those seen in *E. coli*. For example, about half of the mutations in wild type *E. coli* were caused by insertion of transposons when *rpsL* was used as the reporter gene, but these were not observed in the present *Arabidopsis* system (Table 4). In transgenic mice and zebrafish carrying the *rpsL* gene, the predominant mutations (approx. 50%) were frameshifts, which were also found in the *Arabidopsis* system, but apparently at a lower frequency (3/13, or 23%) (Table 4). Sequence substitutions comprised 2 out of the total 13 mutations (15%) in the *Arabidopsis* system, but were not detected in the wild type *E. coli*, mouse and zebrafish systems (Table 4).

EMS is one of the well-known potent mutagens. The frequency of EMS-induced mutations in transgenic *Arabidopsis* was $5.7 \times 10^{-4}$, a frequency which is 20 times higher than that of spontaneous mutations. Most of the mutations, i.e. 24/26 or 92%, were G:C to A:T transitions (Table 2). A strong preference for this transition was also observed in mutations in EMS-treated *Arabidopsis* analyzed with the TILLING method. This type of base change was also predominant (i.e., 26 of 30) in mutant T-lymphocytes at *hprt* gene of rats exposed to EMS, which indicates that these

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**Table 3. Frequency of GC to AT changes at the site of 5’-PuG-3’ motif**

| Site | Sequence | Frequency |
|------|----------|-----------|
| 18   | AGC > AAT | 1         |
| 95   | GCC > GAC | 1         |
| 187  | AGT > AAT | 15        |
| 265  | AGA > AAA | 1         |

| Site | Sequence | Frequency |
|------|----------|-----------|
| 25   | ACG > ATG | 1         |
| 67   | TGC > TAC | 1         |
| 272  | CCG > CTG | 4         |

%: ratio to total GC to AT base changes of 5’-AG-3’ motif (Table 3).

**DISCUSSION**
substitutions were probably caused by mispairing of $O^6$-ethylguanine with thymine. These results therefore indicate that EMS induces $O^6$-ethylguanine also in higher plants.

The mutation spectra in EMS-treated transgenic Arabidopsis showed a strong preference for 5'-PuG-3' sites. Eighteen out of 24 G:C to A:T transitions (75%) occurred at the site of a 3' guanine. This strong preference for 5'-PuG-3' sites is consistent with the results seen in the awd locus in Drosophila germ cells and Chinese hamster fibroblast cells, which was not observed, however, in yeast or in Drosophila vermilion locus. In bacteria, G:C to A:T transitions occurred frequently at a guanine flanked by a G or C, but this bias depended on the concentration of EMS. The TILLING method indicates that the 5'-PuG-3' rule seems to be also the case in Arabidopsis. Moreover, from data with other organisms, the purine at the 5'-PuG-3' site was guanine, but the present study showed that 17 out of 18 (94%) cases contained adenine at the position of 5'-Pu. The TILLING results also indicate the purine bias was stronger for adenine than guanine. The reason for this bias is unclear. It is possible that other factors could have affected the 5'-PuG-3' bias; e.g., concentration of EMS, neighboring sequences at the 5'-PuG-3' sites, and affinity of DNA repair systems such as the mismatch repair and excision repair systems for $O^6$-ethylguanine. To understand the underlying mechanisms, further studies are required for mutation spectra after exposure of the plant to different concentrations of EMS, and by using repair deficient Arabidopsis mutants.

The present system in higher plant was capable of detecting various types of mutations induced by EMS in a manner similar to the studies reported on microorganisms and transgenic animals. Since pML4 carries no eukaryotic elements, it is most likely inactive in the transgenic Arabidopsis as in transgenic mice bearing the same gene. Exogenous DNA is thus expected not to affect selection of the mutants, thereby mutational spectrum. The assay procedure is simple and does not require a large amount of time and labor. These aspects suggest that our mutation detection system could become a powerful tool in investigating DNA damage, DNA repair, and mutagenesis in plants. In the present study, one of several transgenic Arabidopsis strains was used for mutation analysis. Mutations occurring in other strains should be investigated to determine the variation of mutational specificity among strains. It is mentioned, however, that at present, our transgenic plants cannot be used in outdoors due to regulations to grow transgenic plants in Japan.

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