8

EMS Mutagenesis of *Arabidopsis*

YongSig Kim, Karen S. Schumaker, and Jian-Kang Zhu

Summary

A powerful approach for determining the biological functions of genes in an organism is to produce mutants with altered phenotypes and physiological responses. Various approaches for mutagenesis involving chemical, irradiation, and insertional methods have been developed; each has advantages and disadvantages for the study of gene function. In this post-genomic era, the use of reverse genetic approaches to understanding the role of genes in growth and development has become widespread. With development of new techniques such as targeting induced local lesions in genomes (TILLING), ethyl methanesulfonate (EMS) mutagenesis can be used for both forward and reverse genetic studies. Generation of diverse mutant alleles in the same gene provides critical tools to understand the role of these genes in the function of the organism. Here we describe the general method of EMS mutagenesis for the molecular genetic model plant *Arabidopsis thaliana*.

Key Words: EMS; chemical mutagenesis; *Arabidopsis*; mutants.

1. Introduction

Alkylating agents such as ethyl methanesulfonate (EMS) induce chemical modification of nucleotides, which results in mispairing and base changes. Strong, biased alkylation of guanine (G) residues results, forming O^6-ethylguanine, which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with A (adenine)/T ***(1)***. The functions and mechanisms of action of other monofunctional alkylating agents such as diethyl sulfate (DES) and dimethyl sulfate have been extensively reviewed ***(2)***. Comparisons of DES and EMS have shown that they share similar alkylating activities and genetic effects.

The majority (99%) of the time, EMS induces C-to-T changes resulting in C/G to T/A substitutions, whereas methyl methanesulfonate produces T/A to G/C transversion and A/T to G/C transitions ***(1,3,4)***. At a low frequency, EMS generates G/C to C/G or G/C to T/A transversions by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors ***(3)***. Based on codon usage in *Arabidopsis*, the frequency of EMS-induced stop codon and missense mutations has been calculated to be ~5% and ~65%, respectively ***(5)***. EMS mutagenesis generates randomly distributed mutations throughout the genome in *Arabidopsis* ***(1)***. As a result, chemical mutagenesis can be used not only to search for loss- or gain-of-function mutants but also to understand the role of specific amino acid residues in protein function. Results of many studies suggest that use of chemically induced mutants can also provide useful information for understanding the functions of essential genes by generating weak nonlethal alleles. In addition, EMS mutagenesis can be used for generating breeding lines ***(6)***.

To achieve saturation of EMS mutagenesis in *Arabidopsis*, at least 125,000 M1 lines should be generated ***(7)***. Evaluating the degree of saturation requires independent calibration through screening of visible recessive traits such as albinism, embryo lethality, and trichome pheno-

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types ***(8)***. The frequency of mutation depends on the position of the gene in the genome and the treatment conditions during mutagenesis ***(4)***. Effects such as embryonic lethality and chlorophyll mutations are directly related to the length of exposure to a fixed concentration of EMS ***(9)***. High concentrations (over 0.9%) of EMS have been shown to result in a decreased frequency of induced mutations in soybeans ***(10)***.

Use of *Arabidopsis* with amber, opal, and ocher stop codons in an introduced β-glucuronidase (*uidA*) gene determined the spontaneous (natural somatic) mutation rate to be between 10^{-7} and 10^{-8} events per base pair per generation ***(4)***. Using this frequency as a base of comparison, it is clear that significant advantages of chemical mutagenesis include its high efficiency and frequency in achieving saturation mutagenesis, especially as compared to genetic mutagenesis including insertion of transfer DNA (T-DNA) or transposable elements. Isolation of mutants requires the use of relatively small-scale screens with chemically induced mutagenized pools. In addition, chromosomal rearrangements caused by T-DNA or transposon insertion can be avoided with chemical mutagenesis ***(8)***. Compared with irradiation mutagenesis, EMS induces relatively few strand breaks that lead to inversion or deletion mutations ***(9)***. In addition, in Arabidopsis EMS induces four times as many embryonic or chlorophyll mutants as irradiation at the M1 level ***(9)***.

The difficulty in detecting single-nucleotide polymorphisms or substitutions can be overcome by use of **t**argeting **i**nduced **l**ocal **l**esions **in** **g**enomes (TILLING) complemented with denaturing high-performance liquid chromatography (DHPLC; ***5***). These technologies allow chemically induced mutant pools to be used for reverse genetics. With help of automation, robust and rapid detection makes it possible to screen a wide range of mutant pools in a short time and to avoid the often laborious process of forward genetic screening ***(11,12)***.

2. Materials

1. *Arabidopsis thaliana* seeds.
2. Disposable 50-mL plastic tubes.
3. Nutating mixture.
4. 100 m*M* phosphate buffer (pH 7.5).
   a. Prepare 100 mL of 1 *M* K_2HPO_4 and 100 mL of 1 *M* KH_2PO_4.
   b. Pour 70 mL of 1 *M* K_2HPO_4 into a beaker.
   c. Add 20 mL of 1 *M* KH_2PO_4 and mix the solution.
   d. Adjust the pH to 7.5 with the addition of 1 *M* KH_2PO_4.
   e. Dilute the 1 *M* phosphate buffer with water to make 100 m*M* phosphate buffer (*see* **Note 1**).
5. Ethyl methanesulfonate (EMS, F.W. 124.16; *see* **Note 2**).
6. Sterilized water.
7. Squeeze bottle.

3. Methods

3.1. EMS Mutagenesis

1. Weigh 2.5 g of well-dried *Arabidopsis thaliana* seeds (approx 125,000 seeds).
2. Soak the seeds in a 50-mL plastic tube with 40 mL of 100 m*M* phosphate buffer at 4°C overnight.
3. If EMS mutagenized seeds are to be planted in soil, then do not sterilize the seeds. However, if the screening is to be performed with M1 seeds on plates, M0 seeds should be sterilized (*see* **Note 3**).
4. Stand the tube upright to allow the seeds to settle and decant the excess phosphate buffer.
5. Add 40 mL of fresh 100 m*M* phosphate buffer.
6. Add EMS to a final concentration of 0.4% (*see* **Note 4**).
7. Incubate the mixture for 8 h at room temperature with gentle nutation.
8. Wash the seeds thoroughly 20 times with water (40 mL per wash).
9. After EMS mutagenesis, seeds should be planted immediately in soil or on plates. Preserve M1 seeds by drying them on filter paper.

3.2. Planting EMS Mutagenized M1 Seeds

M1 seeds can be planted in soil or on plates for direct screening for dominant mutants. Planting in soil and selfing of the M1 plants will yield M2 seeds for screening for recessive as well as dominant mutants. M1 seeds can be planted in soil uniformly with the use of a squeeze bottle.

1. Prepare lightly wetted soil in flats.
2. Add 1 mL of M1 seeds to 400 mL of water in a squeeze bottle.
3. Squirt the M1 seeds onto the soil.
4. Keep the flat at 4°C for 2 to 4 d for seed stratification.
5. Transfer the flat to a growth chamber at 25°C with a 16/8 light/dark cycle (*see* **Note 5**).
6. Harvest the seeds from approx 500 to 2000 M1 plants and divide the M2 seeds into pools of desired sizes.

4. Notes

1. If EMS mutagenesis requires aseptic conditions, the 100 m*M* phosphate buffer can be sterilized by autoclaving at 121°C for 20 min. Phosphate buffer can be stored at room temperature.
2. Alternate names are methanesulfonic acid ethyl ether, ethyl methanesulfonic acid or ethyl mesylate. Ethyl methanesulfonate liquid can be purchased from Sigma-Aldrich, product number M0880.
3. Phosphate buffer-imbibed M0 seeds can be sterilized by replacing the buffer with 40 mL of 6% sodium hypochlorite. After a 5-min incubation, seeds should be washed with sterilized water at least four times. Add 40 mL of phosphate buffer to sterilized seeds for EMS treatment (**step 6**).
4. **Caution: Steps 6** to **8** should be done in a fume hood because EMS is volatile and highly toxic. The waste containing EMS should be collected separately from that of other chemicals.
5. To prevent contamination by nonmutagenized seeds, use a growth chamber or room dedicated to this purpose.

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