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ARTICLE

Determination of the optimal condition for ethylmethane sulfonate-mediated mutagenesis in a Korean commercial rice, *Japonica cv.* Dongjin

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Abstract Ethylmethane sulfonate (EMS) mutagenesis is a powerful hunting tool to seek novel players for improving agromonic traits. Together with rapid evolution of the next-generation sequencing techniques, the EMS mutagenesis has been revaluated for its utilization to breed crops in practical agriculture and to study functions of key players in valuable agronomic traits. In this study, we systematically investigated conditions for EMS mutagenesis in Dongjin (*Oryza sativa*, *Japonica*) rice plants to make a mutant population. Since the EMS mutagenesis depends on target tissue, EMS concentration and EMS exposure time, we fixed the EMS exposure time as 13 h and treated germinating seeds with various levels of EMS dosage (from 0.25 to 2% EMS concentration). EMS treatment clearly showed negative biological influences including low germination and abnormal seedling development of Dongjin rice plants. Based on the standard of about 50% lethal dose, 0.75 and 1% EMS dosage for 13 h was finally selected as the optimal conditions for EMS mutagenesis of Dongjin germinating seeds.

Keywords Agriculture · Breeding · EMS mutagenesis · Lethal rate · Rice · Survival rate

Introduction

Rice plant is one of the most important crops in the world because of the principal food for over half of the world population. Breeders have been trying to develop new rice varieties that bring high yield and quality [1]; however, there is limitation to obtain valuable genetic diversity from conventional cultivars. To overcome the limitation, mutagenesis has been using as an artificial method to increase genetic variation that can be utilized for breeding in practical agriculture. In addition, rice is a model plant [2] that has broad interests in basic and applied researches because of small genome size, completed genome sequencing, high transformation efficiency and huge genetic resources [3–6]. Thus, mutagen-mediated rice mutants are utilized as the best genetic materials for studying functions of key regulators in enhancing rice agronomic quality.

Three methods are using for rice mutagenesis: insertional, physical and chemical mutagenesis. Insertional mutation is generated by T-DNA or transposable elements. Collection of the insertional mutation has been shared among international research groups and allowed for studying gene function by forward and reverse genetic strategies [7]. The insertional mutagenesis generally provides knock-out effect for a majority of rice genes, but a

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substantial number of rice genes are still missed in the insertional mutant collections [8]. Physical agents such as UV and X-ray radiation are exposed to rice plants that are caused to gene mutations such as point mutation, deletion and chromosome losses [8, 9]. However, the physical agents severely reduce rice viability. The most popular mutagenesis is chemical mutagens such as ethylmethane sulfonate (EMS), methylmethane sulfonate, hydrogen fluoride, sodium azide and methylnitrosourea [10]. Among them, EMS mutagen is widely used because of easy to use and no requirement of special equipment. Therefore, EMS-induced mutant populations have been generated in various plant species [11, 12].

EMS selectively alkylates guanine bases causing misplacing a thymine residue over a cytosine residue opposite to the O-6-ethyl guanine during DNA replication, which results in a random point mutation [13]. The degree of EMS-induced mutation depends on target tissue, EMS concentration and EMS exposure time. The mutation density and lethal doses also vary tremendously for species, genotype and ploidy level [8]. Whatever, long incubation with high EMS concentration causes to generate a large number of point mutations, thereby increasing lethality. Thus, an optimal condition of EMS mutagenesis for a certain cultivar must be determined on the standard to induce approximately 50% lethal dose before starting to make an EMS mutant population [14]. In the optimal EMS condition, the mutation density is expected to be as high as four mutations per Mb in *Arabidopsis* [15–17].

EMS-induced mutations and mutants have several advantages for use in practical breeding and academic research. First, EMS mutant population can be easily produced by any rice cultivars. Second, because of the high density of point mutations, small number of mutant population can be covered for whole rice genome. Third, EMS-induced gene mutation gives rise to various effects of gene regulation such as knock-out, knock-down and dominant effects. Finally, EMS-induced mutants are easily converted to breeding materials. Consequently, the EMS mutagenesis is a powerful hunting tool to seek novel players for improving rice agromonic traits.

Here, we investigated EMS mutagenesis in a Korean commercial rice, *japonica cv.* Dongjin that was aimed at determining an optimal EMS condition according to the standard on induction of ~50% lethal dose. We treated various EMS concentrations to Dongjin brown rice seeds and systematically analyzed survival and lethal rates during germination. The survival rice plants after germination were continuously analyzed for seedling lethality, shoot growth and root growth. Based on these observations, we determined the optimal condition for EMS mutagenesis in the Dongjin rice cultivar.

Materials and methods

Plant materials and EMS mutagenesis

Dongjin rice cultivar (*Oryza sativa Japonica* cv. Dongjin) was chosen for EMS mutagenesis. Outer hulls of Dongjin seeds were removed to make brown rice seeds. After sterilizing, 500 brown rice seeds were aliquoted to eight conical tubes (50 ml Falcon) and 20 ml ultrapure water was added to seeds that were soaked at room temperature overnight. Subsequently, the water was discarded and 20 ml of various EMS concentrations (Sigma, Cat. No. M0880) was added to each conical tube. The seeds were incubated for 13 h at room temperature. After discarding EMS solution, the seeds were rinsed with 40 ml ultrapure water several times. The rinsed seeds were plated on Petri dishes with Murashige and Skoog (MS) media (Duchefa, Haarlem, Netherlands).

Survival and lethal rates

After incubation for 6 days, ~60 seeds for each EMS-treated condition were counted for survival and lethal rates, which were occurred during germination. The standard as survival rice plant was existence of green leaf formation. The survival EMS-treated rice plants after germination were transplanted to soil and grew more for 2 weeks in a greenhouse at 28–30 °C (16-h light/8-h dark cycle). Seedling survival and lethal rates were counted at 3 weeks after imbibition. Mock-treated Dongjin rice plants (0% EMS) were used as control rice plant. Finally, the survival and lethal rates both during germination and during seedling stage were combined to make total survival and lethal rates for each EMS concentration.

Agronomic traits during vegetative growth stage

With the 3-week-old survival rice plants after EMS treatment, the height, leaf number and root length were measured to assess negative effects of EMS on seedling growth and development. Mock-treated Dongjin rice plants were used as control rice plant.

Results

Scheme of EMS mutagenesis

For EMS mutagenesis in rice plant, we chose *Japonica cv.* Dongjin, which is a commercial rice cultivar in Korea. There are several EMS methods currently available with different rice tissues such as germinating seeds, callus and

fertilized spikelets [18]. Among them, we chose the EMS method using germinating seeds. For effective EMS treatment, we removed outer hulls of Dongjin seeds to make brown seeds (Fig. 1A). Before EMS treatment, 500 Dongjin brown seeds were sterilized with 70% ethanol for 20 min followed by 50% chloral hydrate solution for 30 min (Fig. 1B). Five hundred brown rice seeds were aliquoted to eight conical tubes (60 seeds per tube), and 20 ml ultrapure water was added to incubate the seeds for germination at room temperature overnight under dark condition. Subsequently, the water was discarded and 20 ml of mock, 0.25, 0.5, 0.75, 1, 1.25, 1.5 or 2% EMS concentration was added to each conical tube (Fig. 1C). The germinating seeds in each EMS solution were shaken for 13 h at room temperature. After discarding EMS solution, the germinating seeds were washed with 40 ml ultrapure water (10 times, 10 min for each time). The washed seeds were plated on Petri dishes with MS media (Fig. 1D).

Survival and lethal rates caused by EMS treatment during germination

Effect of EMS-induced mutagenesis is easily monitored by survival and lethal rates during germination and vegetative growth stage. For example, high EMS concentration causes to severely reduce seed germination and to distinctly induce seedling lethal phenotype [14]. To test the EMS effect, we first analyzed 6-day-old EMS-treated Dongjin rice plants for survival and lethal rates, which were occurred during germination. We established a standard as the survival rice plant, which produced greening shoot. We saw small number of the albino plants as a typical EMS phenotype, which were categorized into no germination. Two percentage EMS-treated rice plants clearly exhibited low germination rate and slow growth patterns with no root development (Fig. 2). Dependent on applied decreases in EMS concentration, the number of survival rice plants was distinctly increased and seedling growth was gradually active (Fig. 2). 0.25% EMS-treated rice plants showed normal growth patterns as did mock-treated rice plants. Specifically, 2% EMS treatment induced 21% survival and 79% death rates during germination, whereas 0.25% EMS treatment showed 87% survival and 13% death rates (Fig. 3A–B; Table S1). Interestingly, 1% EMS-treated rice plants started to show normal patterns of root growth/development and, specifically, showed 64% survival and 36% lethal rates during germination (Figs. 2, 3A–B; Table S1). These observations indicate that the EMS-mediated lethality positively correlates with EMS concentration during germination.

Seedling survival and lethal rates caused by EMS treatment

To understand survival and lethal rates during vegetative growth stage, the survival EMS-treated rice plants after germination were transplanted to soil and grew more for 2 weeks. All 2% EMS-treated rice plants ($n = 13$) died (Fig. 3C–D; Table S1), indicating that 2% EMS treatment is too much high concentration to keep viability. Dependent on applied decreases in EMS concentration, seedling survival rate was gradually increased, whereas seedling lethal rate was gradually decreased. Although it was small number, the lowest EMS (0.25%) concentration also produced seedling lethal phenotype. To determine the optimal condition of EMS mutagenesis, we combined the survival/lethal rates during germination with seedling survival/lethal rates to make total survival and total lethal rates. The total survival rate was gradually increased dependently on applied decreases in EMS concentration (Fig. 3E; Table S1), whereas the total lethal rate was gradually decreased dependently on applied decreases in EMS concentration (Fig. 3F; Table S1). Since an optimal condition for EMS mutagenesis is generally considered as induction

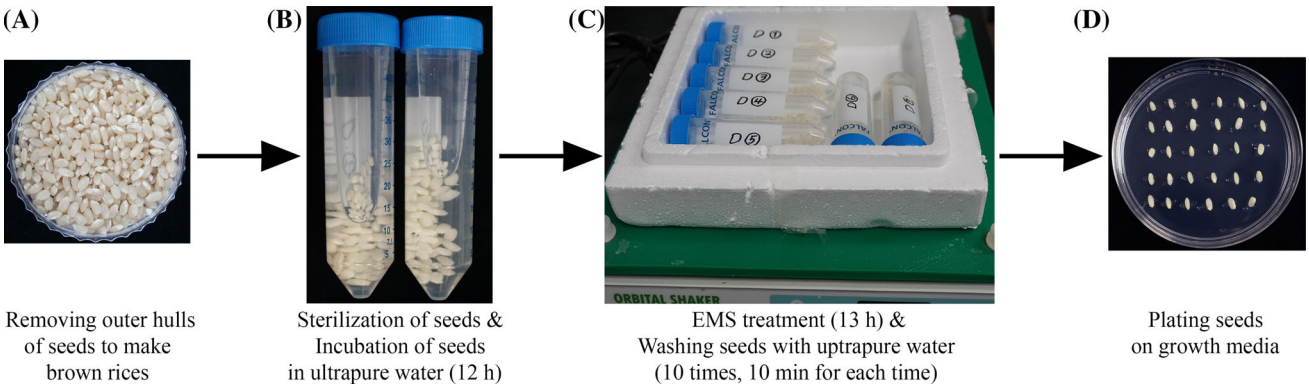

Fig. 1 EMS mutagenesis in Dongjin germinating seeds. (**A**) Dongjin brown seeds after removing outer hulls of seeds. (**B**) Sterilized brown seeds with ultrapure water. (**C**) EMS-soaked brown seeds on a shaking incubator. (**D**) EMS-treated brown seeds on a Petri dish with Murashige and Skoog media

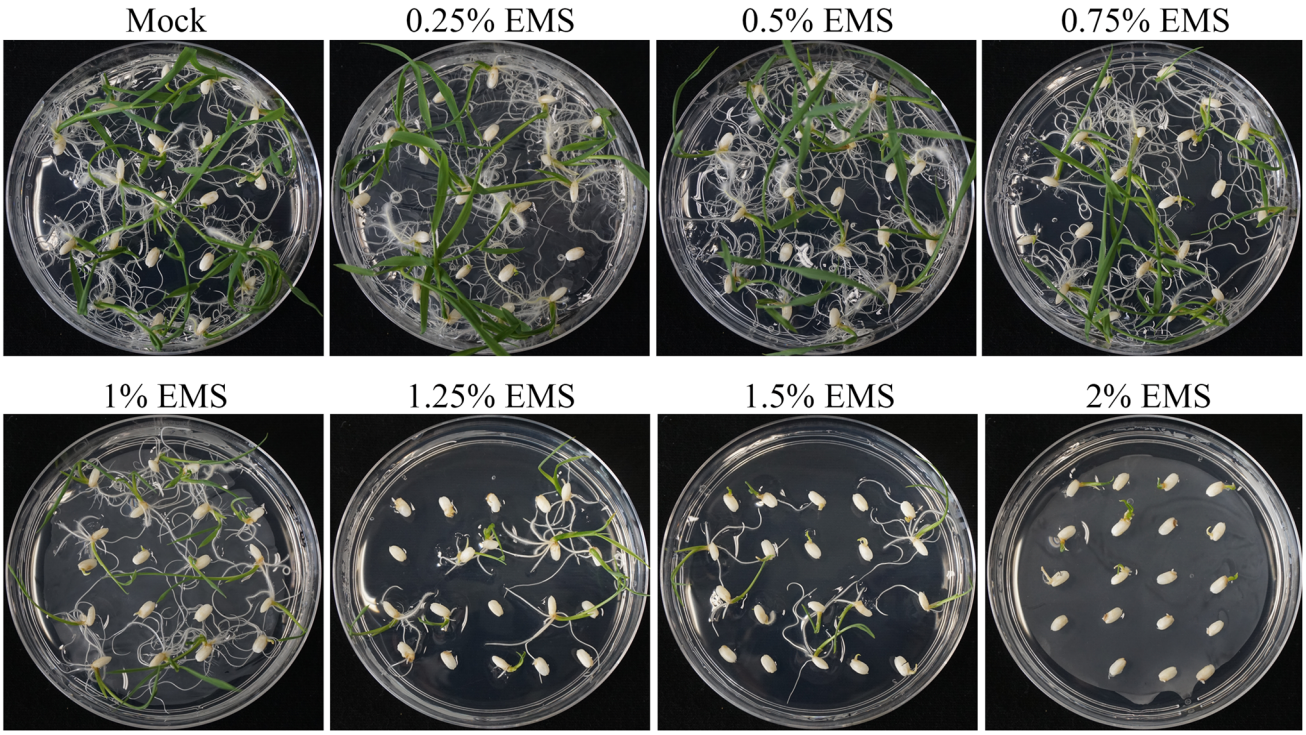

Fig. 2 Germination rate affected by various EMS concentrations. Phenotypes of mock, 0.25, 0.5, 0.75, 1, 1.25, 1.5 and 2% EMS-treated Dongjin rice plants at 6 days after imbibition

of approximate 50% lethal dose, the optimal condition for EMS mutagenesis in Dongjin rice plants was finally determined as 0.75% (44% lethal dose) and 1% (54% lethal dose) EMS concentration.

EMS mutagen negatively affects seedling growth and development

To further assess whether the EMS-treated Dongjin rice plants was affected in seedling growth and development, we first compared shoot growth patterns among all EMS-treated 3-week-old Dongjin rice plants. All 2% EMS-treated rice plants produced albino leaves and finally died (Fig. 4). Thus, we did not further analyze 2% EMS-treated rice plants. Three-week-old Dongjin rice plants produced approximately three leaves between 0.75 and 1.5% EMS treatments, while 0.25% EMS-treated rice plants produced approximately four leaves, which were similar to leaf number of mock-treated rice plants (Fig. 5A). EMS treatment also imposed significant impact on the seedling height (Fig. 4). Three-week-old Dongjin rice plants grew about 15 cm in height between 1 and 1.5% EMS treatments, while 0.25% EMS-treated rice plants grew about 23 cm in height, which were similar to height of mock-treated rice plants (Fig. 5B). We also compared root growth patterns among all EMS-treated 3-week-old Dongjin rice plants. Three-week-old Dongjin rice plants produced about 5 cm in root length between 1 and 1.5% EMS treatments (Fig. 5C). The root length gradually increased after decreasing the EMS concentration, and finally, 0.25% EMS-treated rice plants produced about 9 cm in root length, which were similar to root length of mock-treated rice plants (Fig. 5C). Interestingly, 0.75 and 1% EMS concentrations, which were considered as the optimal EMS condition, showed intermediate defects in seedling growth and development. These observations indicate that EMS mutagen negatively affects seedling growth and development.

Discussion

EMS mutagenesis is a powerful tool in crop improvement, and EMS-induced mutants are considered to be free of the regulatory restrictions imposed to genetically modified organisms [19, 20]. Additionally, together with rapid evolution of the next-generation sequencing techniques, the EMS mutagenesis has been revaluated for its utilization to breed crops in practical agriculture and to study functions of key players in valuable agronomic traits. Here, we assessed EMS effects in a Korean commercial rice, *Japonica cv.* Dongjin, and based on evaluation of the EMS effects, we determined the optimal condition for the EMS mutagenesis of Dongjin rice plant.

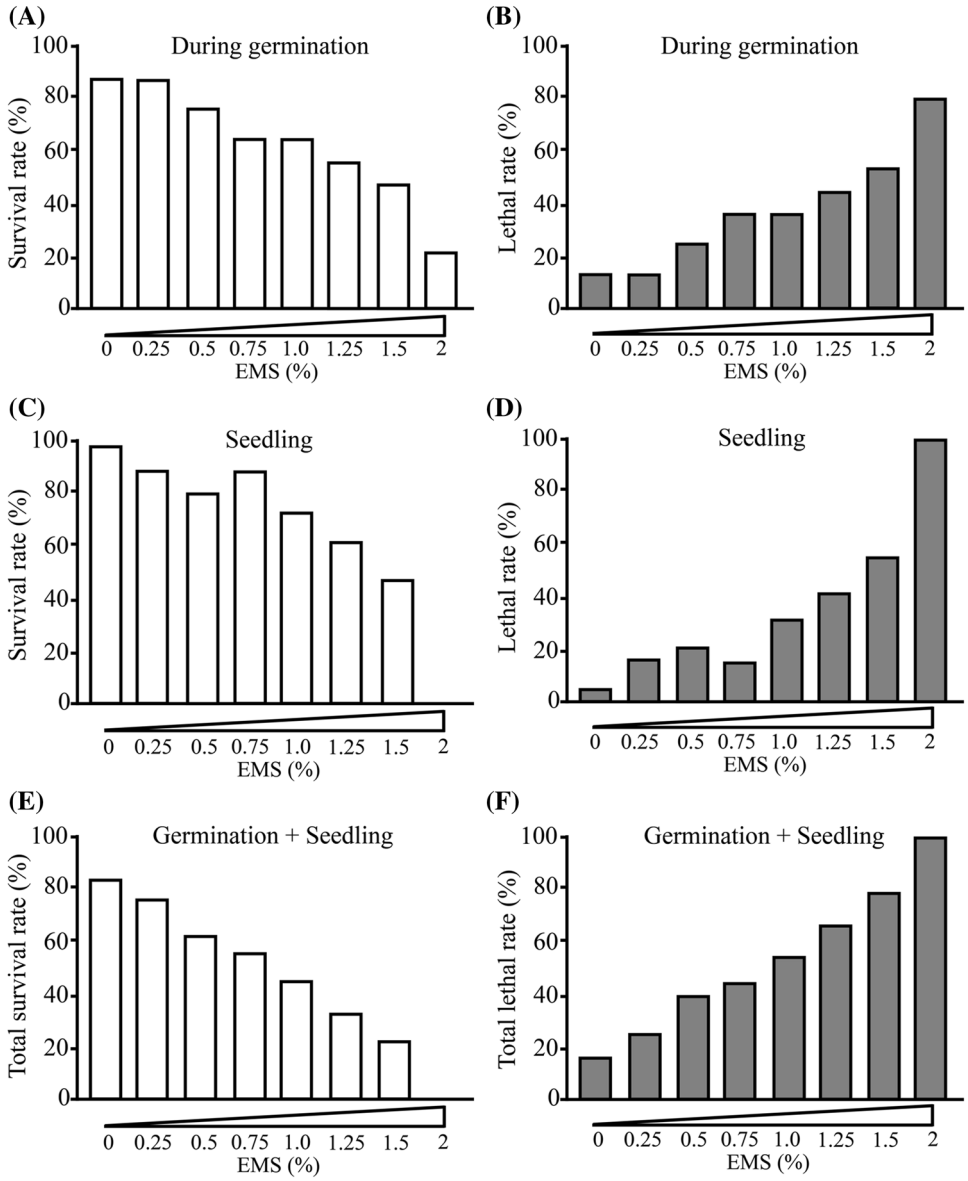

Fig. 3 Effect of various EMS concentrations on survival and lethal rates. (**A**, **B**) Quantitative analysis of survival rate (**A**) and lethal rate (**B**) in EMS-treated Dongjin rice plants during germination. (**C**, **D**) Quantitative analysis of survival rate (**C**) and lethal rate (**D**) in EMS-treated Dongjin rice plants during vegetative growth stage. (**E**, **F**) Quantitative analysis of total survival rate (**E**) and lethal rate (**F**) from germination to 3-week-old of EMS-treated Dongjin rice plants. Sixty brown rice seeds for each EMS concentration were used to analyze survival and lethal rates during germination. 13–52 survival Dongjin rice plants for each EMS concentration after germination were analyzed for seedling survival and lethal rates at 3 weeks after imbibition

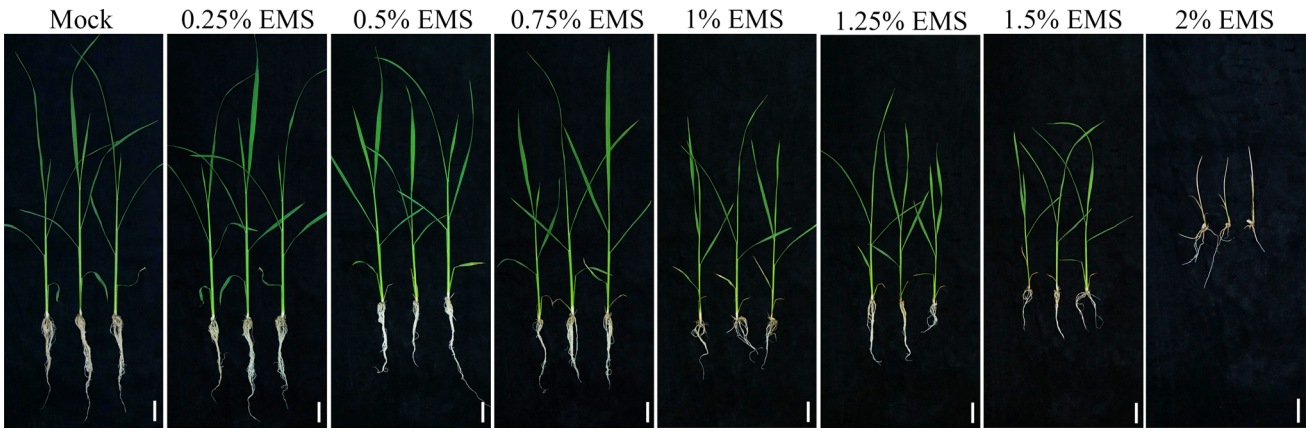

Fig. 4 Seedling phenotypes by exposure of different EMS concentrations. Seedlings of mock, 0.25, 0.5, 0.75, 1, 1.25, 1.5 and 2% EMS-treated Dongjin rice plants at 3 weeks after imbibition. *Size bar* = 2 cm

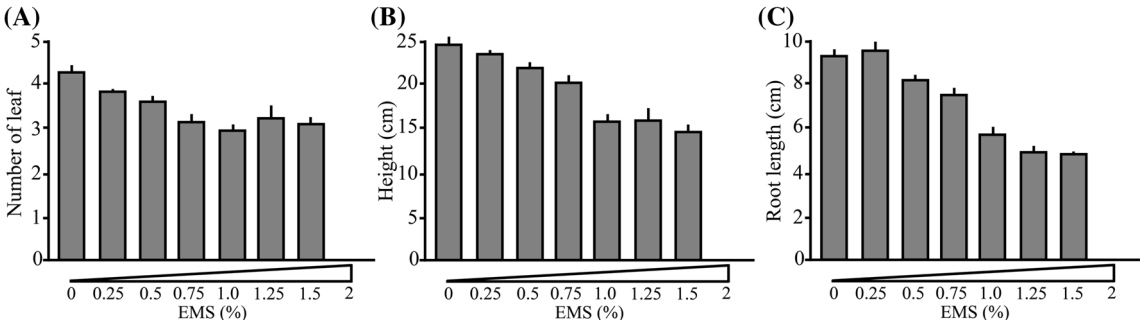

Fig. 5 Effect of various EMS concentrations on seedling growth and development. (**A**) Number of leaf in 3-week-old EMS-treated Dongjin rice plants. (**B**) Shoot height of 3-week-old EMS-treated Dongjin rice plants. (**C**) Root length of 3-week-old EMS-treated Dongjin rice plants. 13–52 survival Dongjin rice plants after germination were analyzed for seedling growth and development at 3 weeks after imbibition

EMS treatment clearly showed negative biological influences including low germination and abnormal seedling development in Dongjin rice plants. The frequency of lethal phenotypes was decreased dependently on applied decrease in EMS concentration during germination. High EMS concentration (2% EMS) caused to severely lose viability during germination. It can be simply explained by high mutation density in genome, which gives rise to damage of major genes for germination. All 2% EMS-treated Dongjin rice plants, which were survival after germination, finally resulted in lethal phenotypes during vegetative growth stage, whereas 0.25% EMS-treated Dongjin rice plants showed small number of seedling lethal phenotypes, suggesting that treatment of high EMS concentration may confer more chance to disrupt key players for seedling growth and development than treatment of low EMS concentration.

In addition to lethal phenotypes, EMS mutagen altered patterns of seedling growth and development. EMS-treated Dongjin rice plants produced small number of leaf and small in height as compared to mock-treated Dongjin rice plants. EMS treatment also changed patterns of root growth and development. Root is a vital organ for plant growth and development, such that root organ physically supports aerial shoot organ, absorbs water and nutrients, stores nutrients and synthesizes plant hormones [21–23]. EMS sensitively affects root growth and development, such that treatment of high EMS concentration caused to seriously impair root development during germination and continuously severe defects in root growth and development during vegetative growth stage, thereby inducing high number of seedling lethality. These observations suggest that a moderate EMS concentration is important to keep viability for generating EMS mutant population.

An optimal condition of EMS mutagenesis is generally determined on the standard to induce approximate 50% lethal dose [14]. The optimal EMS condition was tested in several rice varieties including IR64, IR231 and MR219 [9, 14]. IR231 and MR219 varieties reveal 0.6 and 0.5% EMS dosages as the optimal EMS condition, respectively, which are categorized into the high-sensitive rice to EMS, whereas IR64 variety reveals 1% EMS dosage as the optimal EMS condition, which is categorized into the low-sensitive rice to EMS. We fixed the EMS exposure time as 13 h and added variety to EMS concentrations to Dongjin germinating seeds. 1.25, 1.5 and 2% EMS concentration are too much high EMS dosage to keep viability. There were 67, 78 and 100% lethal rates, respectively. By contrast, treatment of 0.25 and 0.5% EMS concentrations induced weak EMS effects, indicating that the low EMS concentrations keep viability but may induce low mutation density in rice genome. In conclusion, we determined the optimal condition for EMS mutagenesis with Dongjin germinating seed as 0.75 and 1% EMS for 13 h exposure time, which is categorized into the low-sensitive rice to EMS mutagen. The optimal condition gave rise to approximate 50% lethal dose and intermediate EMS effects in seedling growth and development, inferring that the EMS condition induces proper point mutation density in rice genome. We will use the optimal condition to generate an EMS mutant population in Dongjin rice plants.

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References

1. Varshney RK, Bansal KC, Aggarwal PK, Datta SK, Craufurd PQ (2011) Agricultural biotechnology for crop improvement in a variable climate: hope or hype? Trends Plant Sci 16:363–371
2. Shimamoto K, Kyozuka J (2002) Rice as a model for comparative genomics of plants. Annu Rev Plant Biol 53:399–419

3. Gale MD, Devos KM (1998) Comparative genetics in the grasses. Proc Natl Acad Sci USA 95:1971–1974
4. Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun WL, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalma T, Oliphant A, Briggs S (2002) A draft sequence of the rice genome (*Oryza sativa L. ssp. Japonica*). Science 296:92–100
5. Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, Cao M, Liu J, Sun J, Tang J, Chen Y, Huang X, Lin W, Ye C, Tong W, Cong L, Geng J, Han Y, Li L, Li W, Hu G, Huang X, Li W, Li J, Liu Z, Li L, Liu J, Qi Q, Liu J, Li L, Li T, Wang X, Lu H, Wu T, Zhu M, Ni P, Han H, Dong W, Ren X, Feng X, Cui P, Li X, Wang H, Xu X, Zhai W, Xu Z, Zhang J, He S, Zhang J, Xu J, Zhang K, Zheng X, Dong J, Zeng W, Tao L, Ye J, Tan J, Ren X, Chen X, He J, Liu D, Tian W, Tian C, Xia H, Bao Q, Li G, Gao H, Cao T, Wang J, Zhao W, Li P, Chen W, Wang X, Zhang Y, Hu J, Wang J, Liu S, Yang J, Zhang G, Xiong Y, Li Z, Mao L, Zhou C, Zhu Z, Chen R, Hao B, Zheng W, Chen S, Guo W, Li G, Liu S, Tao M, Wang J, Zhu L, Yuan L, Yang H (2002) A draft sequence of the rice genome (*Oryza sativa L. ssp. Indica*). Science 296:79–92
6. Zhang Q, Li J, Xue Y, Han B, Deng XW (2008) Rice 2020: a call for an international coordinated effort in rice functional genomics. Mol Plant 1:715–719
7. Wei FJ, Droc G, Guiderdoni E, Hsing YI (2013) International consortium of rice mutagenesis: resources and beyond. Rice 6:39
8. Parry MAJ, Madgwick PJ, Bayon C, Tearall K, Hernandez-Lopez A, Baudo M, Rakszegi M, Hamada W, Al-Yassin A, Ouabbou H, Labhilili M, Phillips AL (2009) Mutation discovery for crop improvement. J Exp Bot 60:2817–2825
9. Wu JL, Wu C, Lei C, Baraoidan M, Bordeos A, Madamba MRS, Ramos-Pamplona M, Mauleon R, Portugal A, Ulat VJ, Bruskiewich R, Wang G, Leach J, Khush G, Leung H (2005) Cheminal- and irradiation-induced mutants of Indica rice IR64 for forward and reverse genetics. Plant Mol Biol 59:85–97
10. Till BJ, Cooper J, Tai TH, Colowit P, Greene EA, Henikoff S, Comai L (2007) Discovery of chemically induced mutations in rice by TILLING. BMC Plant Biol 7:19
11. Kim Y, Schumarker KS, Zhu JK (2006) EMS mutagenesis of Arabidopsis. Methods Mol Biol 323:101–103
12. Kodym A, Afza R (2003) Physical and chemical mutagenesis. Methods Mol Biol 236:189–204
13. Sikora P, Chawade A, Larsson M, Olsson J, Olsson O (2011) Mutagenesis as a tool in plant genetics, functional genomics, and breeding. Int J Plant Genom 2011:314829
14. Talebi AB, Talebi AB, Shahrokhifar B (2012) Ethyl methane sulphonate (EMS) induced mutagenesis in malaysian rice (cv. MR219) for lethal dose determination. Am J Plant Sci 3:1661–1665
15. Comai L, Henikoff S (2006) Tilling: practical single-nucleotide mutation discovery. Plant J 45:684–694
16. Henikoff S, Comai L (2003) Single-nucleotide mutations for plant functional genomics. Annu Rev Plant Biol 54:375–401
17. Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE (2003) Large-scale discovery of induced point mutations with high-throughput tilling. Genome Res 13:524–530
18. Serrat X, Esteban R, Guibourt N, Moysset L, Nogues S, Lalanne E (2014) EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. Plant Methods 10:5
19. Arisha MH, Shah SNM, Gong ZH, Jing H, Li C, Zhang HX (2015) Ethyl methane sulfonate induced mutations in M_2 generation and physiological variations in M_1 generation of peppers (*Capsicum annuum* L.). Front Plant Sci 6:399
20. Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nat Biotechnol 23:75–81
21. Dolan L, Janmaat K, Willemsen V, Linstead P, Poethig S, Roberts K, Scheres B (1993) Cellular organization of the *Arabidopsis thaliana* root. Development 119:71–84
22. Petricka JJ, Winter CM, Benfey PN (2012) Control of Arabidopsis root development. Annu Rev Plant Biol 63:563–590
23. Robouillat J, Dievart A, Verdeil L, Escoute J, Giese G, Breitler C, Gantet P, Espeout S, Guiderdoni E, Perin C (2009) Molecular genetics of rice root development. Rice 2:15–34