Highly efficient mutagenesis using N-methyl-N-nitrosourea on male germ cells of maize

Huaidong Tian[1*], Zhen Li[1], Pengfei Niu[1], Chenghua Tian[2], Nan Li[1], Feng Yan[1], Xiaohui Shen[1], Hailin Ma[3], Guimei Cui[3]

(1. *Laboratory for Plant Germplasm and Genetic Resources of Crop, School of Life Science, Shanxi University, Taiyuan 030002, China*; 2. *Sorghum Research Institute, Shanxi Agricultural University, Jinzhong 030600, Shanxi, China*; 3. *Shanxi Dafeng Seed Industry, Taiyuan 030031, China*)

Abstract: The mutagenesis with ethyl methanesulfonate (EMS) mixed in paraffin oil on maize (*Zea mays* L.) pollens has been the main means for inducing genic mutants of maize. However, it is necessary to develop a new technique for improving efficiency of random mutagenesis of genes in maize, and to promote mutant creation for functional-genomic research and breeding of maize. In this study, the technique of N-methyl-N-nitrosourea (MNU) mutagenesis on maize male germ cells was developed by establishing the method of MNU treatment of invitro maize pollens and screening the derived progeny populations. The phosphate-buffered protective-solution-1 (PS1) containing 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.4% sucrose was determined as the optimum formula for protecting invitro pollens at 20°C; the effect of MNU concentration, treatment time, pH, and PO_4^{3-} concentration on the survival rate of pollens treated in the MNU solutions containing PS1 formula was clarified, performing the single-factor and orthogonal experiments for pollen viability; the reproductivity of treated pollens was confirmed, examining their germination and insemination; the MNU treatments under each group condition all increased the mutant-character occurrence in the M_1-M_2 populations derived by insemination of mutagenized male germ cells, in contrast to the EMS treatment; when the pollens were treated for 40 min in 1.0 mM MNU solution (pH: 4.8) containing 20 mM PO_4^{3-}, the mutant-character incidence most-significantly reached the maximum value of 10.5%, which was 6.2 times higher than that based on the EMS treatment; the mutant population was obtained by further screening the M_3 populations. The MNU-mutagenic technique remarkably improves the efficiency of broad-spectral induction of maize mutants, which can provide support for the novel functional-gene identification and diversified breeding of maize.

Keywords: *Zea mays* L., inbred line, in vitro pollens, MNU, mutagenization

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1 Introduction

Germplasm, the fundamental genetic component supporting heredity and variation of organisms, is crucial for maintaining biodiversity and human survival. Mutagenesis is an effective way to innovate crop germplasms. Development and application of mutagenic techniques are of great significance for curbing germplasm diversity weakening and cultivation environment deterioration in breeding and production of crops. Maize is the main grain crop. Technological development for mutagenizing maize can open up the path with reference significance for creating genetic resources for genetics and breeding of crops. In the past decade or so, the genetic and breeding science of maize has entered the era of functional genomics and diversified breeding.

Mutation induction includes physical, biological, and chemical mutagenesis. Physical mutagenesis is based on chromosome damage by physical-mutagenic factors such as radiation to induce mutations[1]. Biological mutagenesis, suitable for inducing mutants by some exogenous biofactors such as T-DNA, transposons, and CRIPSR/Cas9[2–4], has been approached to create maize mutants[5–8], but has undergone a noteworthy dispute in biosafety. Chemical mutagenesis, which can randomly induce mutations of endogenous genes with chemical mutagens[9], has advantages such as broad mutant spectrum, higher genic-mutant rate, and non-dispute on biosafety, and thus is appropriate for creating the genic mutants for functional-genomic researches and breeding of crops.

The EMS, an alkylating mutagen, has been used for inducing mutations in plants. Since the EMS was reported to possess mutagenic action on maize[10], the mutagenic method based on pollen treatment with EMS mixed in paraffin oil has been the main means for creating genic-mutants of maize[11–13]. Maize that possesses complex and large genome with 2.3 gigabase was estimated to contain at least 59 000 genes including more than 39 000 potential protein-coding genes and over 12 000 publicly available genes[14–17]. Recently, a pan-genomic map of maize containing 43 000 gene families was constructed by integrating the genomes of 12 temperate maize inbred lines and B73/Mo17[18]. The utilization of maize mutants has promoted identification of partial

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Biographies: Zhen Li, MS candidate, research interest: mutagenesis, Email: 451030473@qq.com; **Pengfei Niu**, MS candidate, research interest: mutagenesis, Email: 2369311582@qq.com; **Chenghua Tian**, Associate Researcher, research interest: breeding, Email: tianchenhua521@163.com; **Nan Li**, MS candidate, research interest: mutant creation, Email: 527916938@qq.com; **Feng Yan**, MS candidate, research interest: mutant creation, Email: 370584277@qq.com; **Xiaohui Shen**, MS candidate, research interest: mutant creation, Email: 437638145@qq.com; **Hailin Ma**, Associate Researcher, research interest: breeding, Email: ymsmhl@163.com; **Guimei Cui**, Experimenter, research interest: breeding, Email: dfzycgm@163.com.

***Corresponding author: Huaidong Tian**, PhD, Associate Professor, research interest: crop breeding and germplasm innovation, identification of functional genes in crops. School of Life Science, Shanxi University, Taiyuan 030002, China. Tel: +86-351-7010599, Email: huaidongt@sxu.edu.cn.

genes in maize. However, biofunctions of most genes in maize are still unknown, for which a main reason is deficiency of abundant mutants due to low mutagenic efficiency. It is necessary to develop new technology for improving efficiency of mutagenesis of maize genes to create enough abundant mutants for functional-genomic research and breeding of maize.

The MNU, a nitroso-compound inducing mononucleotide changes[9], possesses the mutagenic action distinct from the EMS. This mutagen has been approached for the induction of rice mutants[19–22], but the research for its mutagenic effect on maize has not been reported. In this study, the technique of MNU mutagenesis on maize male germ cells was developed to solve the low-efficiency issue for random mutagenesis of endogenous genes in maize. In comparison with the EMS mutagenesis of maize, this technology significantly contributes to enhancing mutagenic intensity and treatment efficacy, increasing mutant occurrence and broadening the mutational spectrum, thus achieving a leap in mutagenic efficiency of endogenous genes in maize.

2 Materials and methods

2.1 Plant materials and growth conditions

An inbred line, A378, isolated from hybrid progenies between two maize blood lineages (Lancaster and Reid), was used as the wild type material for mutagenesis. Fresh mature pollens were collected from the A378 and refrigerated for the following treatments. M_1-M_3 populations were derived by the insemination of mutagenized pollens to self-plant embryosac. All the materials were planted in the experimental field of this laboratory.

2.2 Scheme for MNU treatments of invitro pollens

The mutagenic-scheme design, including mutagen selection, determination of tissue/cells to be treated, and configuration of treating-liquid system, is the prerequisite for establishing mutagenic technique. The MNU, a water-soluble mutagen, possesses the mutagenic function distinct from the EMS; the latter has been used in the treatment of maize pollens with the EMS mixed in paraffin oil mixture[17]. In this study, the invitro pollens were subjected to the MNU-mutagenic treatments in suitable aqueous environment, to improve the mutagenic effect on a large number of male germ cells. Considering that invitro cells are prone to absorb water and burst to death when exposed in aqueous environment, it is first necessary to establish a method for protecting invitro pollens in solutions. Some protective reagents such as glycerol, ethylene glycol, dimethyl sulfoxide, and sucrose, which can increase the viscosity coefficient of aqueous solution and stabilize cells in solution, have been used for protecting invitro cells in cryosolution[23,24]. The PBS (pH 4.8) and three PBS-buffered PSs containing glycerol, ethylene glycol, dimethyl sulfoxide, and/or sucrose were designed (Table 1) to analyze the effect of solutions on the invitro pollen vitality at room temperature. An appropriate amount of pollens were subjected to the non-mutagenic immersion treatment in PBS, PS1, PS2, and PS3 for 20 min, followed by the vitality analysis, respectively. The immersion in each solution was repeated three times.

Table 1 Scheme for protection of invitro maize pollens

Solutions	Gl/%	EG/%	DS/%	Su/%
PS1	30	15	15	0.4
PS2	35	20	0	0.6
PS3	50	0	0	1.5
PBS	0	0	0	0

Note: Gl-glycerol; EG-ethylene glycol; DS-dimethyl sulfoxide; Su-sucrose; PBS-phosphate buffer solution; PS-protective solution.

MNU concentration, treatment time, pH and phosphate concentration, set as the total treatment factors, were subjected to single-factor and orthogonal experiments, respectively, to analyze the effects of the factors on the viability of pollens treated with MNU in optimum PS. In the single-factor experiment, five levels of each factor were set as the variable conditions, respectively, and the other factors were set as the invariants (Table 2), according to the design reported by Tian et al.[19] The orthogonal experiment was performed according to the design with four factors and three levels. Based on the mutagenic-treatment scheme, the MNU solutions were prepared by dissolving appropriate amounts of MNU in the optimum PS at an appropriate pH and phosphate concentration, respectively. The appropriate amount of pollens were subjected to the MNU treatment under each group condition at 20°C in a dark environment, followed by the vitality analysis, respectively. The treatment under each group condition was repeated three times.

Table 2 Scheme for MNU treatments of invitro maize pollens

TRT	MNU/mM	Time/min	pH	PO_4^{3-}/mM
MNU TRT	0.5	20	3.6	5
	1.0	40	4.2	10
	1.5	60	4.8	20
	2.0	80	5.4	30
	2.5	100	6.0	40

Note: TRT, treatment.

2.3 Analysis of viability of treated invitro pollens

According to Abdelgadir et al.[25], the pollens immersed without MNU treatment and the pollens treated in the MNU solution containing the optimum PS formula under each group condition were subjected to vitality staining with 0.5 M triphenyltetrazolium chloride solution, respectively. After staining, the viability of pollens was examined using a biomicroscope (CH20BIMF200, Olympus). The total, surviving, and dead pollens were counted respectively. The viability of immersed pollens was statistically analyzed by comparing the significance of difference between survival/bursting rates of pollens treated under PBS and PS immersions. The viability of MNU-treated pollens subjected to single-factor experiment was statistically analyzed by comparing the significance of the difference between survival rates of pollens treated under different levels of each variable. Statistical analysis for orthogonal experiment was performed to clarify the degree and optimal level group of MNU treatment factors affecting pollen viability.

2.4 Electron microscopy of immersed invitro pollens

The pollens without treatment, PBS-immersed pollens, and optimum PS-immersed pollens were fixed in 2.5% (v/v) glutaraldehyde solution, dehydrated in ethanol solutions, substituted with tert butanol, and observed with a scanning electron microscope (JSM-6490LV, JEOL), respectively.

2.5 Reproductive examination of MNU-treated invitro pollens

The culture solution containing 15% (w/v) sucrose, 0.05% (w/v) boric acid, 0.3% (w/v) $CaCl_2$, 0.2% (w/v) $MgCl_2$, 0.1% (w/v) KNO_3, and 0.035% (w/v) gibberellin was prepared, as described by Cui et al.[26] After MNU treatment, the pollen samples were gently washed in 8% sucrose solution and incubated in the culture solution for 10 min at 20°C, to terminate mutagenic effect and enable pollen to germinate.

After incubation, the pollens were stained in the solution containing 0.05% aniline blue, sectioned, and subjected to observation of invitro germination with a fluorescent microscope

(IX51, Olympus). The pollens were pollinated on stigma and subjected to the observation of germination and elongation of pollen tubes. After pollination, the ovary was sectioned and observed with the microscope.

2.6 Construction and screening of M_1–M_3 populations

The levels of each MNU-treatment factor, which maintained higher treated-pollen vitality, were set as variables, respectively, to analyze the mutagenic effect of MNU treatments. The 0.067% (v/v) EMS mixed in paraffin oil was set as the condition for control treatment, according to the method described by Lu et al.[17]. The pollens mutagenically treated under each group condition and incubated in the culture solution were pollinated to self-stigma, to derive the corresponding M_1 kernel population. The M_1 kernel population was planted to derive the corresponding M_1 plant population. The M_2 kernel population was derived by self-pollination of each M_1 plant, and planted to derive the corresponding M_2 plant population. The M_3 kernel population was derived by the self-pollination of candidate mutants selected from M_2 population and planted to derive the M_3 plant population.

Growth and development of M_1 population derived under each group condition were analyzed by investigating the seedling rates of M_1 kernel population and the adult plant rates of M_1 seedling population. Candidate mutants were selected by investigating the characteristics of M_1–M_2 seedlings and the characters of culm, leaf, tassel/ear, and kernel in M_1–M_2 adult populations. The analysis of standard deviation and difference significance for seedling/adult plant rates of M_1 and incidence of mutant characters in M_1–M_2 populations was performed to verify the mutagenic effect of the MNU treatments compared with the control treatment. The heritability of mutant characters in M_3 populations was analyzed to identify homozygous mutants.

3 Results and analysis

3.1 Optimum PS for protecting invitro pollens

The immersion treatments with PBS (pH 4.8) and three types of PBS-buffered PSs were applied to the mature pollens from the inbred line A378 respectively, and the viability of immersed pollens was analyzed by investigating their survival and bursting rates. In comparison with the PBS that significantly reduced the survival rate to 35.7%, the PS1, PS2, and PS3 all increased the survival rates and decreased the bursting rates to varying degrees (Figure 1). The PS1 containing 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide, 0.4% sucrose, and 10 mM phosphate most obviously increased the survival rate to 56.7%.

The PBS-immersed pollens were observed to burst in large quantities and lose plump surface structure, compared with the pollens without immersion. In contrast to the PBS-immersed pollens, the PS1-immersed surviving pollens showed the complete surface structure with normal germination pore and 3D structure (Figure 2). The PS1 was thus determined as the optimum formula for protecting the invitro pollens.

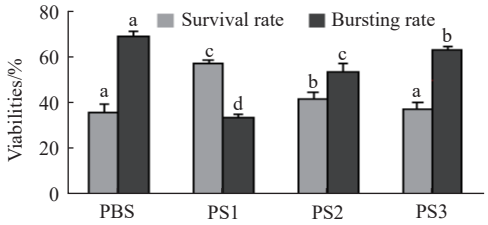


Figure 1 Viability of invitro pollens immersed in PBS and PBS-buffered PSs respectively

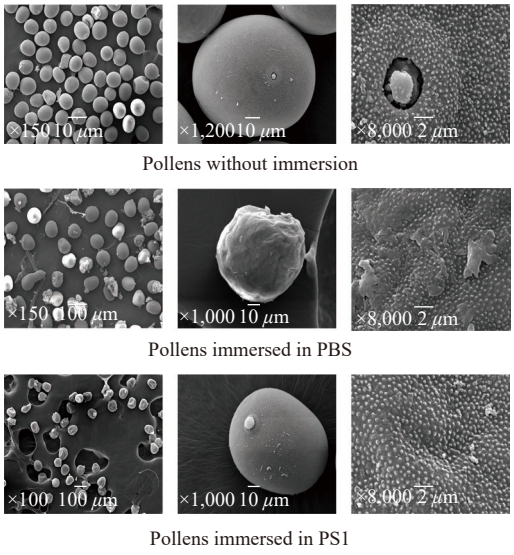


Figure 2 Scanning electron micrographs of pollens without immersion, immersed in PBS, and immersed in PS1

3.2 Effect of MNU treatments on invitro pollen viability

Based on the scheme of MNU-mutagenic treatments, the invitro pollens were treated in the MNU solution containing the PS1 formula under each group condition, respectively. The effects of MNU concentration, treatment time, pH, and PO_4^{3-} concentration on the survival rates of treated pollens were analyzed by the single-factor experiment for pollen viability, respectively. The survival rates presented a decreasing trend with the increase of MNU concentration and treatment time, and the characteristic of first increasing and then decreasing with the increase of pH and PO_4^{3-} concentration (Figure 3). Overall, the pollen survival rates were able to be maintained at a higher level under the three levels among 0.5–1.5 mM MNU, 20–60 min, pH 4.2–5.4, and 10–30 mM PO_4^{3-}, respectively. The orthogonal experiment result showed that the affecting degree of MNU-treatment factors on the pollen survival rates was MNU concentration > treatment time > pH > PO_4^{3-} concentration, and that 0.5 mM MNU/40 min/pH4.8/20 mM PO_4^{3-} was the optimal group for maintaining the highest survival rates (Table 3). The effect of MNU treatment on the treated pollen viability was thus clarified by the single-factor and orthogonal experiments.

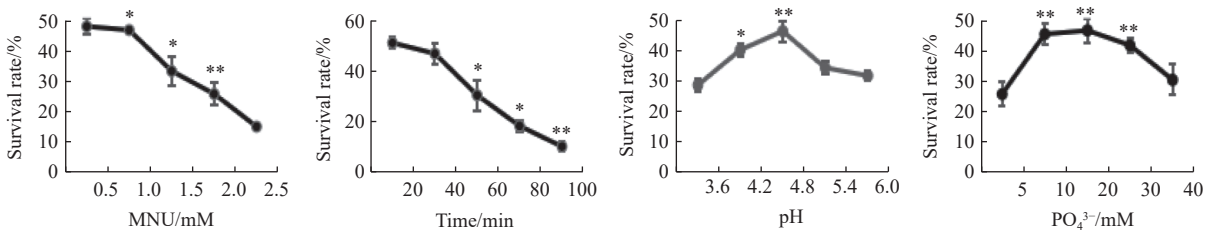


Figure 3 Viability of invitro pollens treated with MNU in single-factor experiment

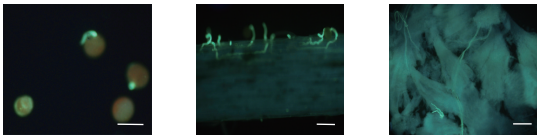
Table 3 Viability of invitro pollens treated with MNU in orthogonal experiment

MNU/mM	Time/min	pH	PO_4^{3-}/mM	Survival rates/%
0.5	20	4.2	10	43.1
0.5	40	4.8	20	49.5
0.5	60	5.4	30	33.6
1.0	20	4.8	30	36.8
1.0	40	5.4	10	40.8
1.0	60	4.2	20	27.4
1.5	20	5.4	20	21.1
1.5	40	4.2	30	22.8
1.5	60	4.8	10	13.2

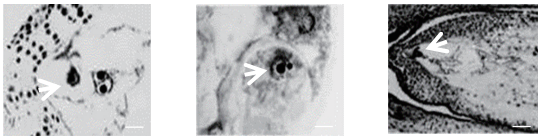
Statistical indicators	MNU conc	Time	pH	PO_4^{3-} conc.
k1 mean	42.1	33.7	31.1	32.4
k2 mean	35.0	37.7	33.2	32.7
k3 mean	19.0	24.7	31.8	31.1
Range	23.1	13.0	2.1	1.6
Square sum	835.4	264.2	6.6	4.3
Mean square	417.7	132.1	3.3	2.2
F	192.5	60.9	1.5	
p	0.005	0.016		

3.3 Reproductivity of MNU-mutagenized male germ cells

After being treated optimally in the MNU solution containing PS1 formula and washed in 8% sucrose solution, the pollens were incubated in the culture solution containing 15% sucrose, 0.05% boric acid, 0.3% $CaCl_2$, 0.2% $MgCl_2$, 0.1% KNO_3, and 0.035% gibberellin. After incubation, the pollens were observed to possess the ability to germinate invitro and germinate in stigma, and subsequently the pollen tubes were observed to mature and elongate to embryo sac (Figure 4). The pollens were further observed to be able to complete the insemination of their male germ cells into egg cell, and the zygote was shown to be generated (Figure 4). These results confirm that the mutagenized male germ cells are able to complete their reproduction.



Germination and elongation of pollen tubes



Insemination of mutagenized male germ cell

Note: Arrows indicate egg before insemination, egg being inseminated, and zygote from left to right, respectively. Bars in upper images = 100 μm; bars in lower images = 50 μm.

Figure 4 Reproductive process of MNU-treated invitro pollens

3.4 Conditions for MNU-mutagenesis of male germ cells

To explore the mutagenic effects of MNU-treatment factors on maize male germ cells, the three levels of each MNU-treatment factor that could maintain higher viability of treated pollens were selected as variables and the other factors were set as invariants respectively, according to the single-factor experiment scheme. An appropriate amount of pollens were treated under each group condition and subsequently incubated in the culture solution. The progeny populations were derived by the insemination of mutagenized male germ cells. The growth and development of M_1 population were analyzed by investigating the seedling rates of M_1 kernel populations and the adult plant rates of M_1 seedling populations, compared with the growth/development indices of M_1 populations derived from the control treatment with 0.067% EMS mixed in paraffin oil. In comparison with the control treatment, almost all the MNU treatments resulted in a significant decrease in seedling rates and adult plant rates (Table 4). The growth/development indices presented the decreasing trend with the MNU-concentration increase and treatment-time extension, and the characteristic of first decreasing and then increasing with the increase of pH and PO_4^{3-} concentration (Table 4). The changes are related to the occurrence of malformed embryo, dysplastic endosperm, and albino/etiolated seedlings in M_1 populations.

Table 4 Effect of each MNU-mutagenic factor on growth and development of M_1 populations

TRT	Variables		K QTY	Seedling rates/%	Adult plant rates/%
MNU TRT	MNU/mM	0.5	543	74.4±1.1^a	76.3±1.4^b
		1.0	564	65.1±1.1^b	69.2±0.5^c
		1.5	558	53.2±1.2^c	61.4±0.9^d
	Time/min	20	579	79.2±0.9^a	77.3±1.9^a
		40	564	65.1±0.7^b	69.2±1.7^b
		60	571	59.0±1.0^c	63.3±1.0^c
	pH	4.2	567	70.2±1.1^b	74.3±1.2^b
		4.8	564	65.1±1.2^c	69.2±0.8^c
		5.4	580	71.5±0.8^b	72.4±1.3^b
	PO_4^{3-}/mM	10	566	72.6±0.6^b	71.3±1.3^b
		20	564	65.1±1.3^d	69.2±1.7^c
		30	598	68.1±0.8^c	72.4±0.9^b
0.067% EMS in PO			560	74.7±0.5	78.5±2.1^a

Note: TRT-treatment; K-Kernel; QTY-quantity; PO-paraffin oil. The same as below.

The M_2 populations were derived by the self-pollination of each M_1 plant. The effect of each MNU-treatment factor on character occurrence was analyzed by evaluating the phenotypical alteration and segregation of characters in M_1–M_2 populations. The MNU treatment under each group condition caused the partial seedling death and the occurrence of mutant characters for culm type, leaf form/color, tassel/ear form/color, and kernel form/color in M_1–M_2. All the MNU treatments led to an increase in mutant character incidence, compared with the control treatment (Table 5). The culm,

Table 5 Effect of each MNU-mutagenic factor on character occurrence in M_1–M_2 populations

TRT	Variable		Quantity of mutant characters						Incidence/%
			Seedling	Culm	Leaf	T/E	K	Total	
MNU TRT	MNU/mM	0.5	4	6	8	3	16	37	6.9±0.2^c
		1.0	5	9	15	4	26	59	10.5±0.6^d
		1.5	1	5	5	1	11	23	4.1±0.2^b
	Time/min	20	2	3	4	1	6	16	2.8±0.3^a
		40	5	9	15	4	26	59	10.5±0.7^c
		60	3	6	6	-	12	28	4.9±0.6^b
	pH	4.2	2	3	5	-	9	19	3.4±0.4^b
		4.8	5	9	15	4	26	59	10.5±0.1^d
		5.4	3	4	6	1	15	30	5.2±0.4^c
	PO_4^{3-}/mM	10	1	6	6	1	13	27	4.8±0.5^b
		20	5	9	15	4	26	59	10.5±0.8^c
		30	2	3	5	2	13	25	4.2±0.4^b
0.067% EMS in PO			-	2	2	1	4	9	1.7±0.2^a

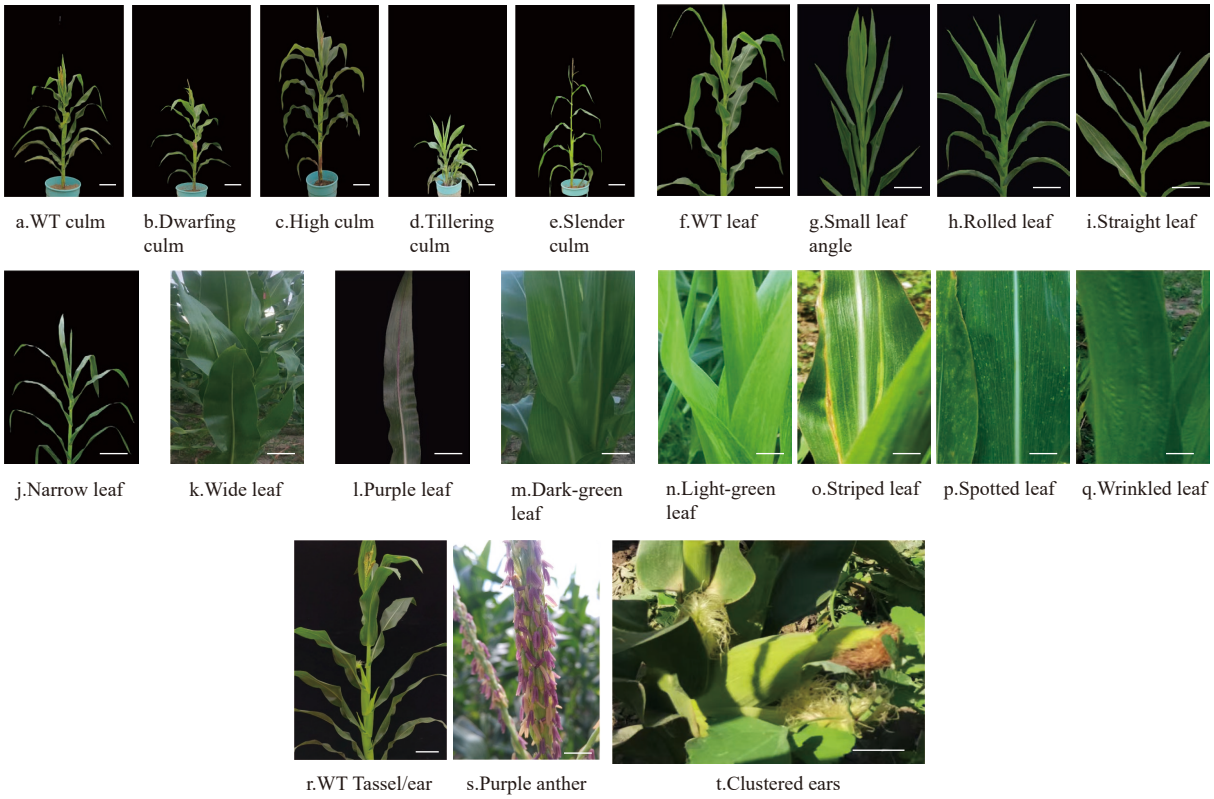
Note: T/E, tassel/ear.

leaf, tassel, ear, and kernel mutants exhibited various character alterations, in comparison with the wild type of inbred line. When the pollens were treated in the MNU solution (pH 4.8) containing 1.0 mM MNU and 20 mM phosphate for 40 min, the incidence of mutant characters reached the maximum value of 10.5% (Table 5). This group condition was determined to be optimal for MNU mutagenesis on maize male germ cells.

3.5 *ZmMM* mutant population

The candidate mutants for plant and kernel characters were determined by screening the M_1-M_2 populations. The M_3 kernel/plant populations were derived by the self-pollination of viable and fertile candidate-mutants. A total of 150 homozygous mutants for culm, leaf, tassel/ear, and kernel characters were obtained, further analyzing the genetic stability of candidate-mutant characters in the M_3 population, compared with the wild type of inbred line. The mutant population was referred to as *ZmMM*, given that the mutants were induced by MNU-mutagenesis on maize male germ cells.

In contrast to the wild type of inbred line, the homozygous mutants for culm characters possessed the dwarfing culm, high culm, multi-culm, and/or slender culm; the homozygous mutants for leaf characters possessed the small leaf angle, the rolled leaf, the straight leaf, narrow leaf, wide leaf, purple leaf, deep-green leaf, light-green leaf, striped leaf, spotted leaf, and wrinkled leaf, respectively; the homozygous mutants for tassel/ear characters possessed the purple anther and clustered ears, respectively (Figure 5). The character alteration possibly resulted from heritable mutations related to metabolism, development, and yield formation of maize plant.



Note: Bars in a–e = 20 cm; bars in f–k and r = 10 cm; bars in l–q, s and t = 2 cm.

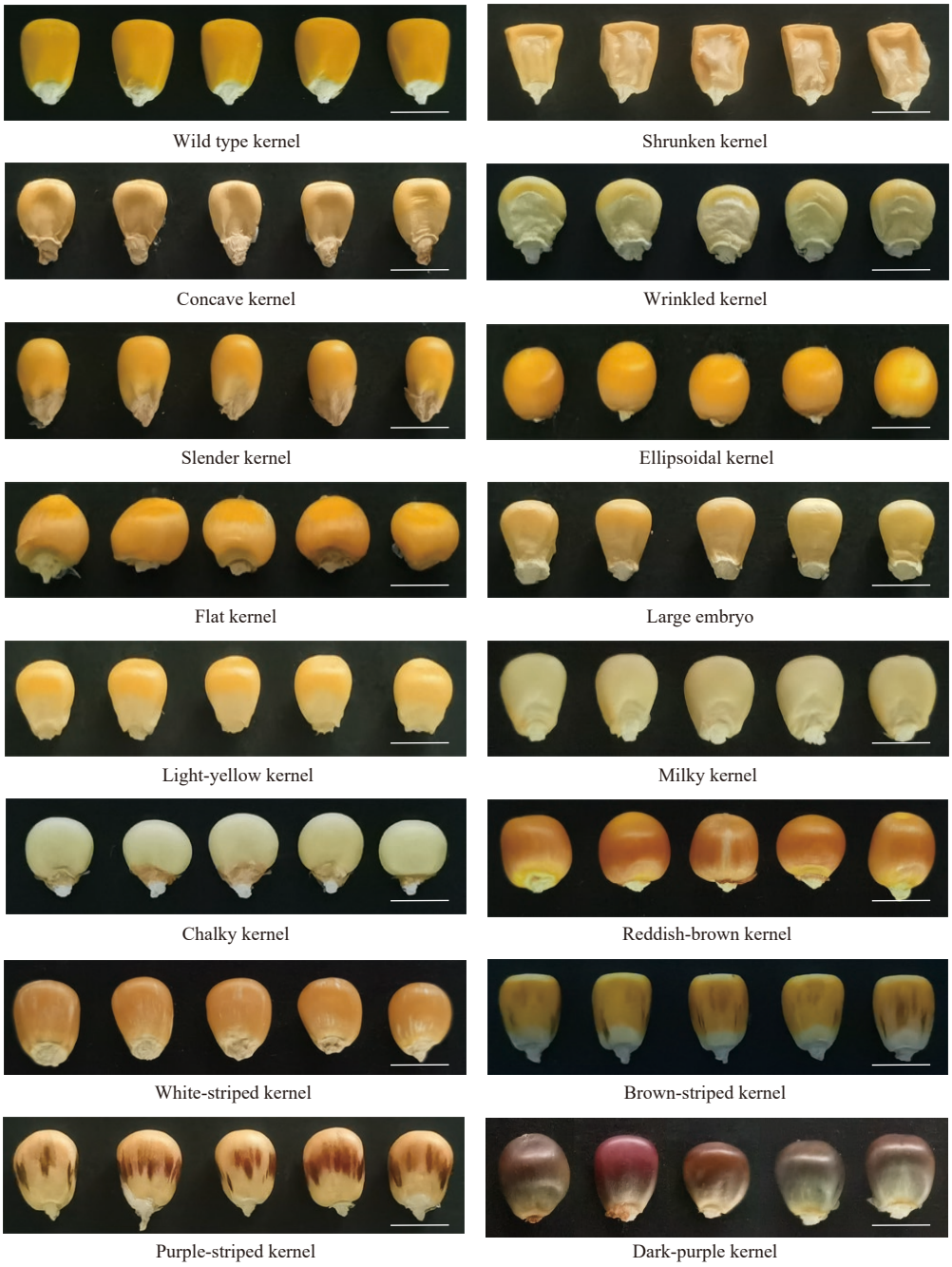
Figure 5 Mutant characters of culm, leaf, and tassel/ear in ZmMM induced by MNU mutagenization of maize male germ cells

In contrast to the wild type inbred line, the kernel mutants possessed the shrunken, concave, wrinkled, slender, ellipsoidal, flat, big-embryo, light-yellow, milky, chalky, red-brown, white-striped, brown-striped, purple-striped, and dark-purple kernels, respectively (Figure 6). Additionally, the kernel mutants exhibited significant changes in length, width, thickness, and/or weight of seeds (Table 6). These mutants are the meaningful genetic materials related to yield and quality of maize kernel.

4 Discussion

For over sixty years, the mutagenesis of maize pollens with EMS/paraffin oil mixture has been the main means for inducing genic mutants of maize. The EMS-induced mutants have been utilized in genetic research and breeding of maize. In recent years, genetic and breeding science of maize has entered the era of functional genomics and diversified breeding. However, the functional-genomic research and diversified breeding of maize are being confronted with the following two key scientific issues. One is that biological functions of most maize genes are still unknown, and the other is that a wealth of valuable genic-mutant materials need to be created and utilized. To create more diverse mutants with genic mutations in maize, it is necessary to develop a new mutagenic technique for improving the efficiency of random mutagenesis of endogenous genes in maize. In this study, the technique of MNU mutagenesis on maize male germ cells was developed by the comprehensive innovation in mutagenizing process.

The invitro pollens from the wild type of maize inbred line A378 were subjected to the MNU treatment in aqueous environment, to establish the method of optimized mutagenic-treatment of suitable tissue cells. Unlike the pollen mutagenization with EMS/paraffin oil mixture with organic solvent property, the MNU-treatment solution with aqueous property possesses the sufficient permeability of mutagen into the pollen cells to be treated, and can bring the strong bursting impact to the cells. The systematic experiments were conducted as follows, to establish the technical



Note: Bars = 1 cm.

Figure 6 Mutant characters of kernel in ZmMM induced by MNU mutagenization of maize male germ cells

architecture for comprehensively resolving the conflict between the positive and the negative effects of MNU-treatment solution on the treated pollen cells: The PBS-buffered PS1 was first determined as the optimum formula for protecting the pollens immersed in aqueous environment at 20°C. The three levels of each MNU treatment factor were then clarified to be appropriate for maintaining higher viability of the pollens treated in the MNU solutions containing PS1 formula by the single-factor experiment. The degree and optimal level group of the factors for maintaining pollen viability were further defined by the orthogonal experiment, and the reproductivity of optimally-treated pollens was finally verified (Figures 1-4; Table 3). The MNU-treatment method not only clearly contributes to increasing the mutagenic intensity, but also enables a large amount of treated active pollens to be suitable for the efficient construction of progeny populations by insemination of mutagenized male germ cells due to the prominent enhancement of mutagenic-treatment efficacy.

The previous related researches indicated that the mutagenic rate of MNU was obviously higher than that of EMS on rice genome[27,28]. In this study, the three levels of each MNU-treatment factor that maintained higher viability of treated pollens were selected as variables to perform the single-factor experiment for clarifying the variable effect on the mutant character occurrence in maize. The seedling and adult plant rates of M_1 populations derived by the MNU treatments were negatively correlated with MNU concentration and treatment time, and showed a decrease followed by an increase with the increase of pH and PO_4^{3-} concentration (Table 4). The changes in the M_1 growth/development indices were manifested in the occurrence of phenomena such as malformed embryo, dysplastic endosperm, and albino/etiolated seedlings in M_1 populations, due to the MNU toxicity or the lethal effect of dominant mutation. The various mutant characters of plant and

Table 6 Quantization of typical kernel mutant characters in *ZmMM*

Character types	Length/mm	Width/mm	Thickness/mm	Weight per 100 grains/g
Wild type kernel	13.8 ± 0.7	10.9 ± 0.7	4.5 ± 0.3	31.9 ± 0.5
Shrunken kernel	10.2 ± 0.2**	8.6 ± 0.4**	3.5 ± 0.2*	21.7 ± 0.8**
Concave kernel	13.2 ± 0.8	8.1 ± 0.4**	3.7 ± 0.5*	25.9 ± 0.6**
Wrinkled kernel	12.2 ± 0.3**	11.6 ± 0.8	3.9 ± 0.3*	27.4 ± 0.2**
Slender kernel	14.2 ± 0.3	7.6 ± 0.6**	4.2 ± 0.3	29.8 ± 1.0**
Ellipsoidal kernel	8.7 ± 0.4**	8.2 ± 0.7**	6.9 ± 0.6**	28.8 ± 0.4**
Flat kernel	9.1 ± 0.2**	13.5 ± 0.9**	6.2 ± 0.3**	36.1 ± 0.4**
Big-embryo kernel	12.9 ± 0.5	8.5 ± 0.5**	4.3 ± 0.2	29.6 ± 0.4**
Light-yellow kernel	9.4 ± 0.2**	8.3 ± 0.6**	6.5 ± 0.7**	29.9 ± 1.2**
Milky kernel	13.6 ± 0.8	11.0 ± 0.8	4.4 ± 0.3	31.3 ± 0.4
Chalky kernel	12.3 ± 0.9*	10.8 ± 1.1	4.8 ± 0.1	32.5 ± 0.4
Reddish-brown kernel	11.1 ± 0.7**	10.7 ± 0.4	6.8 ± 0.5**	34.3 ± 0.6**
White-striped kernel	11.2 ± 0.5**	10.4 ± 0.6	4.8 ± 0.2	28.5 ± 0.4**
Brown-striped kernel	13.5 ± 0.7	10.6 ± 0.6	4.3 ± 0.2	29.0 ± 0.4**
Purple-striped kernel	12.9 ± 0.6	9.8 ± 0.6	4.7 ± 0.1	30.3 ± 0.2**
Dark-purple kernel	10.7 ± 0.6**	9.6 ± 1.0	4.8 ± 0.3	30.0 ± 0.5**

kernel occurred in the M_1-M_2 populations. In comparison with the control treatment with EMS/paraffin oil mixture, the MNU treatments under each group condition all significantly resulted in an increase in mutant character incidences in M_1-M_2 populations (Table 5). Among them, the MNU treatment under the condition with 1.0 mM MNU, 40 min, pH 4.8, and 20 mM PO_4^{3-} induced the mutant characters with the highest incidence, which was about 6.2 times higher than that based on the control treatment. This group condition was determined to be optimal for MNU mutagenesis on male germ cells. These results indicate that the MNU mutagenesis significantly improved the induced rate of maize mutants compared to the EMS mutagenesis, and that the optimal conditions are necessary for maximizing the mutagenic efficiency.

The mutagenic mechanisms of MNU and EMS have been understood by the researches about mutagenesis of mammalian and microbial cells[29–33]. The EMS, a S_N2 type mutagen with higher Swain-Scott substrate constant, attacks the highly-nucleophilic sites such as ring nitrogen atoms in DNA, rarely causes alkylation at oxygen atom site, and relies on the error-prone pathway of repair system. In contrast to the EMS, the MNU, a S_N1 type of mono-functional alkylating agent with lower S-S constant, has higher reactivity to low-nucleophilic centers such as oxygen atom site in DNA, and is able to induce the point mutation by base substitution independently from the error-prone pathway of repair system. The independence of MNU from the repair system helps to increase the occurrence of heritable mutations in mutagenized cells. Since the MNU has the mutagenic function distinct from EMS, the MNU-mutagenesis on maize male germ cells can also contribute to broadening the mutation spectrum and inducing novel mutants. The mutant population, referred to as *ZmMM*, was created by the MNU mutagenesis and the progeny population screening (Figures 5, 6 and Table 6). The *ZmMM* is a valuable resource pool for novel functional-gene identification and breeding of maize.

5 Conclusions

The highly efficient mutagenic technique using MNU on male germ cells of maize was developed by establishing the optimum PS protecting invitro pollens, determining the levels of MNU treatment factors for maintaining higher vitality of treated pollens, and ascertaining the optimal conditions for inducing mutant character occurrence; the new mutant population referred to as *ZmMM* was created by this technique. This technique can significantly enhance mutagenic intensity and treatment efficacy, increase mutant incidence, and broaden the mutational spectrum, and thus contribute to the remarkable improvement of mutagenic efficiency of endogenous genes in maize. The utilization of *ZmMM* is expected to promote the functional-genomic research and diversified breeding of maize.

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