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Comparison between Germinated Seed and Isolated Microspore EMS Mutagenesis in Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Abstract: Mutagenesis is an important tool for breeding and genomic research. In this study, the germinated seeds and isolated microspores of a double haploid line 'FT' were treated with EMS, respectively, with the aim of comparing the effects of the two approaches on generating mutants in Chinese cabbage. For microspore EMS mutagenesis, the isolated microspores were treated with 0.12% EMS for 20 min, a total of 1268 plantlets were obtained, and 15 M_1 mutants were screened with a mutation frequency of 1.2%. For seed EMS mutagenesis, 7800 germinated seeds were treated with 0.8% EMS for 12 h, and a total of 701 M_2 mutants were screened, with a mutation frequency of 18.78%. In total, 716 mutants with heritable morphological variation including leaf color, leaf shape, leafy head, bolting, and fertility, were obtained from the EMS mutagenesis experiments. Homozygous mutant plants could be screened from M_1 lines by microspore mutagenesis, and M_2 lines by seed mutagenesis. The mutation frequency was higher in seed mutagenesis than in microspore mutagenesis. Based on these results, we propose that seed EMS mutagenesis is more suitable to generate a large-scale mutant library, and the microspore EMS mutagenesis is conducive to rapidly obtaining homozygous mutants.

Keywords: Chinese cabbage; EMS; mutagenesis; isolated microspore culture; mutants

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1. Introduction

Plant mutants are ideal materials for discovering new genes and revealing their functions, which are widely used in functional genomics researches [1–6]. Plant mutant libraries are typically constructed by artificial mutagenesis, including chemical mutagenesis [7,8], physical mutagenesis [9,10], and insertion mutagenesis [11–13]. Ethyl methanesulfonate (EMS) is a widely used chemical mutagenic agent [14,15]. EMS mutagenesis has various advantages over other methods, including high mutation rates, low chromosomal aberrations, simple operation, and no requirement for genetic transformation [16].

EMS mutagenesis has been applied to construct the mutant libraries in a wide range of crops, such as rice [17,18], *Arabidopsis thaliana* [7,19], wheat [20–23], tomato [24,25], soybean [26–29], maize [30], peanut [31], and *Brassica napus* [32,33]. Regarding EMS mutagenesis of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*), there are two approaches, seed and microspore mutagenesis. For seed mutagenesis, Lu et al. [34] treated an inbred line 'A03' seeds with 0.4% EMS for 16 h and constructed a mutant library, containing 4253 M_1 families. For microspore mutagenesis, Lu et al. [35] mutagenized the buds with different concentrations of EMS solution, and the isolated microspore culture was conducted where a total of 142 mutants were identified. Huang et al. [36] treated the microspores of a

double haploid (DH) line 'FT' with different concentrations of EMS solution, and then the microspores were cultured. A total of six stably inherited mutants were obtained, with a mutation rate of 0.46%. The concentration of the solution, duration of action, and the starting material (seeds, bulbs, embryos, tissues, pollen, etc.) used for mutagenesis all affected the efficiency of mutagenesis. The varieties of Chinese cabbage used in the above studies were different, so it could not provide a basis for us to accurately compare the mutagenesis efficiency.

The objective of this study was to compare the effects of these two approaches. Seed EMS mutagenesis and microspore EMS mutagenesis were applied to create mutants in a Chinese cabbage DH line 'FT', which was used as the mutagenic material. Our results not only provide valuable germplasm resources for Chinese cabbage but also guide future work involving the generation of mutants.

2. Materials and Methods

2.1. Plant Materials

The DH line 'FT' derived from the Chinese cabbage variety 'Fukuda 50' was used as the experimental material. This variety is characterized by heat resistance, early maturity, white flowers, folded green leaves, and an ovoid leafy head (Figure 1).

Figure 1. Wild-type 'FT' plant. (**a**) floral organ; (**b**) leafy head.

2.2. Microspore EMS Mutagenesis

A total of 50 germinated 'FT' seeds were vernalized at 4 °C for 15 days and sown in trays in a greenhouse (15 °C–26 °C) at Shenyang Agricultural University from September to December 2016. Four hundred and fifty unopened flower buds containing late uninucleate spores were selected for isolated microspore culture, following the methods of Zhang et al. [37]. The isolated microspores were treated with EMS solutions of 0.12% for 20 min. Based on the previous research of Huang et al. [36], we set up a pre-experiment. EMS concentration of 0.12% and 0.16%, which were treated for 10 min, 15 min and 20 min, respectively, and the embryo rate was calculated. Finally, the EMS concentration was determined to be 0.12%, and the treatment for 20 min was a semi-lethal dose, which was conducive to the creation of mutants.

The ploidy levels of the regenerated plants from the microspore culture were checked using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry was performed according to the methods of Huang et al. [36].

2.3. Seed EMS Mutagenesis

In total, 7800 'FT' seeds were germinated at 25 °C for 12 h. The germinated seeds were immersed in 0.8% EMS and placed in a 50-turn shaker for 12 h. The treated seeds were then thoroughly washed in flowing water for 12 h and sown in trays in a greenhouse (12–26 °C) in December 2017. The live plants (M_0) were self-pollinated, and the M_1 seeds were harvested.

3. Results

3.1. Microspore EMS Mutagenesis

In total, we obtained 1339 embryoids after EMS mutagenesis treatment and 1268 regenerated plants (M_0 plants) after rooting culture. Regenerated plants were divided into haploids, diploids, and tetraploids (Figure 2). Among them, a total of 1034 DH plants were obtained to further identify the mutants.

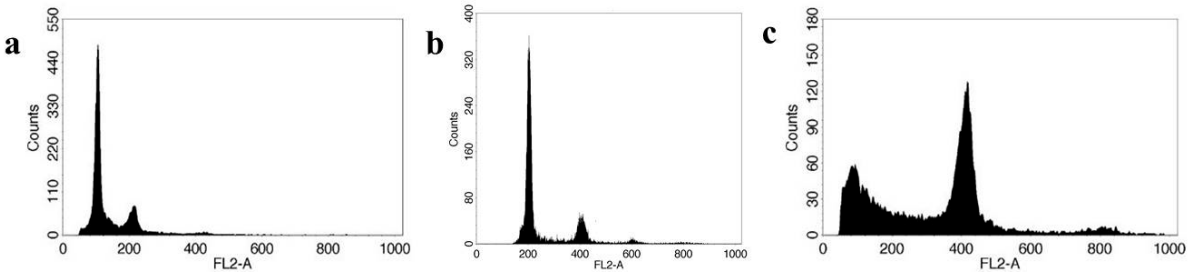

Figure 2. Determination of ploidy levels in regenerated plants by flow cytometry. (**a**) Haploid plant; (**b**) double haploid plant; (**c**) tetraploid plant. Note that the X-axis represents the DNA content, and the Y-axis represents relative cell counts.

In M_0 generation, a total of 30 DH plants exhibited mutant phenotypes, including leaf shape, leaf color, leafy head, and fertility mutants (Table 1). In the M_1 generation, a total of 15 mutants were identified, and the mutation frequency was 1.2% (Table 2).

Table 1. Characterization of mutation types in M_0 plants of Chinese cabbage (microspore EMS mutagenesis).

Trait	Variant Characteristics	No. of Mutant Plants (M_0)	Mutation Frequency (%)
Leaf shape	Crinkled leaf	4	0.32
Leaf color	Leaf etiolation	5	0.40
	Partial leaf etiolation	5	0.40
Leafy head	Small leafy head	6	0.48
	Vertical leafy head	7	0.56
	Non-heading	2	0.16
Fertility	Male sterile	1	0.08
	Total	30	2.40

Table 2. Characterization of mutation types in M_1 plants of Chinese cabbage (microspore EMS mutagenesis).

Trait	Variant Characteristics	No. of Mutant Plants (M_1)	Mutation Frequency (%)
Leaf shape	Crinkled leaf	2	0.16
Leaf color	Leaf etiolation	4	0.32
	Partial leaf etiolation	4	0.32
Leafy head	Small leafy head	3	0.24
	Vertical leafy head	2	0.16
	Total	15	1.20

Mutants were mainly classified as leaf traits (leaf color and leaf shape) and leafy head mutants. A total of 10 leaf trait mutants were detected in the M_1 generation. The mutation frequency was 0.8%. Leaf color is the main type of leaf trait mutation, such as complete leaf etiolation (Figure 3a right) and partial leaf etiolation (Figure 3b,c right). In addition, two leaf shape mutants were detected. Compared to wild-type 'FT' plants, these two crinkled leaf

mutants (Figure 4a,b right) both exhibited the crinkled leave characteristics, and one of them developed slowly at all stages.

Figure 3. Three leaf color mutants. (**a**) The wild-type 'FT' plant (left) and complete leaf etiolation mutant (right); (**b**,**c**) the wild-type 'FT' plant (left) and partial leaf etiolation mutant (right).

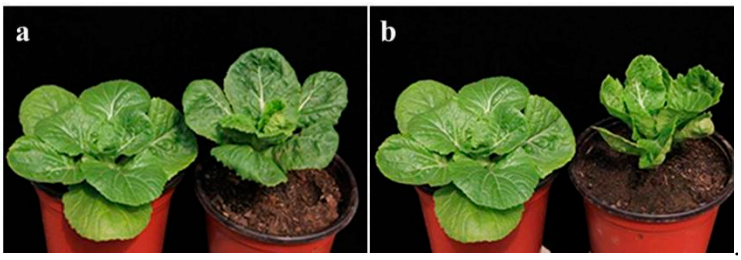

Figure 4. Two leaf shape mutants. (**a**,**b**) The wild-type 'FT' plant (left) and crinkled leaf mutant (right).

Five leafy head mutants were detected in the M_1 generation, and the mutation frequency was 0.4%. Two vertical leafy head mutants (Figure 5a,b right) and three small leafy head mutants (Figure 5c right) were identified.

Figure 5. Three leafy head mutants. (**a**,**b**) The wild-type 'FT' plant (left) and vertical leafy head mutants (right); (**c**) the wild-type 'FT' plant (left) and small leafy head mutant (right).

3.2. Seed EMS Mutagenesis

A total of 7800 seeds were treated with EMS solution, 4920 seeds germinated, and 3990 plants survived in the M_0 generation. After self-pollination, the seeds (M_1 generation) of 3731 lines were harvested. The germination rate and seedling survival rate were 63.1% and 51.2%, respectively.

Among 3731 M_1 lines, 1121 lines exhibited mutant phenotypes as compared to the wild-type 'FT' phenotype, and the mutation rate was 30.04% (Table 3). We obtained 701 stably inherited mutations in the M_2 generation, indicating an 18.78% mutation frequency (Table 4). The mutants with variation in multiple traits were also screened, including those with chlorophyll-deficient and flat leaves, and chlorophyll-deficient and crinkled leaves. The major types of mutants were leaf color, leaf shape, leafy head, bolting, and fertility traits.

Table 3. Characterization of mutation types in M_1 plants of Chinese cabbage (seed EMS mutagenesis).

Trait	Variant Characteristics	No. of Mutant Lines (M_1)	Mutation Frequency (%)
Leaf color	Leaf etiolation	386	10.34
	Partial leaf etiolation	73	1.95
	Leaf gloss variation	15	0.40
	Dark-green leaf	15	0.40
	Light-green leaf	10	0.26
	Anthocyanin accumulation	15	0.4
	Stay-green leaf	23	0.62
Leaf shape	Crinkled leaf	189	5.06
	Leaf thickness variation	12	0.32
	Petiole length variation	22	0.59
	No mesophyll	7	0.19
	Leaf margin variation	22	0.59
	Cracked leaf	16	0.43
	Slender leaf	17	0.46
	Leaf senescence	16	0.43
	Entire leaf	20	0.54
	Abnormal leaf	36	0.96
Leafy head	Non-heading	61	1.63
	Vertical leafy head	32	0.86
	Earlier leafy head formation	5	0.13
	Later leafy head formation	4	0.11
	Small leafy head	13	0.34
	Large leafy head	3	0.08
	Abnormal leafy head	4	0.11
Bolting	Early bolting	44	1.18
Fertility	Male sterile	58	1.55
	Female sterile	3	0.08
	Total	1121	30.04

Table 4. Characterization of mutation types in the M_2 plants of Chinese cabbage (seed EMS mutagenesis).

Trait	Variant Characteristics	No. of Mutants (M_2)	Mutation Frequency (%)
Leaf color	Leaf etiolation	221	5.92
	Partial leaf etiolation	65	1.74
	Leaf gloss variation	8	0.21
	Dark-green leaf	8	0.21
	Light-green leaf	5	0.13
	Anthocyanin accumulation	8	0.21
	Stay-green leaf	8	0.21
Leaf shape	Crinkled leaf	143	3.83
	Leaf thickness variation	9	0.24
	Petiole length variation	18	0.48
	No mesophyll	2	0.05
	Leaf margin variation	18	0.48
	Leaf crack	10	0.26
	Slender leaf	10	0.26
	Leaf senescence	10	0.26
	Entire leaf	12	0.32
	Abnormal leaf	18	0.48

Table 4. *Cont.*

Trait	Variant Characteristics	No. of Mutants (M_2)	Mutation Frequency (%)
Leafy head	Non-heading	46	1.23
	Vertical leafy head	20	0.54
	Earlier leafy head formation	3	0.08
	Later leafy head formation	3	0.08
	Small leafy head	7	0.18
	Large leafy head	2	0.05
	Abnormal leafy head	4	0.11
Bolting	Early bolting	16	0.43
Fertility	Male sterile	25	0.67
	Female sterile	2	0.05
	Total	701	18.78

In total, 8.65% of mutations affected leaf color, which was the most frequent mutation type. Among the leaf color mutations, leaf etiolation was most common, including complete leaf etiolation (Figure 6a–c right) and partial leaf etiolation (Figure 6d–f right).

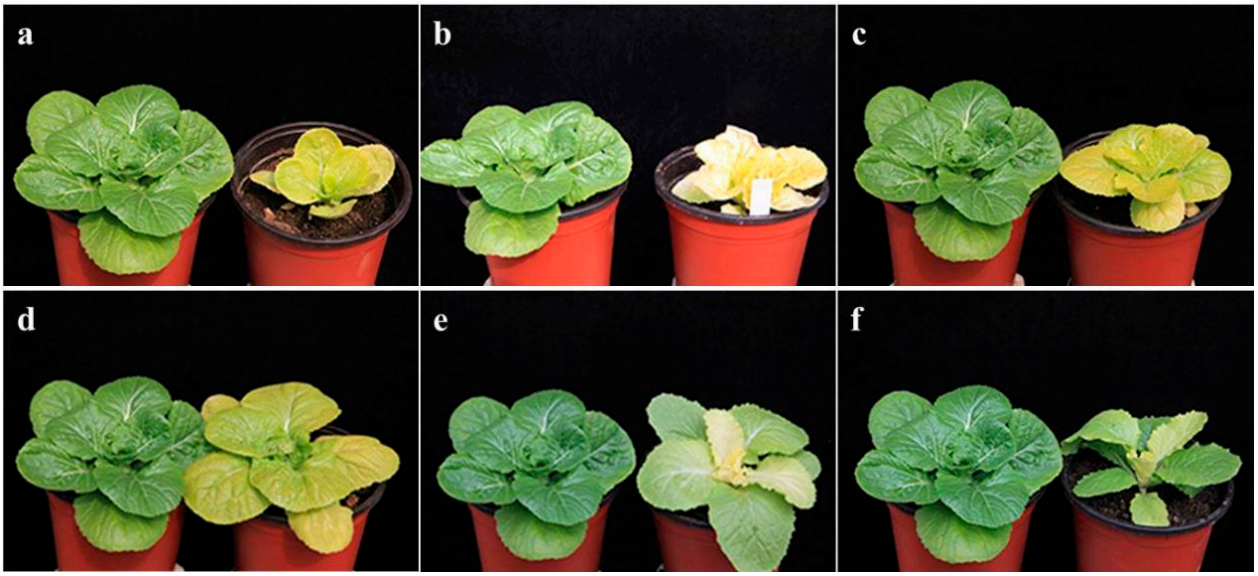

Figure 6. Six leaf color mutants. (**a**–**c**) The wild-type 'FT' plants (left) and complete leaf etiolation mutants (right); (**d**–**f**) the wild-type 'FT' plants (left) and partial leaf etiolation mutants (right).

A total of 250 leaf shape mutants were obtained in the M_2 generation. The mutation frequency was 6.7%. The mutations included changes in leaf margin (Figure 7a,b right), mesophyll (Figure 7c right), and crimp degree (Figure 7d–f right). The no mesophyll mutants were rare, and their leaves were twisted into strips (Figure 7c right).

The leafy head of wild-type 'FT' was ovoid, and the heading leaves were folded and shriveled. A total of 85 leafy head mutants were verified in the M_2 generation, and the mutation frequency was 2.27%. Forty-six non-heading mutants were identified (Figure 8). Twenty vertical leafy head mutants were observed at a frequency of 0.54%.

A total of 16 early-bolting mutants were observed (Figure 9 right), and the flowering time was about 25 days earlier than the wild-type 'FT' plants.

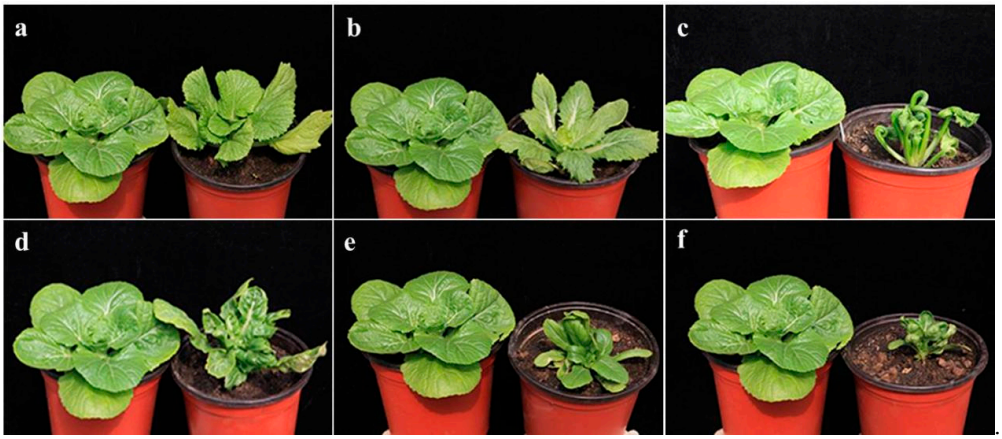

Figure 7. Six leaf shape mutants. (**a**,**b**) The wild-type 'FT' plant (left) and leaf margin mutants (right); (**c**) the wild-type 'FT' plant (left) and no mesophyll mutant (right); (**d**–**f**) the wild-type 'FT' plant (left) and crimple leaf mutants (right).

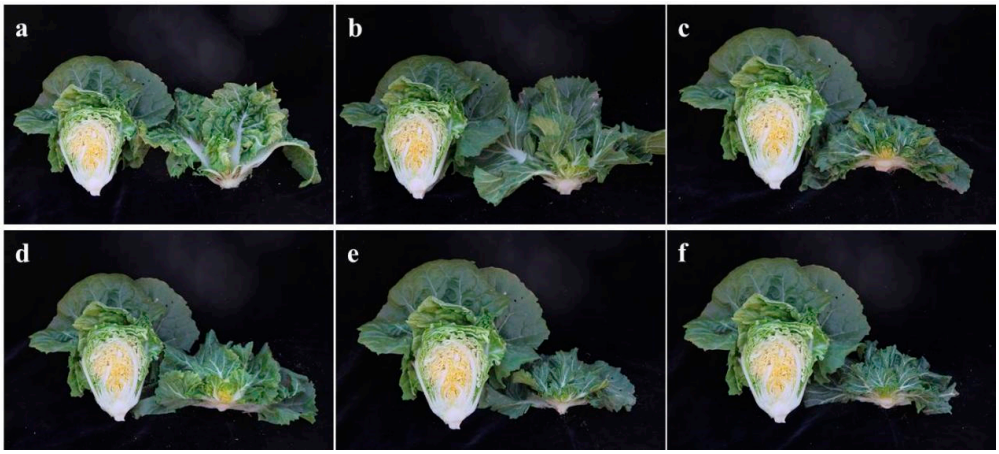

Figure 8. Six leafy head mutants. (**a**–**f**) The wild-type 'FT' plants (left) and non-heading mutants (right).

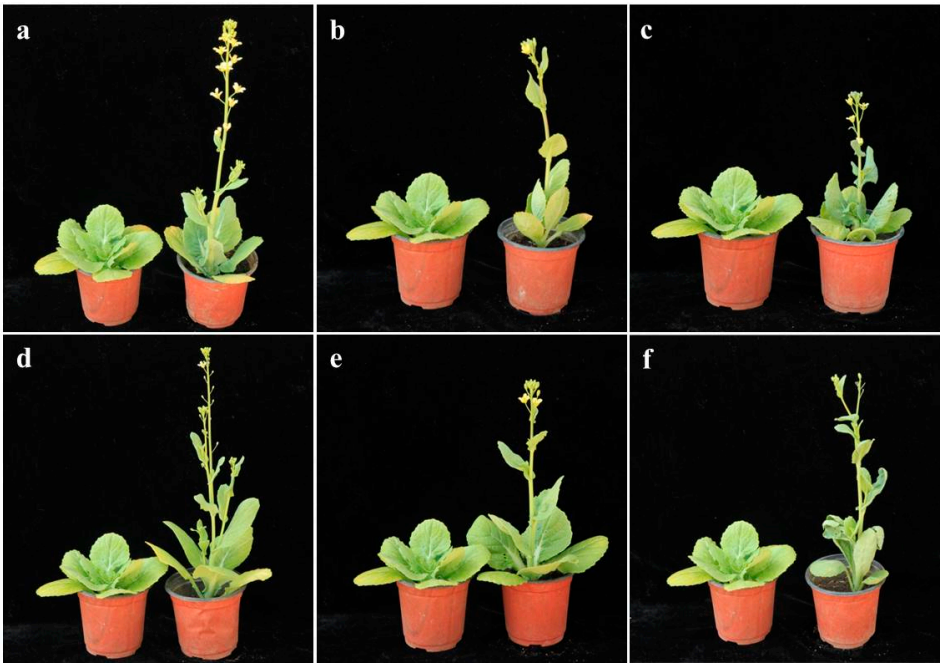

Figure 9. Six bolting mutants. (**a**–**f**) The wild-type 'FT' plants (left) and early bolting mutants (right).

The frequency of fertility mutations was 0.72%, including male-sterile mutant (Figure 10) and female-sterile mutant (Figure 11). The stamens of male-sterile mutants completely degenerated without pollen. The female-sterile mutants showed pistil abortion and smaller floral organs.

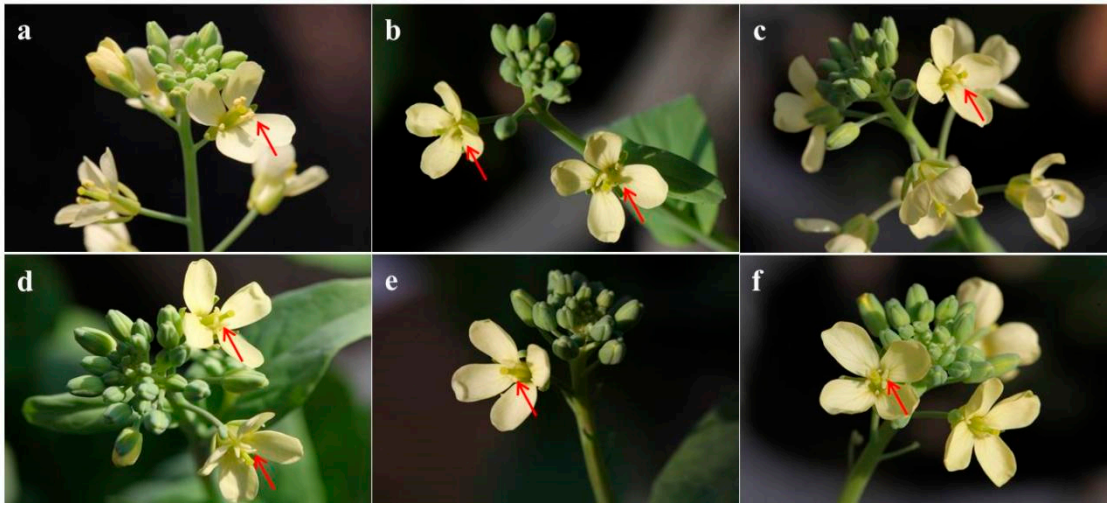

Figure 10. Five male-sterile mutants. (**a**) wild-type 'FT' plants; (**b**–**f**) male-sterile mutants. The red arrow points to the stamens.

Figure 11. Two female-sterile mutants. (**a**) wild-type 'FT' plants; (**b**) female-sterile mutants; (**c**) pistils of wild-type 'FT' (left) and female-sterile mutants (right).

3.3. Comparison of the Two Mutagenesis Approaches

As shown in Table 5, for the EMS mutagenesis of seeds, 701 stably inherited mutations were screened in the M_2 generation, with a mutation frequency of 18.78%. For the EMS mutagenesis of microspores, 15 stably inherited mutations were screened from the M_1 generation, with a 1.2% mutation frequency.

Table 5. Comparison of two mutagenesis approaches.

Mutagenesis Approach	No. of Mutants	Mutation Frequency (%)	Homozygous Generation	Technical Operation
Seed mutagenesis	701	18.78	M_2	Easy
Microspore mutagenesis	15	1.2	M_1	Complicated

4. Discussion

Mutants are important for the investigation of gene function in crop plants. Wang et al. [18] obtained *zebra-15* mutants from the restorer line *Jinhui10* (*Oryza sativa* L. ssp. *indica*) by treatment with EMS for studies of chlorophyll. Ansari et al. [22] treated the seeds of diploid wheat *Triticum monococcum* with 0.25% EMS to obtain brittle culm mutants

(*brc1*, *brc2*, and *brc3*), therefore the cloning of mutant genes could contribute to wheat improvements. Li et al. [29] constructed a mutant library by treating 80,000 seeds of the soybean *Glycine max* cv. Zhongpin661 (Zp661) with 50 mmol/L EMS for 9 h and identified a yellow pigmentation mutant (*gyl*) with a significantly decreased chlorophyll (Chl) content and abnormal chloroplast development. Similarly, the construction of a Chinese cabbage mutant library by EMS mutagenesis has important implications for studies of the functional genome.

The precise mutagenesis materials and methods are crucial for the successful construction of a mutant library. Inbred lines have been usually used as mutagenesis materials in *Brassica* crops [34,38–40]. In this study, a Chinese cabbage DH line was employed as the mutagenesis material to create a mutant library. The genetic background of the DH line was homozygous, which was a benefit to screen the mutants. Moreover, the genetic background was highly consistent between the wild-type and the mutants, and the genetic differences only occurred at the mutation sites, which was helpful to further study the functional genomics in Chinese cabbage.

Various methods for EMS mutagenesis are used to create Chinese cabbage mutants and the treatment of microspores and seeds are the most common approaches. In a comparative analysis of these two methods, we found that the mutation rate was higher for seed mutagenesis. We could easily obtain a substantial number of mutants in a small-scale mutagenesis experiment. Wang et al. [32] treated seeds of a *Brassica napus* L. cv. Ningyou7 DH line with 0.6% EMS for 18 h and obtained 1652 mutants from 7110 plants (mutation frequency, 23.23%). Zhang et al. [33] treated seeds of NJ7982 (*Brassica napus* L.) with 0.4% EMS for 12 h and obtained a large number of mutants in the M_2 generation, with a mutation rate of 18.51%. Lu et al. [34] treated 12,000 seeds of the Chinese cabbage inbred line 'A03' with 0.4% EMS for 16 h and obtained various types of mutants in the M_2 generation at a frequency of 37.62%. In our experiment, 701 mutants were obtained by the treatment of 7800 seeds with 0.8% EMS. The mutants exhibited altered leaf color, leaf shape, leafy head, bolting, and fertility. The potential breeding value of the mutants was revealed, such as stay-green mutants, male-sterile mutants, non-heading mutants and bolting mutants, which have important significance for the improvement of these traits. Seed mutagenesis is simple and easy to implement. The mutant rate was satisfactory, but stably inherited mutations could only be screened in the M_2 generation. Therefore, seed mutagenesis was more suitable for large-scale functional genomics, genetic diversity, and the innovation of germplasm resources.

Microspore mutagenesis is a rapid approach for creating the homozygous mutants, which can be screened in M_1 generation and can accelerate the functional genomic studies. Huang et al. [36] treated microspores of the Chinese cabbage DH line 'FT' with 0.04%, 0.08%, and 0.12% EMS for 10 min and obtained six mutants from among 1304 regenerated plants in the M_1 generation (mutation rate, 0.46%). In our experiment, isolated microspores were treated with 0.12% EMS for 20 min, yielding 15 stably inherited mutations in regenerated plants, with a mutation frequency of 1.2%. Microspore mutagenesis had a lower mutation rate, and it relied on the mature isolated microspore culture system. We speculated that the lower mutation rate might be due to the majority of mutant microspores with the inability to form embryoids.

In both mutagenesis methods, a large number of variant features, such as male sterility, early-bolting, and leaf etiolation, cannot be stably inherited. We speculated that these variations might be related to environmental conditions or physiological injury, and similar results have been reported in our previous research [36]. In addition, it is possible that some EMS-induced mutations were unstable and may recover its original genotype in some cases.

In conclusion, we compared and analyzed the EMS mutagenesis efficiency between two different approaches. In total, we obtained 716 mutants by EMS mutagenesis of seeds and microspores, and the mutation frequency was lower using microspores of Chinese cabbage (1.2%) than using seeds (18.78%). Based on our results, we propose that seeds of

DH lines or other homozygous strains are more suitable for EMS mutagenesis to generate a large-scale mutant library, and microspores are conducive to rapidly obtain homozygous mutants. In addition, these mutants could be used to investigate gene function by gene mapping and cloning, and further reveal the molecular mechanism underlying important traits. The Chinese cabbage mutants derived in this study also provide new germplasm resources for genetic studies and breeding.

Author Contributions: H.F., S.H. and Y.G. designed the experiments. Y.G. conducted the experiments, performed the data analysis, and wrote the manuscript. G.Q., M.Z., W.F. and Z.L. participated in creating the mutants. H.F., S.H. and J.R. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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