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ETHYLMETHANESULFONATE MUTAGENESIS OF CUCUMBER FOR LARGE-SCALE MUTANT SCREENS

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Abstract

To broaden the genetic resources for cucumber (*Cucumis sativus* L.) breeding, and to accelerate the systematic functional analysis of cucumber genes, a cucumber mutant library was constructed by using Ethylmethanesulfonate (EMS) mutation in this study. A total of 2,200 seeds were treated with EMS solutions, and 421 M_2 families were obtained, which showed a mutant frequency of 19.1%. Some visible mutant phenotypes, such as having few spines, long fruits, short fruits, pale green fruit color, and plants without flowers were identified in this study. This study will be helpful for the identification of causal genes and for the determination of interesting mutant phenotypes of cucumber in the future.

Key words: Breeding, Fruit, Mutant, Spine, Tilling.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops in the world. A number of genomic resources, including transcriptome (Wu *et al*., 2010), are available for the dissection of the developmental pathways in cucumber (Li *et al*., 2009). In recent years, the genome sequencing program of cucumber (Huang *et al*., 2009) has led to the availability of a large number of gene sequences in public databases, which has consequently encouraged the development of reverse genetics approaches in cucumber (Shang *et al*., 2014). A future challenge will be to determine the function of all of the genes in cucumber. One of the best ways to ascertain function is to disrupt genes and determine the phenotype of the resulting organism. However, compared with other crops, such as Arabidopsis and rice (Uraguchi *et al*., 2011; Mary *et al*., 2015), the development of functional genomics study in cucumber is slow due to partial reasons of low and unstable genetic transformation efficiency (Nanasato *et al*., 2013), narrow genetic base (Plader *et al*., 2007), and lack of mutant plants. If the cucumber mutant library could be constructed in a larger scale, and combined with genome information and techniques, such as map-based cloning (Tabata *et al*., 2013) and targeting induced local lesions in genome (Tilling) (McCallum *et al*., 2000; Uraguchi *et al*., 2011), the systematic functional analysis of cucumber genes with these approaches could be realistic.

Mutant is important for the functional genomics study (Coelho *et al*., 2000), especially for the plants with known genome information. By using mutant plants, the function of many important genes that regulate different developmental processes or agronomic traits, has been identified (Gao *et al*., 2015; Wu *et al*., 2015). There are many strategies to build mutant library, such as T-DNA or transposon insertion (Alonso *et al*., 2003; An *et al*., 2005), and chemically induced mutagenesis (Greene *et al*., 2003). Considering the low and unstable genetic transformation efficiency, the long growth cycle, and the large plant type, it will be difficult to build the cucumber mutant library by using techniques involved transgenosis, such as T-DNA and transposon insertional mutagenesis. Compared with insertion mutation, chemically-induced mutation such as ethylmethanesulfonate (EMS)-mediated mutagenesis has been shown some advantages such as high efficiency because each individual line can bear single point missense and nonsense substitutions in hundreds of genes (Greene *et al*., 2003). More interestingly, some other techniques, such as tilling and map-based cloning (McCallum *et al*., 2000; Uraguchi *et al*., 2011; Tabata *et al*., 2013), can work together with EMS mutated library, if some genes with candidate important functions, or some plants with interesting mutant phenotypes were identified.

Some EMS mutant collections in cucumber have been generated and different kinds of phenotype alterations had been described (Fraenkel *et al*., 2012; Boualem *et al*., 2014), which has constituted another important resource for cucumber genetic studies. However, most mutant phenotypes were identified during the seedling stage (Fraenkel *et al*., 2012; Boualem *et al*., 2014), and few reports are related to the fruit phenotype, which is of high significance for cucumber production. To broaden the genetic resources for cucumber breeding, and to accelerate the systematic functional analysis of cucumber genes, a cucumber mutant library was constructed by using EMS mutation in the present study. A total of 2,200 seeds were treated with EMS solution, and 421 M_2 families were obtained. Some mutant phenotypes such as a few spines, long fruits, short fruits, pale green fruit color, and plants without flowers, were also identified in the present study. The work described in the present study will be helpful in causal gene identification for interesting mutant phenotypes in the future.

Material and Methods

Plant material and EMS concentration selection: Experiments were carried out using the cucumber cultivar 'Dongnong 649', which was kindly provided by Prof. Zhiwei Qin from Northeast Agricultural University, Harbin, China. To determine the best EMS concentrations, 100

cucumber seeds for each treatment were soaked in 100 mL of distilled water for 4 h at room temperature and were then mutagenized with different concentrations (0.5%, 1%, 1.5%, 2%, and 2.5%) of EMS (Sigma, USA) in 0.2 M phosphate buffer (pH=7). These seeds were treated in EMS solution for 18 h at room temperature with gentle shaking. The treated seeds were then washed with 10 mL of 1 M $Na_2S_2O_3$ once, with 20 mL of 100 mM $Na_2S_2O_3$ thrice and with 20 mL of distilled water thrice. The seeds were sown in compost in 96 trays, which allowed for seedling emergence frequency to be determined accurately. Control seeds, which were exposed to phosphate buffer (pH=7) treatment, were treated in the same manner. The best EMS concentrations were selected by seedling emergence frequency determination of cucumber seeds.

EMS-mediated mutagenesis and mutant library construction: Mutant population was constructed based on the following protocols. Wild type seeds (M_0) were treated with two different concentrations (1.5% and 2%) of EMS solution for 18 h. The treated seeds were planted in a greenhouse on the farm at the Northeast Agricultural University, China, in accordance with the standard cucumber agronomic practice. Each M_1 plant was self-pollinated separately and M_2 seeds were collected from individual M_1 plants and kept separately. Six to eight seeds belonging to each M_2 family were sown in cultivation pots and the corresponding seedlings were transplanted in greenhouse. Each M_2 plant was self-pollinated separately and M_3 seeds were collected from single M_2 plants.

Mutant screening: Mutants were screened by observing the plants through the whole growth stage in all generations. Each mutant candidate was characterized according to 7 classes and 34 subclasses (Table 1). Data were then collected and recorded using a Canon 600D digital camera.

Measurement of spine number: The spines of mutant and wild type fruits with same developmental stage were marked by marker pen at 15 d after pollination. The number of spine was then calculated by the calculator. Data were presented as means ± SD. At least three fruits were subjected to statistical analysis in each case. Tukey's test was conducted for statistical analysis ($p<0.05$).

Results

Construction of cucumber mutant library: To optimize the EMS mutagenesis, a 'kill-curve' analysis was first conducted by using a dose ranging from 0.5% to 2.5% EMS (Table 2). Seedling emergence frequency was used as the evaluation index for suitable EMS concentrations. Results revealed that the seedling emergence frequency was greatly reduced by the incremental increase of EMS concentrations (Table 2). At 0.5% and 1% EMS concentrations, 77% and 79% of the treated seedlings emerged. At 1.5% and 2.0% EMS concentrations, the seedling emergence frequencies were 61% and 58%. At 2.5% EMS concentration, only 56% of the treated seedlings emerged. When the seeds were treated with phosphate buffer (pH=7), the seedling emergence frequency was 100% (Table 2).

Table 1. List of phenotype classes and subclasses.

Class	Subclass	Class	Subclass
Cotyledon	Morphology	Fruit	Shape
	Shape		Length
	Color		Color
	Tricotyledon		Stripe color
Leaf	Color		Surface gloss
	Shape		Fruit tumor size
	Leaf edge		Fruit tumor number
	Length		Spines color
	Width		Spines number
	Tip shape		Bloom
	Trichome density	Plant	Dwarf
	Petiole length		Small plant
Flower	Sepals shape	Tendrils	Number
	Sepals number		Shape
	Petal number	Stem	Thickness
	Petal color		Internodes length
	Sex differentiation		Trichome density

Table 2. Impact of EMS concentration on seedling emergence.

EMS concentration	Number of seeds treated with EMS	Seedling emergence (%)
CK	100	100
0.5%	100	77
1%	100	79
1.5%	100	61
2%	100	58
2.5%	100	56

To construct a large-enough cucumber mutant library while obtaining a certain number of desired mutants at the same time and considering the lethal character of EMS mutation, 1.5% and 2% concentrations of EMS were selected for the subsequent experiment. A total of 800 seeds were then treated with 1.5% EMS, and 900 seeds were treated with 2% EMS. The EMS-treated seeds were sown in soil, and seedlings were grown to fruit maturity in the greenhouse from April to August 2014. The 500 EMS-treated seeds from the kill-curve analysis were also sown in soil and used for generating M_1 plants. For the M_1, 878 out of 2,200 seeds were germinated and grown into plants. Individual M_1 plants were self-pollinated to produce M_2 seeds. From the 2,200 EMS-treated seeds, 421 M_2 seed stocks were obtained due to low fertility, which showed a mutant frequency of 19.1%. Six to eight M_2 seeds from 421 individual lines were sown in soil, and seedlings were grown in the greenhouse from May to September 2015. Individual M_2 plants of each line were self-pollinated to produce M_3 seeds, and 1,807 M_3 seed stocks were finally obtained.

Mutant phenotype identification: In the previous experiment, cucumber mutant phenotypes, such as cotyledon number and morphology, leaf shape, and plant architecture, were described (Fraenkel *et al*., 2012; Boualem *et al*., 2014). The mutant phenotypes of M_1 and M_2 plants were also identified in the present study (Fig. 1; Tables 3 and 4). Besides the mutant phenotype described by other reports (Fraenkel *et al*., 2012; Boualem *et al*., 2014), some new mutant phenotypes related to cucumber fruit development were also identified in the present study.

Table 3. Class of observed mutant phenotypes of M_1 plants.

Major category	Subcategory	No. of families
Plant	Dwarf	6
	Small plant	11
	Trichome	1
	Color	1
Leaf	Color	14
	Shape	11
	Petiole	2
Flower	Shape	3
	Color	1
Fruit	Shape	4
	Color	2
	Spine number	1
Tendril	Number	1

Table 4. Class of observed mutant phenotypes of M_2 families.

Major category	Subcategory	No. of families
Cotyledon	Color	8
	Shape	5
	Number	7
Leaf	Color	14
	Shape	8
Fruit	Color	4
	Spine number	2
	Length	2
Flower	Shape	4
Plant	Dwarf	5
	Small plant	5

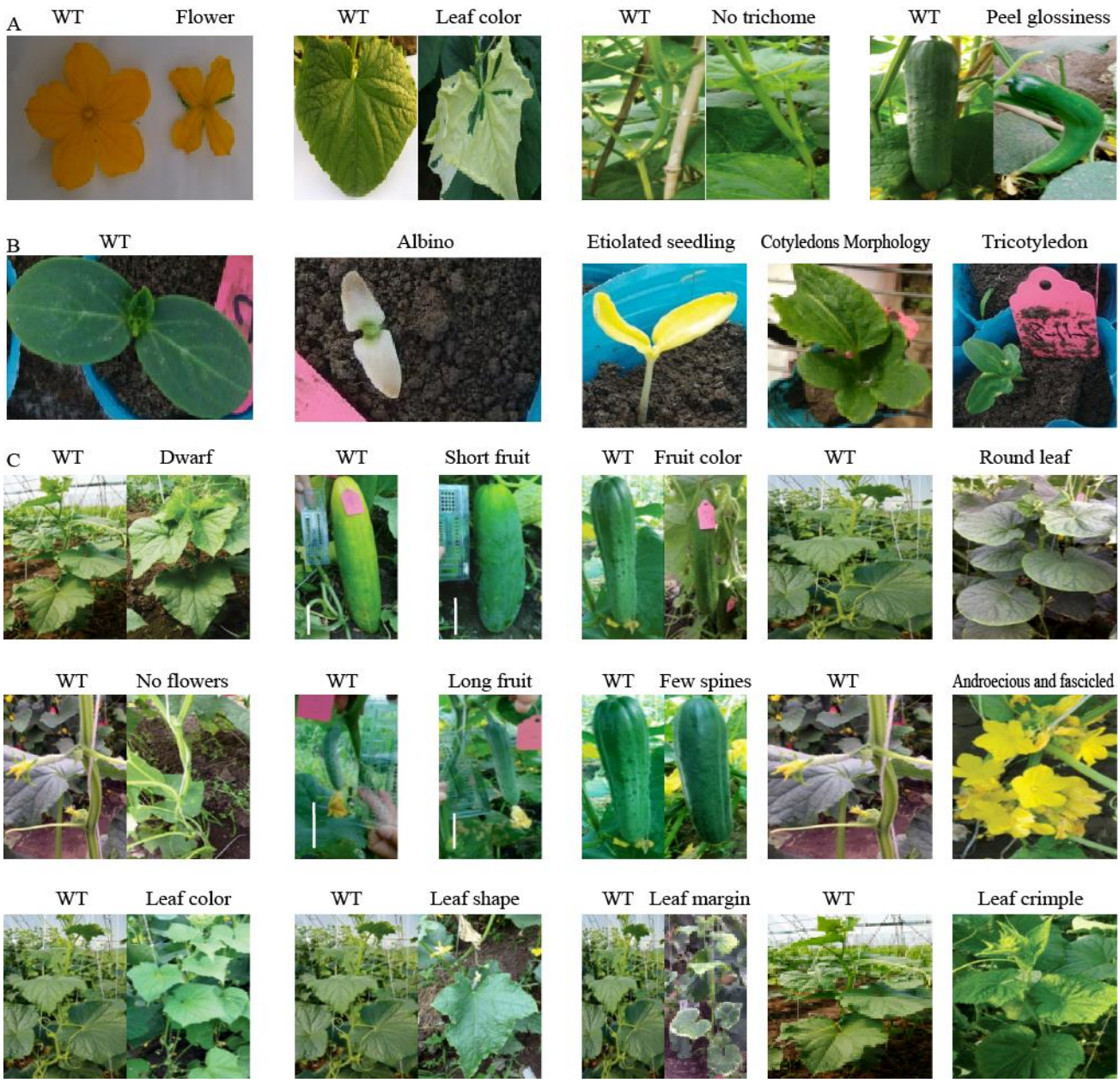

Fig. 1. Mutant phenotypes identified from the EMS-treated wild type (WT) seeds. (A) Mutant phenotypes observed in the M_1 plants; (B) Mutant phenotypes bserved in the cotyledon seedling stage of M_2 families; (C) Mutant phenotypes observed in the individual plant of M_2 families.

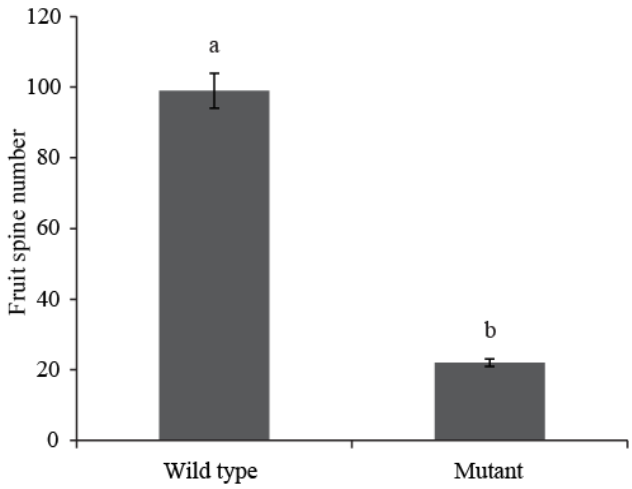

Fig. 2. Number of fruit spine in wild type and mutant cucumber plants. Values are expressed as means ± SE (n = 3). Letters represent significant differences at the 0.05 level based on Tukey's test.

Fifty eight out of 878 lines of M_1 plants showed mutations in plant architecture, color of leaf stalk and fruit, leaf shape, flower and fruit, and mutant phenotype of tendril, as opposed to other wild type plants (Fig. 1A; Table 3). Seeds from the 421 M_1 plants were obtained, whereas the other 457 M_1 plants were not able to grow seeds because of their low fertility rate.

Six to eight seeds belonging to each M_2 family were sown to produce M_3 seeds. Sixty four out of 421 M_2 lines showed mutations, with a frequency of 15.2%. Some mutant phenotypes in seedling stage were present, such as albino, etiolated seedling, and tricotyledon (Fig. 1B; Table 4). The important mutant phenotype candidates were dwarf, short fruit, long fruit, few spines, light green fruit color, round leaf, androecious, numerous numbers of petals, plants with no flowers, leaf shape, leaf crimple, and loss of green margins on leaves (Fig. 1C; Table 4).

Phenotype analysis of few spines mutant: Considering the high significance of fruit phenotype for cucumber breeding, the present research paid more attention to mutant phenotypes of few spines (Fig. 2) in the next study. Fruit spines in mutant and wild type cucumber fruits were counted and compared. The result showed that the number of spines in mutant cucumbers were significantly smaller than that of wild type. Each mutant cucumber tended to have 21.6 ± 4.6 fruit spines, whereas wild types of cucumber had 98.8 ± 10.9 (Fig. 2).

Discussion

The genetic background of cucumber is narrow, and this serves as a problem for genetic resources innovation and breeding of cucumbers (Plader *et al*., 2007). In the present study, a cucumber mutant library was constructed by using EMS mutagenesis. A total of 421 M_2 lines derived from 2,200 EMS mutated seeds were obtained, and 64 out of 421 lines (mutant frequency of 15.2%) showed visible mutant phenotype, which might be useful for cucumber genetic resources innovation and breeding in the near future. Considering the long growth cycle, large growth habit, and low transformation efficiency of cucumbers (Nanasato *et al*., 2013), the data of this study showed that EMS mutagenesis is an efficient way for mutant library construction of cucumber. In addition, EMS mutagenesis might also be suitable for other *Cucurbitaceae* plants such as pumpkin, watermelon, and melon (Dahmani-Mardas *et al*., 2010).

Some important mutant phenotype candidates, such as spine number, fruit length, and fruit color, were identified in this study (Fig. 1). Fruit is the most important commercial character of cucumber, and breeding objectives for cucumber improvement are often focused on fruit quality. The spine number of cucumber fruit is one of the important qualities of a cucumber fruit. The *numerous spines* (*ns*) locus was described; the results showed that few spines on the fruit is dominant to many *ns* from Wis. 2757 (Fanourakis & Simon, 1987; Pierce & Wehner, 1990). Another locus *ss* for small spines had also been reported (Fanourakis & Simon, 1987; Pierce & Wehner, 1990). Large, coarse fruit spines were dominant to small, fine fruit spines. The *ss* locus for small spine size was linked to the *ns* locus for numerous spines and to *u*, *Tu*, *D*, *pm*, and *te* with the probable order of *ns-ss-Tu-D-u-pm-te* (Fanourakis & Simon, 1987; Pierce & Wehner, 1990). The *s* locus for spine size and frequency was also reported (Strong, 1931; Tkachenko, 1935), and the results showed that many small fruit spines, a characteristic of European cultivars, is recessive to the few large spines of most American cultivars (Strong, 1931; Tkachenko, 1935; Pierce & Wehner, 1990). Another two locus, *s-2* for spine-2 and *s-3* for spine-3, had also been described (Pierce & Wehner, 1990). The locus *s-2* acts in a duplicate recessive epistatic fashion with *s-3* to produce many small spines on the fruit (Pierce & Wehner, 1990). Some reports concentrate on spine color. The *B* locus for black or brown spines is dominant to white spines on cucumber fruit (Wellington, 1913; Strong, 1931; Tkachenko, 1935). The *gl* locus was described as cucumber fruit without spines (Robinson & Mishanec, 1964). Although there were a number of reports mentioned above, the gene responsible for few spines on cucumber fruit has yet to be identified. The mutant phenotype caused by EMS is always discrete, considering the typical characteristic of EMS mutagenesis as a single nucleotide mutation (Greene *et al*., 2003). With the development of sequencing and map-based cloning technique, combined with the known genome information of cucumber and different kinds of markers (Huang *et al*., 2009; Shang *et al*., 2014), the causal genes of mutant phenotype caused by EMS mutagenesis can be identified within a short time. The researchers are currently doing the genetic analysis of the mutant phenotype, and they plan to identify the causal genes of few spines of cucumber in the near future.

The 64 lines that showed visible mutant phenotype were identified under normal cultivation. There is a possibility that there are still some other mutants that could be identified by other forms of treatments, such as heavy metal tolerance, diseases resistance, and fruit quality determination. Among others, the heavy metal contamination in vegetable crops, including cucumber, has become a serious problem. Our plan is to identify the mutant candidate with a high capacity for heavy metal

tolerance or sensitivity to heavy metal by using the mutant library constructed in this study. The researchers are also working on the identification of crucial mutant candidate genes related to heavy metal tolerance by using the Tilling technique combined with the mutant library. The extraction of genomic DNA from the M_2 and M_3 populations for tilling analysis is currently underway. The researchers will be increasing the EMS-mutagenized M_2 population to about 5,000 and will continue the phenotyping of the EMS-mutagenized cucumber plants.

In summary, this study demonstrated that EMS-mediated mutagenesis is an efficient approach to induce cucumber mutants. A large amount of cucumber mutants has been identified by using EMS mutagenesis. These mutant lines may contribute to the future cucumber breeding and genetic resources innovation.

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