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SCREENING OF EMS-INDUCED NACL-TOLERANT MUTANTS IN *CUCURBITA MOSCHATA* DUCHESNE EX POIR.

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Abstract

Salinity is one of the major constraints that impair plant growth and ultimately limits crop productivity. Growing salt-tolerant crops is thought to be an efficient strategy to make use of the saline land. Pumpkin (*Cucurbita moschata*) is an economically important and worldwide cultivated vegetable. EMS (ethyl methanesulfonate), as a mutagenic agent, has been widely used to increase genetic variability in crop breeding. In this study, we designed a screen of NaCl-tolerant mutants induced by EMS in *Cucurbita moschata*. Two NaCl-tolerant mutants were screened under NaCl stress and further identified using physiological and biochemical parameters. As the result of genetic analysis, we deduced that the NaCl-tolerant mutant phenotype was controlled by QTLs. This research provides the protocol of mutant screening and new sources for salt-tolerant cultivar breeding in pumpkin. The mutants can also be used to better understand the salt-tolerance mechanism in plants.

Key words: Pumpkin, Salinity, Tolerance, Ethyl Methanesulfonate

Introduction

Salinity is a significant abiotic stress that severely limits the growth, development and productivity of crops (Jan *et al.*, 2017) as well as causes the continuous loss of arable land (Pons *et al.*, 2011). Soil salinization is estimated to affect ~20% of the irrigated areas and is an alarming situation limiting crop productivity worldwide (Yeo, 1999). Breeding salt-tolerant plant cultivars can therefore be understood as an efficient strategy to make use of the saline land.

Pumpkin (*Cucurbita moschata* Duchesne ex Poir.) is a widely cultivated and economically important vegetable in the world. It is rich in mineral elements, vitamins, amino acids, carotenoid and carbohydrates (Acosta-Patino *et al.*, 2001; Kim *et al.*, 2016; Yang *et al.*, 2007). The health efficacy of pumpkin seeds have been well documented (Grzybek *et al.*, 2016; Stevenson *et al.*, 2007). Pumpkin seedlings is widely used as stock for other cucurbitaceous plants to improve resistance against both biotic and abiotic stresses. As a glycophytic plant, pumpkin displays a low level of salinity tolerance and to date, there is limited germplasm that possesses considerable salinity tolerance.

Mutagenesis strategies are considered as significant steps towards generation of novel and valuable genetic variations and mutation-derived varieties have been released in many important crops such as rice, wheat, cotton, sesame, grapefruit and banana (Ahloowalia *et al*., 2004). Among various mutagenesis methods, the chemical agents which have the ability to bring about genetic variability in crops, have gained popularity for many years (Talebi *et al*., 2012). They are easy to use, do not require any specialized equipment and can provide a very high mutation frequency (Vicente-Dolera *et al.*, 2014a). Amongst mutagenic agents, ethyl methanesulfonate (EMS) is currently most favored and widely used. EMS tends to cause single base pair changes (G switch to A, or C switch to T) rather than deletions and translocations and provides a random point mutation (Hoffmann, 1980). EMS-based mutagenesis has been used to generate special alleles, such as extremely small in size, purple leaf color and early ripening fruit in tomato (Saito *et al.*, 2011). Through EMS mutagenesis, researchers have developed TILLING (Targeting Induced Local Lesions IN Genomes) population containing various mutant phenotypes in *Cucumis sativus* and *Cucurbita pepo* (Fraenkel *et al.*, 2014; Vicente-Dolera *et al.*, 2014b). In the aspect of salt-tolerant mutant, three rice mutants with increased tolerance to salt have been screened by gamma-ray irradiation and further identified through physiological experiments (Domingo *et al.*, 2016; Takagi *et al.*, 2015). Through EMS mutagenesis, Japanese researchers have screened salt-tolerant mutant in rice and identified a salt-tolerant related gene (Takagi *et al.*, 2015). So far, however, no salt-tolerant mutant of pumpkin has been reported based on our knowledge.

The goal of this research was to screen NaCl-tolerant mutants in *Cucurbita moschata* in order to enrich germplasm source helping salt-tolerant breeding. Salt-tolerant mutants, as excellent research materials, may further be used to identify salt-tolerant related genes and better understand salt-tolerance mechanism in plants.

Materials and Methods

Materials: The original inbred line coded '360-3' was used for EMS-induced mutagenesis in this research, donated from Cucurbita Germplasm Resources Center at our Institute. This inbred line which shows obvious typical traits of *Cucurbit moschata* has been detected with strong fertility and growth vigor previously.

EMS mutagenesis: The preliminary experiment included two parts, which was carried out to determine the optimal EMS (Sigma, USA) concentration and treatment time. In the first part, seeds of the original inbred line were soaked in distilled water for 1h, and then EMS was added to make

a final concentration of 0% (Control), 0.5%, 1%, 1.5% and 2% for 24h at low-speed shaker. Each treatment contained 50 seeds and was repeated three times. The treated seeds were washed with 1M $Na_2S_2O_3$ and subsequently with distilled water and were incubated in growth chamber at 28°C. Germination rates were calculated and were thereafter sown in growing mix (peat: perlite = 1: 1). Young seedlings were grown in climate-controlled chambers under long day conditions (16h light/8h dark cycle) at 25°C. In the second part, the 1% EMS was employed to soak '360-3' seeds for 0h, 8h, 16h, 32h and 48h. Each treatment (50 seeds) was repeated three times. Other experimental conditions were the same to the first part.

In large-scale mutagenesis, about 1000 seeds were mutagenized with 1.5% EMS for 24h. Other procedures are the same as the preliminary experiment. Survived plants (14-day-old) were transplanted to the field (Gardening Practices Base attached to School of Horticulture Landscape Architecture, Xinxiang, Henan) after 2 days' acclimatization. For each plant, the main vine was retained and all lateral vines were removed. Manual self-pollinations (early in the morning) were attempted on each plant. Female flowers were protected before anthesis to avoid the transfer of pollen by insects. About 60 days post self-pollination, M1 fruits were harvested and M2 seeds were extracted 30 days later.

Genetic screen: After germination, seeds of M2 and '360-3' were sown in growing mix (peat: perlite = 1: 1), one seed for each pot. Seedlings were grown in climate-controlled chambers under long day conditions. Plants with three true leaves were applied with 200mM NaCl for 5 days. Lethal data (Plants showing totally dried-up stem are deemed to die) were recorded to estimate segregation ratio of mutant phenotype. Survival rates lower than '360-3' at M2 generation were discarded while the survived lines were transplanted to the field after washing off salt. Field management and self-pollination were practiced as stated above. The M3 seeds were harvested and sown in next growing season. Similarly, M3 and M4 seedlings were applied with NaCl and survival rates were recorded.

14-day-old plants of '360-3' and two target mutants were applied with 100mM NaCl for 5 days to further identify NaCl-tolerant phenotype and fresh weight, total chlorophyll and proline were measured. Chlorophyll of 0.1g mature leaf tissues was extracted with 80% acetone solution and then determined through spectrophotometer method (UV-1100 spectrophotometer, MEIPUDA, Shanghai, China). The equation for calculating the total chlorophyll was used according to Sumitahnun (Chunthaburee *et al.*, 2016). Proline content was estimated by the modified procedure of Bates (Bates, 1973). About 0.5g of leaf tissues was ground using 5 mL of 3% sulfosalicylic acid and then boiled at 100°C for 10min. Centrifuged for 10min at 3000r/min after cooling at room temperature. The resulting solutions were extracted with toluene, and the absorbance of the toluene phase was measured at 520nm. Proline concentration was estimated with reference to the standard curve. Seeds of '360-3' and two target mutants were soaked with 100mM NaCl solution for 6h, and then transferred to petri dish covered with filter paper which was kept wet with 100mM NaCl all the time. Petri dish was placed in darkness at 25°C and germination rates were recorded 72h later. All the experiments were repeated at least three times.

Data analysis and figure construction: The statistical analyses (Student's *t* test) were conducted using Microsoft Excel 2013. All the figures were constructed by Microsoft Excel 2013 and Illustrator CS5.

Results

Production of mutant population: Seeds of '360-3' were mutagenized by EMS to produce M1 seeds (Supplementary Fig. S1). The M2 seeds were harvested separately from M1 plants, discarding fruitless or seedless plants. The M2 family was screened under high concentration of NaCl to discard non-resistant plants. The homozygous NaCl-tolerant plants showing no segregation at M3 generation were considered to be target mutants. The heterozygous plants were screened further at M4 generation until NaCl-tolerant phenotype was stable genetically (Supplementary Fig. S1).

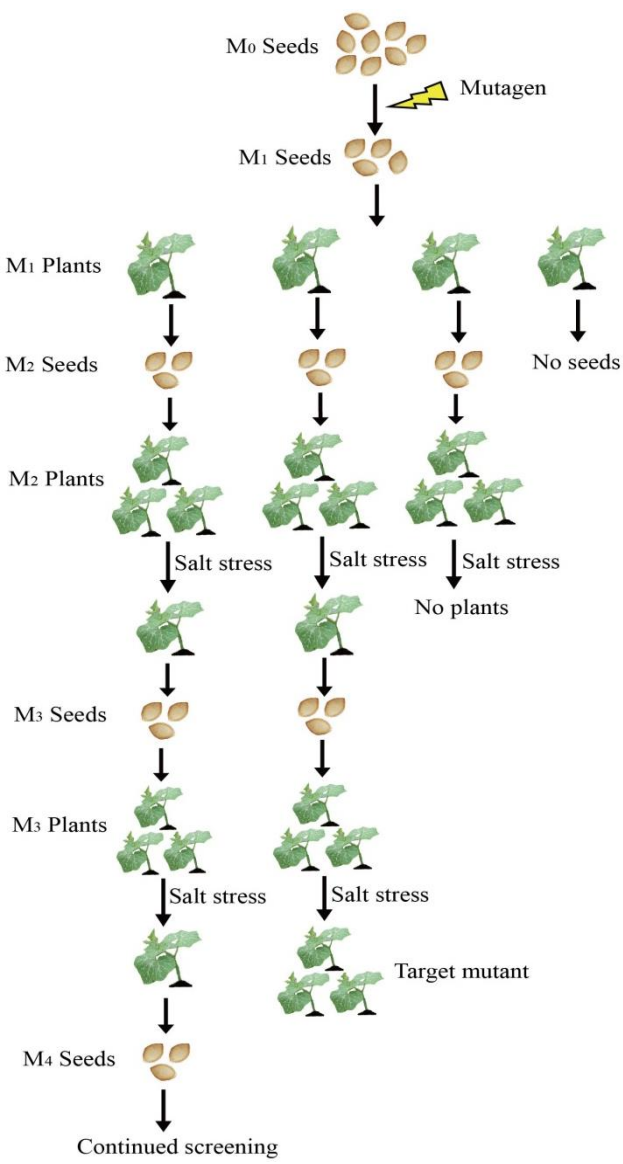

Fig. S1. Schematic diagram of NaCI-tolerant mutant screen.

To choose the optimal EMS concentration and mutagenesis time that are likely to produce appropriate mutations but will not heavily affect germination and fertility, we performed two preliminary experiments. Seeds of '360-3' were exposed to 0%-2% EMS for 24h and the germination rates were calculated. The 1% EMS was employed to soak '360-3' seeds for 0h-48h and germination rates were calculated subsequently. Fig. 1 displays the results of the experiment, in which the germination rates decreased progressively as the dose and treatment time increased. The median lethal dose is usually thought to be an important reference using EMS mutagenesis. Our results exhibited that 2% (24h) and 1% (48h) can lead to median lethality (Fig. 1).

The germination rates ranging from 70% to 85% are believed to be moderately affected by the mutagen (Vicente-Dolera *et al.*, 2014a). Therefore, we finally chose 1.5% EMS (24h) as the treatment condition for large-scale mutagenesis considering the optimum germination time (48h, 28°C) for pumpkin seeds. About 1000 seeds that were mutagenized with 1.5% EMS were sown. With the addition of the mutagenized seeds in preliminary experiments, about 600 seedlings were obtained and transplanted to the field. About 50% of the M1 plants could survive under field condition and only 18% of M1 plants could bloom. About 20 M1 plants produced seeds. Seeds were harvested from M1 plants (Manual self-pollination) separately as M2 families. The mutation populations were established employing the above-mentioned strategy.

Phenotypic evaluation of the M1 population: Generally speaking, EMS dose harms to plant growth and affects growth vigor negatively. Here, we assessed the morphological variation resulted from EMS mutagenesis. Compared with the control (Fig. 2A), mutagenized seedlings exhibited slow and weak growth (Fig. 2B). Some germinated seeds cannot develop into seedlings since each pot was sown with one seed in growing mix (Fig. 2B). EMS can induce sorts of mutation randomly, including vegetative growth variation, which might have an impact on yield or other important agronomic traits. In this research, some mutations resulted in vegetable growth and modifications in the normal architecture of the plants (Fig. 2).

To produce mutant populations, all survived plants were transplanted to the field since mature plants require large space for growing and setting fruits. We assessed morphological variation of mutant plants at the mature plant stage because yield condition differed in temperature, humidity, light intensity, etc., compared with growth chamber. Control plants displayed stronger growth vigor and healthier morphology in leaf color and shape (Fig. 3A). Mutations resulted in light colored leaf which were observed in few mutagenized plants (Fig. 3B). We speculated these mutations affected chlorophyll formation. According to the boundary between dark and light green on some leaves, we supposed those plants were chimera resulted from mutations (Fig. 3C, D). Some dwarf plants were too weak to survive under field condition. Other mutagenized plants showed no obvious variation at vegetative growth as compared with the control (Data not shown). Mutations that altered reproductive development, mostly affected morphologic floral organs in both male and female flowers, finally decreasing fertility (Data not shown). Some mutations delayed flowering time while in some cases, no floral organs were observed from few plants which were finally discarded.

To obtain M2 seeds, we conducted self-pollination for each M1 plants. About 80 M1 plants were able to set fruit which were labelled numerically. Among these, only ~25% could grow and develop to mature fruits. Compared with the control group, mutations relatively affected fruit shape, color and decreased fruit size (Fig. 4). Some fruits lost epidermis powder and some exhibited delayed ripening time (Fig. 4C). The M2 seeds were obtained through cutting post-mature fruits after harvesting from No.2 to No.78 (Fig. 4C) and were designated as M2 families.

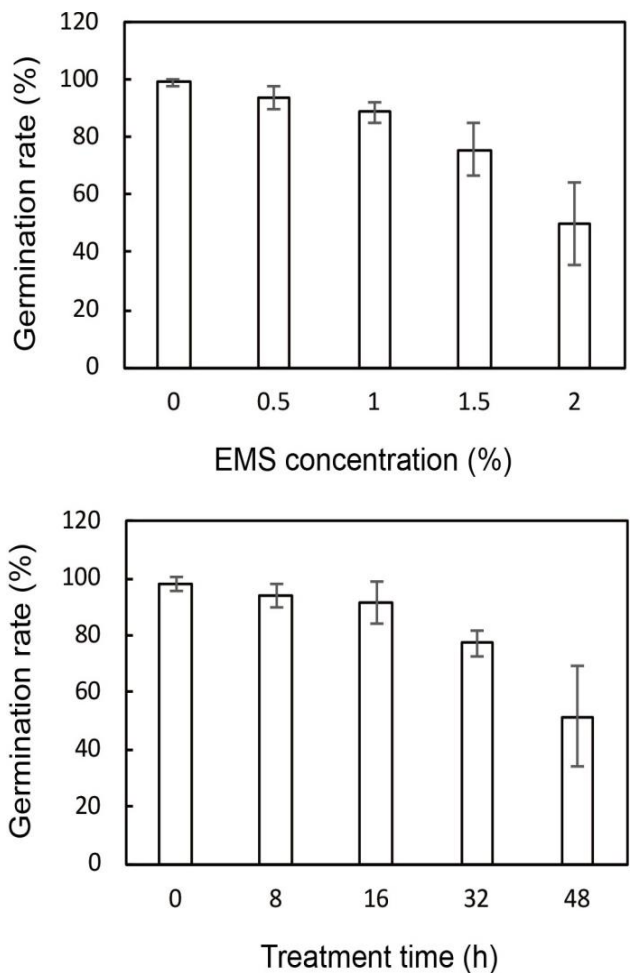

Fig. 1. Germination rates under different EMS dose and treatment time.

Screen and identification of NaCl-tolerant mutants: NaCl-tolerant mutant phenotypes were screened from M2 families. We first investigated the germination rates since EMS might affect seed weight, quality and germination. Fruits containing less than 10 seeds were discarded from the family group, such as No.11, No.31 and No.60 (Table 1). Few families displayed delayed germination or winkled seed coat which might decrease germination potential (Table 1). All germinated seeds were sown in growing mix and one seedling was transplanted to each pot. Subsequently, seedlings with three true leaves were treated with high concentration NaCl to screen target mutant. Each seedling was applied with 200mM NaCl and most plants died

eventually. Survival rates were recorded from each M2 family (Table 1). Survival rates less than 3.9% ('360-3' as control) is designated to be NaCl-sensitive. Two families (No.35 and No.62) exhibited the potential to possess NaCl-tolerant mutation (Table 1). We tested segregation ratio of the two families, none of them followed Mendel inheritance, so we deduced the NaCl-tolerant mutant phenotype was controlled by QTLs (Quantitative Trait Loci).

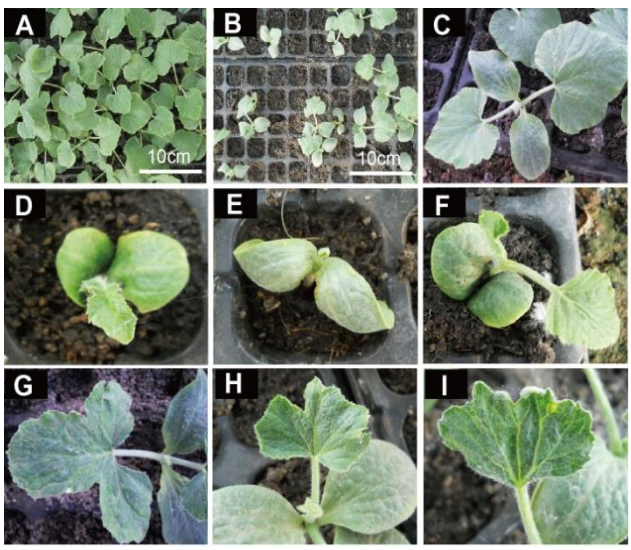

Fig. 2. Morphological changes of M 1 mutagenic seedlings
Morphological variation of M 1 mutant seedling. (A) 10-day-old wild-type plants after sowing, (B) 10-day-old mutagenized plants after sowing, (C) Wild-type plant, (D) Dwarfism, (E) Curly cotyledon, (F) extended petiole, (G-I) Variation of leaf shape.

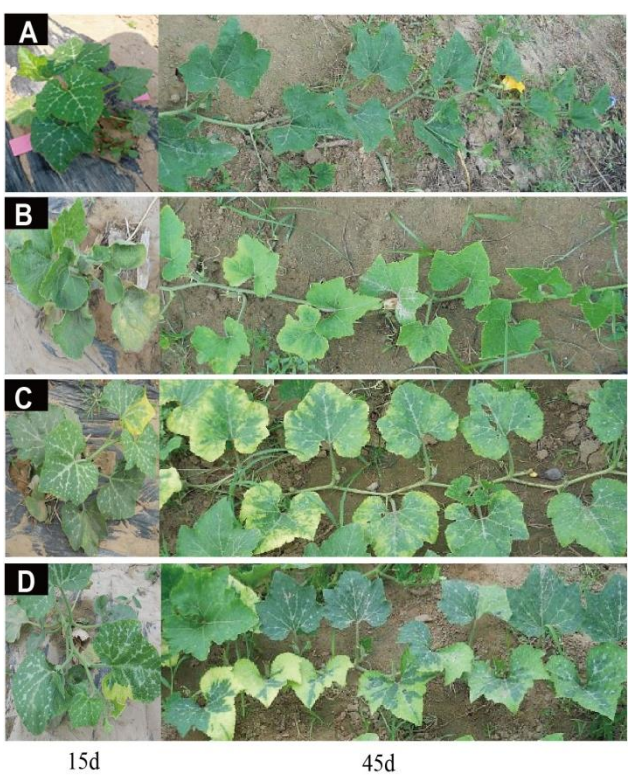

Fig. 3. Morphological changes of M 1 mutagenic plants in the field.
The picture was taken at 15d and 45d after transplant. (A) '360-3' wild-type plant, (B) M1 plant with light color leaf, (C and D) chimera plant.

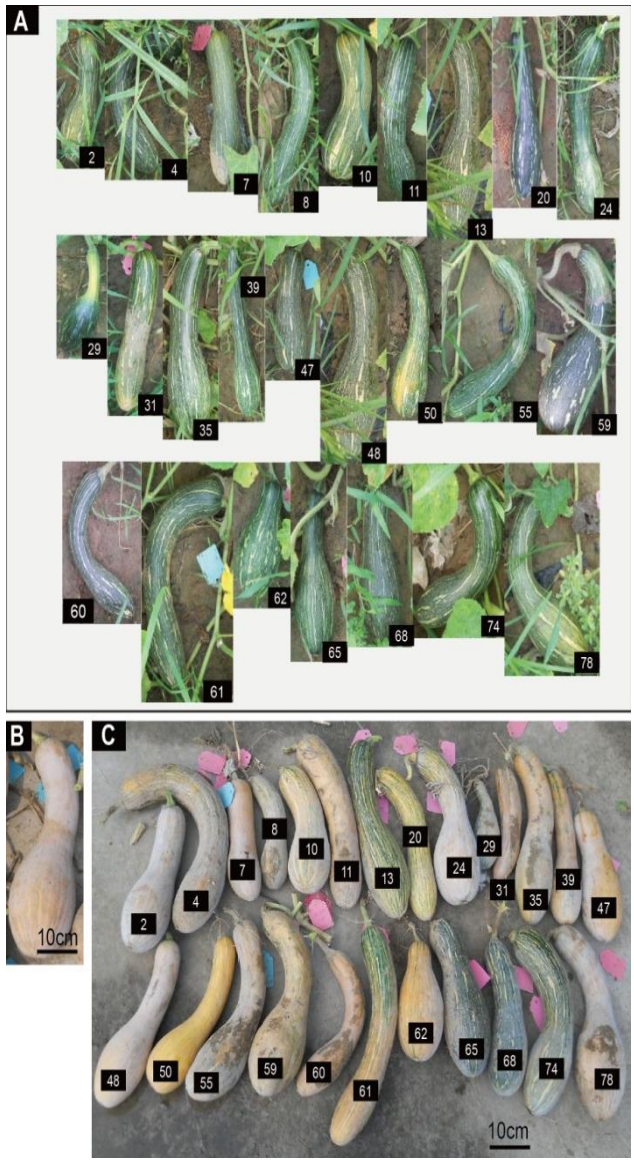

Fig. 4. Morphological changes of M1 fruit.
(A)Fruits of 14 days after self-pollination, 80 M1 fruits were labeled numerically and non-development fruits were excluded from the picture. (B) Mature fruit of '360-3' (C) Mature M1 fruits after harvest.

To produce M3 lines, seeds were harvested separately from individual M2 plants of family 35 and 62. In the M3 lines, offspring of family 35 and 62, were applied with the same salt stress and survival rates were calculated. Based on the data, 35-3 and 62-1 displayed nearly 100% survival rates so we speculated their NaCl-tolerant phenotype to be genetically stable (Table 2). Seeds of the two M3 lines were harvested to produce M4 generation and the same screen was carried out. There was no segregation of NaCl-tolerant phenotype at M4 generation, so 35-3 and 62-1 were considered to be homozygous mutant. The two mutants were then named as ST35-3 and ST62-1 (Salt Tolerance), respectively. For genetic analysis, the cross between wild type '360-3' and homozygous mutants were carried out. All of the F1 seedlings were not tolerant to NaCl, so the two mutants were genetically recessive.

To further identify the NaCl-tolerant phenotype, morphological, physiological and biochemical parameters

related to salt stress were investigated. Under salt stress, original inbred line '360-3' showed yellow leaf color in comparison with mutants, which showed consistency in total chlorophyll content (Fig. 5). Plant growth evaluated from fresh weight was also inhibited in '360-3' compared with mutants. Salt stress generally results in proline accumulation in plants, however, the proline accumulation observed from ST35-3 and ST62-1 were lower than '360-3' (Fig. 5) after salt stress treatment. We also tested the germination rates since salt stress has an inhibiting effect on seed germination. The results indicated that the germination of '360-3' was significantly inhibited by NaCl compared with the mutants (Data not shown). In conclusion, our data showed that the two mutants were NaCl-tolerant at seed germination and seedling stages.

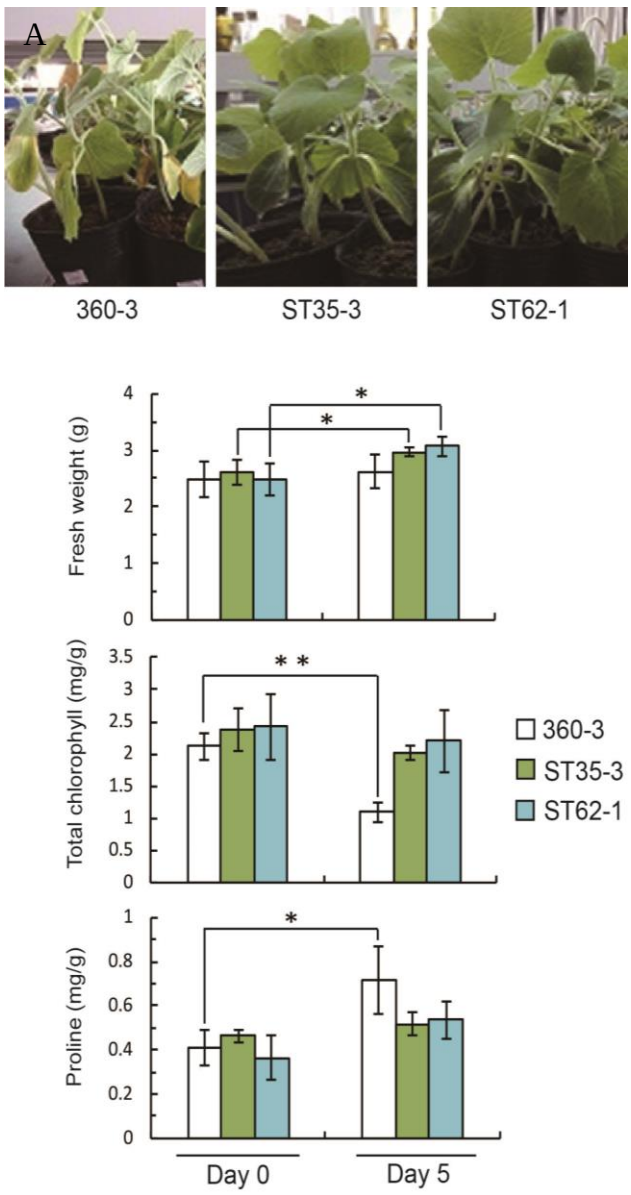

Fig. 5 Response to salt stress of the NaCI-tolerant mutants.
(A) Morphology of '360-3', ST35-3 and ST35-3 and ST-62-1 after salt stress treatment. The picture was taken from plants when they were 20 days old. (B) Different responses to salt stress in '360-3' ST-35-3 and ST-62-1. Asterisks indicate significant differences analysis based on Student's test, $p<0.05$(*), $p<0.01$(**).

Discussion

Chemical mutagenesis which requires a simple technical condition and possesses relatively high mutation frequency, has been applied to increase genetic variability in crop plants. Abundant variation materials could be created by chemical mutagenesis and moreover, some mutants have played an important role in crop breeding. One of the most frequently used chemicals is EMS, causing point mutation (G-C switch to A-T). Since EMS has a negative effect on germination, growth and development (AB Talebi, 2012), the optimization of EMS treatment dose and time should be determined in advance. A strict correlation was observed between the EMS dose and treatment time with the toxicity. Our results were consistent with previous study (Roychowdhury *et al.,* 2011) which demonstrated that with increasing EMS levels, germination and survival rates decreased. Plants may differ widely in EMS tolerance, for example, high mutation rates were achieved by 0.25-0.5% EMS in *Arabidopsis thaliana* (Greene *et al.,* 2003), tomato TILLING population was constructed through 0.7% and 1% EMS (Minoia *et al.,* 2010), whereas to produce TILLING population in cucumber, 1.5-2% EMS was applied (Fraenkel *et al.,* 2014). Among the plants mentioned above, the bigger the seed size, the higher EMS concentration is required to achieve certain mutation rates. In this study, the most suitable treatment was 1.5% EMS (24h) in *Cucurbita moschata* D. based on the germination rates. The M1 plants showed delayed and weak growth compared with the control. Less number of M2 families were obtained because many weak plants could not survive in the field and only few plants were able to set seeds. So the mutations induced by EMS, in most cases, are harmful on plants in the aspect of farming.

EMS can induce various mutant phenotypes. In our study, vegetative growth and modifications in the normal architecture of the plants such as, dwarfism, curly cotyledon, extended petiole and variation of leaf shape were observed in M1 seedlings due to mutations. Some M1 plants exhibited chimera resulting from recessive gene mutation. Some of the M1 plants displayed light leaf color, altered floral organs and delayed flowering time at the mature plant stage. Similar mutant phenotypes induced by EMS were reported previously in *Cucumis sativus* and *Cucurbita pepo* (Fraenkel *et al.,* 2014; Vicente-Dolera *et al.,* 2014b). Change of fruit color and shape were also observed in M1 plants which are in agreement with fruit variation induced by EMS reported in tomato (Minoia *et al.,* 2010). Since EMS is able to induce fruit morphological variation, we believe EMS mutagenesis may have a potential value in fruit quality.

EMS can induce point mutation randomly which may generate target phenotype. Based on this principle, we carried out a genetic screen of NaCl-tolerant mutant under high concentration of NaCl solution. Previously, salt-sensitive and salt-tolerant mutants in *Arabidopsis thaliana* have been screened through EMS mutagenesis and salt-tolerant related genes identified (Quesada *et al.,* 2000; Wu *et al.,* 1996; Zhu *et al.,* 1998). The Root-bending assay is the screening condition for salt sensitive mutants (Wu *et al.,* 1996; Zhu *et al.,* 1998). However, the method is hard to apply to pumpkin because of its long and thick root. To screen salt-tolerant mutants, seeds were sown on agar

medium supplemented with 250mM NaCl and germination assays were carried out (Quesada *et al.,* 2000). However, it is inconvenient to follow the method since the seeds of pumpkin are much bigger. Usually, salt injury index is used to identify salt-tolerance in crop plants. However, this method is based on morphology (Mujtaba *et al.,* 2018) and is too subjective. Not clear, we employed survival rates at the seedling stages to quantify the salt-tolerant phenotype. Our trial suggested this screening method was convenient and effective to large horticultural crops.

When it comes to the genetic basis of salt-toleranance, many researchers believe the traits, as well as the resistance to other abiotic stresses, are quantitative loci (Du *et al.,* 2016; Hartman *et al.,* 2014; Thoen *et al.,* 2016; Tiwari *et al.,* 2016; Zhang *et al.,* 2016). Based on our data in *Cucurbita moschata*, the survival rates under salt stress did not follow the segregation of 3:1 or 15:1 ratio, suggesting the NaCl-tolerant mutant phenotype is caused by QTLs. However, salt-tolerant mutants caused by single nuclear mutations in *Arabidopsis* were reported previously (Quesada *et al.,* 2000), revealing the complexity of genetic basis of salt resistance in plants.

Many studies have been conducted to understand plant stress response to salinity, suggesting osmolytes, osmoprotectants, radical detoxification, ion transport systems, and changes in hormone levels and hormone-guided communications are all relevant to salt stress (Park *et al.,* 2016). Our mutants showed a relatively high level of fresh weight and total chlorophyll content (compared with '360-3') after NaCl treatment, suggesting that the mutants were less effected by NaCl stress. As a common response to salt stress, proline accumulation contributes in removing ROS, stabilizing subcellular structures and modulating cell redox homeostasis (Sharma *et al.,* 2011). In tomato, the proline accumulation increases greatly within the tolerant genotype, mainly in leaves and when compared to the most sensitive genotype, suggesting proline accumulation varies between tolerant and sensitive genotypes (Gharsallah *et al.,* 2016). However, early in 1999, Lutts *et al.*, demonstrated that over-accumulation of proline was related to a symptom of salt injury rather than an indicator of salt tolerance in rice (Lutts *et al.,* 1999). Our findings from the result of a slight increment in proline accumulation of two NaCl-tolerant mutants (compared with '360-3') revealed that increased level of proline accumulation were not consistent with NaCl resistance. The complexity of salt tolerance in plants (Park *et al.,* 2016) and limited experimental data in our research, the mechanism of NaCl tolerance in our mutants needs further investigation.

Salt-tolerant crops have played an important role on using saline and alkaline land for agriculture production (Takagi *et al.,* 2015). On one hand, our research provides a reference of mutant screen based on EMS mutagenesis, while on the other hand, our mutants become new sources for salt-tolerant breeding in pumpkin. As significant research material, the mutants could be used for further research work in order to better understand salt-tolerant mechanism in plants.

Table 1. NaCl-tolerant mutant phenotype screen at M2 generation.

M2 family or Control	M2 Seed amount [a]	TKW [b] (g)	Seed quality	M2 seedlings for screen	Survived seedlings	Survival rates (%)
360-3	134	149	Good	129	5	3.9
2	93	107	Normal	89	2	2.2
4	91	87	Good	90	1	1.1
7	15	66	Normal	12	0	0
8	24	89	Normal	20	0	0
10	76	94	Delayed germination	71	0	0
11	7	98	Normal	NA [c]	NA	NA
13	87	56	Normal	85	1	1.2
20	18	101	Normal	16	0	0
24	79	110	Good	77	0	0
29	28	46	Wrinkled seed coat	21	0	0
31	0	NA	NA	NA	NA	NA
35	**103**	**96**	**Good**	**98**	**8**	**8.2**
39	41	56	Normal	38	0	0
47	120	97	Delayed germination	114	2	1.8
48	104	114	Delayed germination	92	2	2.2
50	57	96	Normal	55	0	0
55	119	99	Good	113	4	3.5
59	120	130	Delayed germination	109	0	0
60	0	NA	NA	NA	NA	NA
61	21	125	Wrinkled seed coat	18	0	0
62	**94**	**136**	**Good**	**88**	**7**	**7.9**
65	87	78	Normal	79	2	0
68	64	96	Normal	60	2	3.3
74	72	98	Normal	68	0	0
78	115	142	Good	107	3	2.8

[a] Seed amount was counted per fruit
[b] TKW: Thousand kernel weight
[c] NA: Not available

Table 2 NaCl-tolerant mutant phenotype screen at M3 generation.

M2 family or control	M3 lines	Seedlings for screen	Survived seedlings	Survival rates (%)
360-3	NA	108	4	3.7
35	1	124	60	48.4
	2	98	26	26.5
	3	**151**	**142**	**94.0**
	4	135	19	14.1
62	**1**	**97**	**95**	**97.9**
	2	85	22	25.9

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