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Research Paper

An EMS mutant library for carrot and genetic analysis of some mutants

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Carrot is among the top-ten most economically important vegetable crops in the world. However, the gene pool of cultivated carrot is narrow and the collection and preservation of carrot germplasm are very limited in China. In this study, seeds of carrot inbred line "17005" were treated by ethyl methyl sulfone (EMS) to construct a mutant library. The conditions of EMS mutagenesis on inbred line "17005" were optimized, including treatment of seeds at 0.5% EMS for 6 h. We obtained a number of mutant lines showing inheritable morphological mutations in cotyledon, growth point, leaf, taproot in the vegetative phase and mutations in bolting time, primary stem height and color, secondary branch, flower and seed in the reproductive phase through M_2 and M_3. The F_2 segregating population from the cross of yellow taproot and purple-red epidermis taproot with wild type showed that these two mutants were controlled by single recessive gene respectively. The inheritable mutants can not only enrich the carrot germplasm, but also provide resources for the function research of carrot gene.

Key Words: carrot, EMS, mutagenesis, mutant.

Introduction

Carrot (*Daucus Carota* L.) is the most widely grown member of the Umbelliferae and is listed as one of the healthy functional foods. Area harvested of carrot in the world has reached 1,147,155 ha, of which area in China is 405,620 ha, accounting for 35.4% of the world (FAO 2017). However, the gene pool of carrot cultivars in China is very narrow (Zhuang 2005), and only 389 varieties has been collected and preserved, which accounts for less than 1/15 of carrot germplasm in the world (Zhu *et al.* 2008). Meanwhile, this situation has seriously restricted the breeding of excellent carrot varieties, and has also led to the lack of resource available for genetic research and gene functional study of important traits for carrot.

Mutant generating is an important way for germplasm innovation, and also an important content for genetics and functional genomics research (Yan *et al.* 2004). A large-scale mutant library is very effective for high-throughput research of gene function in plants. The mutant library can be generated by spontaneous mutation, physical and chemical mutation, and insertion mutation. Among them, EMS is widely used in chemical mutagenesis because of its simple operation, high mutagenic efficiency, point mutation and stable heredity (Greene *et al.* 2003). A saturated mutant library has been constructed for Arabidopsis (McCallum *et al.* 2000) and rice (Abe *et al.* 2012). Mutant libraries have been also constructed for various vegetables such as tomato (Matsukura *et al.* 2007), cucumber (Chen *et al.* 2018), pepper (Yang *et al.* 2016), Chinese cabbage (Lu *et al.* 2014), watermelon (Hou *et al.* 2016) and melon (Galpaz *et al.* 2013). Some traits such as super compact (Wang *et al.* 2017), glabrousness (Xu *et al.* 2015) and curly leaf (Rong *et al.* 2019) of cucumber and mature fruit color (Huh *et al.* 2001) of pepper have been mapped. However, mutant library construction and gene mining of related important traits for carrot are still very limited. Moreover, a high-quality carrot genome (Iorizzo *et al.* 2016) confers additional perspective and value of mutant library of carrot for functional genomic analyses. Inbred line "17005" is one of the typical types of carrot cultivated in Shanxi Province, taproot of which shows uniform orange color, smooth skin and early bolting resistance in spring. In this study, we carried out EMS mutagenesis in carrot "17005" to construct a mutant library and obtained mutants related to cotyledon, leaf, growth point and taproot in the vegetative phase and mutants related to bolting time, primary stem height, seed stalk color, secondary branch, flower and seed in the reproductive phase. In addition, we also proved that yellow taproot and purple-red epidermis taproot mutations were respectively controlled by single recessive gene.

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Materials and Methods

Plant materials

The wild type carrot used for EMS mutagenesis was

inbred line "17005" self-pollinated from an outcrossed cultivar "Nongjiahong" for four generations that showed orange and cone-shape roots in the vegetative phase, and a green stalk, 4–6 secondary branches and semi-bell umbel in the reproductive phase. Seeds of inbred line "17005" were provided by the horticultural college of Shanxi Agricultural university, China.

Optimization of EMS treatments for mutagenesis

Seeds of inbred line "17005" were treated with four EMS concentrations (0%, 0.5%, 1%, and 1.5%) for 6 h, 8 h, 10 h and 12 h, respectively. Each EMS concentration was treated by three replications with 100 seeds per replication. The treated seeds were placed in an incubator at 25°C for germination. Seed germination rate was recorded for 15 days. The germinated seeds were sowed in nursery trays with the ratio 3:1 of peat and vermiculite for seedlings. The emergence rate was recorded for 15 days as well. The median lethal dose (LD_{50}) was used to screen the optimized condition for mutagenesis.

Development of EMS mutagenesis population

In July 2016, 8000 carrot seeds of inbred line "17005" were treated with 0.5% EMS for 6 h. The germinated seeds were sowed in nursery trays and the seedlings were transplanted to the Horticultural Station of Shanxi Agricultural University three weeks later. The M_1 plants were self-pollinated and 1088 M_2 families (10–1000 seeds/family) were obtained. In July 2017, we planted 60 seeds for each M_2 family and obtained about 15,140 M_2 taproots that were vernalized in a cooler at 0–3°C for 3 months. A total of taproots of 980 M_2 families were planted in the greenhouse of the Horticultural Station of Shanxi Agricultural University in February 2018, 3 taproots for each family. Mutant plants were self-pollinated to obtain M_3. Traits observation in the vegetative and reproductive phase of M_2 and M_3 plants were based on the description and data standard of carrot germplasm (Zhuang and Zhu 2007). Segregation of wild and mutant plants was counted in each M_2 family to infer the inheritance mode of the mutation. A schematic process describing the development of mutant library was showed in **Fig. 1**.

Genetic analysis of yellow and purple-red epidermis taproot mutant

In spring 2018, we crossed yellow taproot and purple-red epidermis taproot mutants with wild type "17005" respectively, to produce F_1. In summer of 2019, F_2 was obtained by self-pollination of F_1 seed stalks. In that autumn, two F_2 populations were planted in the field of the Horticultural Station of Shanxi Agricultural University to investigate the inheritance modes of mutants. In carrot, the *Y* and Y_2 loci explain most of the phenotypic variation among white, yellow, and orange taproots. Total RNA was extracted from the leaves of the yellow taproot mutant and wild type using Trizol. cDNA was then synthesized using a PrimeScript™ RT Reagent Kit (TaKaRa) following the manufacturer's instructions. The coding sequences of the candidate gene DCAR_032551 (Iorizzo *et al.* 2016) for *Y* locus from the yellow taproot mutant and wild type were sequenced. Full-length cDNA sequences were analyzed using DNAMAN software. Two CAPs markers, 4135^{ApoI} and 4144^{ApeKI}, linked to Y_2 were also used as described by Ellison *et al.* (2017). Digestion products were separated on 7% non-denaturing polyacrylamide gels.

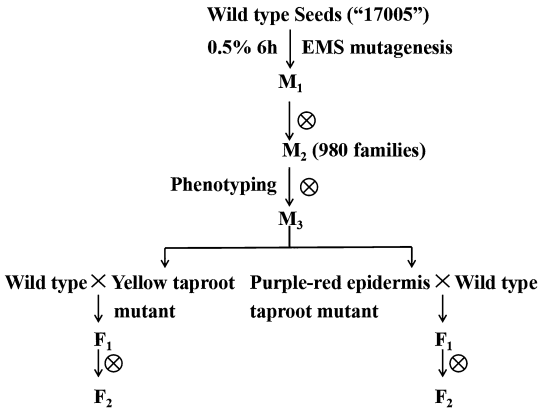

Fig. 1. Schematic process of mutant library construction for carrot.

Results

Identification of optimal EMS treatments for mutagenesis in inbred line "17005"

We tested sixteen EMS combinations and examined their effects on the germination of carrot seeds and the emergence of germinated seeds. The lethal rate of germination and emergence increased gradually with the increase of EMS concentration and treat time. The LD_{50} treatment for seeds mutagenesis in inbred line "17005" was determined as 0.5% EMS for 6 h (**Table 1**). Therefore, this treatment was used for large-scale EMS treatment of inbred line "17005" seeds to construct the mutant library.

Phenotypic variations among M_2 Mutagenesis population

Among 15,140 plants from 1088 M_2 families, 11,024 exhibited significant mutant phenotypes in the vegetative phase with a phenotypic mutation rate around 72.81%, which included mutations in cotyledon, growth point, leaf and root. In the reproductive phase, 768 plants showed significant mutant phenotypes with a phenotypic mutation rate around 5.07%, which included mutations in bolting time, seed stalk color, primary stem height, secondary branch, flower and seed (**Table 2**). Plants carrying multiple mutations were also observed.

Cotyledon mutants observed firstly in the vegetative phase of M_2 mutation population accounted for about 20.31% of all morphological changes and showed changes of cotyledon number and notch. The types of cotyledon number mutations included monocotyledon, tricotyledon

Table 1. Effects of concentration of EMS and treatment time on germination lethal rate and emergence lethal rate of carrot

EMS/%	Treat time (h)	Germination lethal rate/%	Emergence lethal rate/%
0.0	6	0.0	8.3
	8	0.3	9.0
	10	0.0	10.7
	12	0.0	9.0
0.5	6	54.0	52.2
	8	58.7	57.3
	10	58.3	60.8
	12	54.3	67.9
1.0	6	57.7	66.9
	8	90.0	90.0
	10	99.7	100.0
	12	100.0	100.0
1.5	6	86.3	95.1
	8	99.3	100.0
	10	99.7	100.0
	12	100.0	100.0

and tetracotyledon mutants. Some of monocotyledon and tricotyledon mutants were also associated with shallow notches or deep notches (**Fig. 2**). Changes in growth point numbers varied from one to four. Each growth point showed different vigor when growth point numbers were more than two (**Fig. 3**). Among all morphological mutations observed in the vegetative phase, leaf mutants had the highest proportion which was 50.45%. The leaf mutations included changes in leaf angel, shape and color. Leaf angel mutants had flat and erect leaves (**Fig. 4B**, **4C**). Changes in leaf shape included thin and broad leaves (**Fig. 4E**, **4F**).

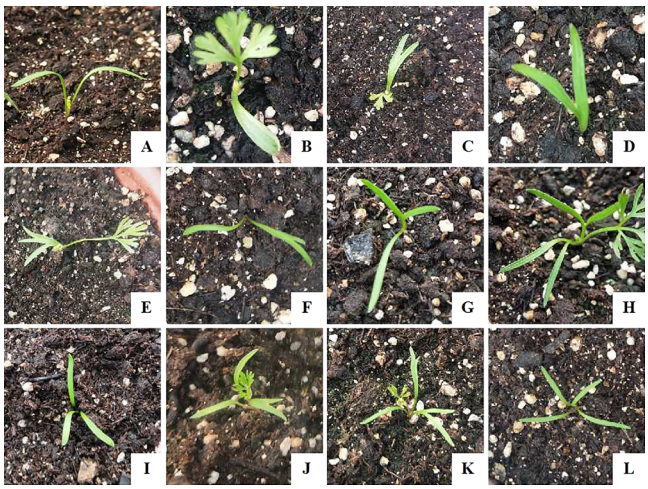

Fig. 2. Phenotypes of cotyledon mutants of M_2 population. A: Wild cotyledon; B: Monocotyledon; C: Monocotyledon with shallow notch; D: Monocotyledon with deep notch; E: Monocotyledon with two notches; F: Dicotyledon with shallow notch; G: Dicotyledon with single deep notch; H: Dicotyledon with two deep notches; I: Tricotyledon; J: Tricotyledon with shallow notch; K: Tricotyledon with deep notch; L: Tetracotyledon.

Fig. 3. Phenotypes of growth point mutants of M_2 population. A: Wild type; B: Double growing points; C: Three growing points; D: Four growing points.

Table 2. Statistics on mutation types of M_2 population

Traits	Phenotypes	No. of mutations	Percentage of mutation (%)
Vegetative phase			
Cotyledon	Number of cotyledon (single, three, four)	2395	20.31
Growth point	Number (double, three, four)	549	4.66
Leaf	Leaf angle (flat, upright)	2207	50.45
	Leaf shape (wide leaf, thin leaf)	218	
	Leaf color (complete mutation, chimeric mutation)	3524	
Taproot	Root shape (long cone, short cylinder, oval)	1033	18.07
	Root color (white, yellow, dark orange, light orange, red, purple-red epidermis, purple)	1098	
Reproductive phase			
Bolting time	Early or late bolting	202	1.71
Seed stalk color	Chlorotic	16	0.14
Primary stem color	Purple-green chimerism	97	0.82
Secondary branch	Number (none, less, multiple)	162	1.37
Primary stem height	Dwarfing	8	0.07
Flower	Umbel shape (flat, spherical, irregular)	162	1.51
	Structure (umbel like leaf)	8	
	Stylopodium color (orange)	8	
Seed	Seed length (small, medium)	105	0.89

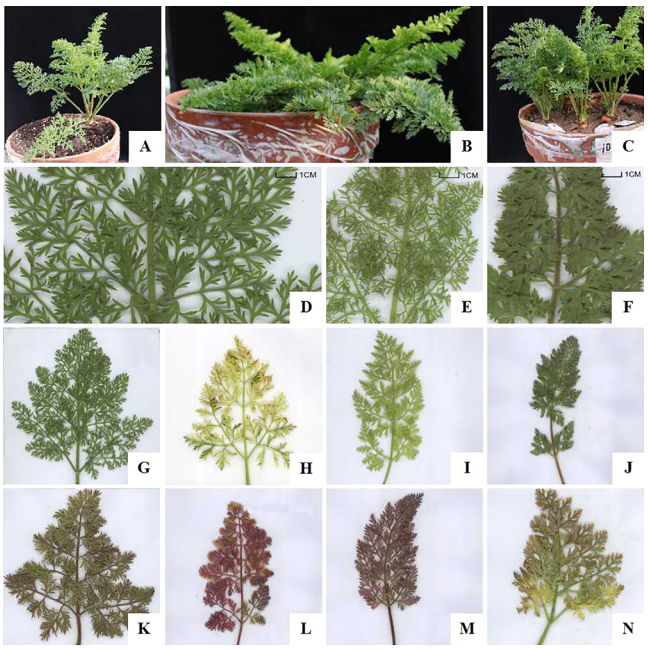

Fig. 4. Phenotypes of representative leaf mutants in the vegetative phase in M_2 generation. A: Wild type (semi-erect); B: Flat leaf mutant; C: Upright leaf mutant; D: Wild type; E: Narrow leaf mutant; F: Broad leaf mutant; G: Wild type leaf; H: Yellow leaf mutant; I: Light green leaf mutant; J: Deep green leaf mutant; K: Purple-green leaf mutant; L: Purple-red leaf mutant; M: Purple leaf mutant; N: Mosaic leaf mutant.

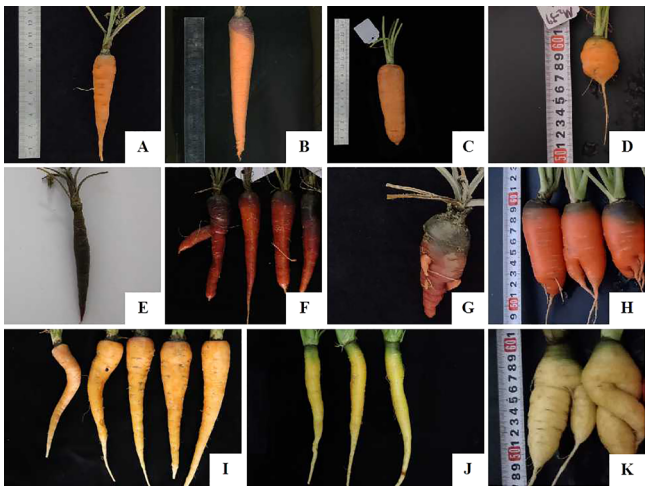

Fig. 5. Phenotypes of typical taproot mutants in M_2 generation. A: Wild type taproot; B: Long conical mutant; C: Short cylindrical mutant; D: Oval mutant; E: Purple taproot mutant; F: Purple-red taproot mutant; G: Red taproot mutant; H: Dark orange taproot mutant; I: Light orange taproot mutant; J: Yellow taproot mutant; K: White taproot mutant.

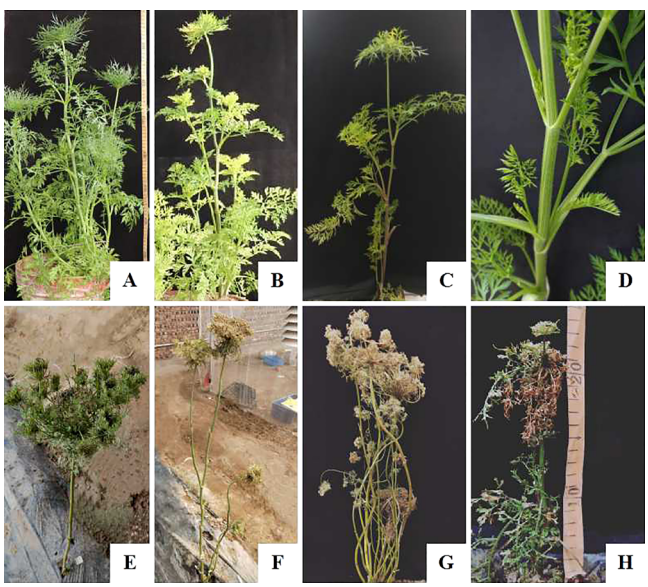

Fig. 6. Phenotypes of stalk mutants in M_2 population in the reproductive phase. A: Wild type stalk; B: Yellow-green mosaic stalk mutant; C: Purple-green primary stem mutant; D: Secondary branch like leaf mutant; E: No secondary branch mutant; F: Less secondary branch mutant; G: Multiple secondary branch mutant; H: Dwarfing mutant.

The leaf color mutants showed yellow, light green, dark green, purple-green, purple-red, purple and mosaic leaves. Some of them were associated with changes of leaf veins (**Fig. 4H–4N**). Taproot mutations included mutations in shape and color. Taproot shape mutants exhibited long cones, short cylinders and oval shapes. The taproot color mutants included purple, purple-red, red, dark orange, light orange, yellow and white taproots (**Fig. 5**).

Bolting time mutants included early bolting (bolting 15 days earlier than wild type) and late bolting (bolting 15 days later than wild type). Seed stalks of wild type line "17005" were green with 4–6 secondary branches and the heights of primary stems were 80 cm–120 cm. Some mutants manifested purple-green chimeric primary stems and etiolated stalks. In secondary branch mutants, branch numbers varied from none to a few, to multiple (more than 10 branches). Dwarfing mutants (lower than 30 cm) with no secondary branches were identified in M_2 family (**Fig. 6**). Most of the chloric, no or less branching and dwarf mutants exhibited poor fertility and obtained none or very few seeds. Floral mutations included umbel shapes and flower organs. The umbel shape mutation showed flat, spherical and irregular umbels. The umbels of some primary stalks turned into green-yellow leaves. We also identified several floral mutants with orange stylopodia that had low fertility and only obtained a dozen seeds (**Fig. 7**). Changes in seed size were mainly observed in seed mutation and some seeds were significantly smaller than seeds of wild type line "17005" (**Fig. 8**). However, the germination rates of these small seed mutants were not affected (data not showed).

Based on segregation among M_3 plants, we discovered that some mutations observed in M_2 were inheritable which contained most mutations related to cotyledon, growth point, leaf, taproot in the vegetative phase and with bolting

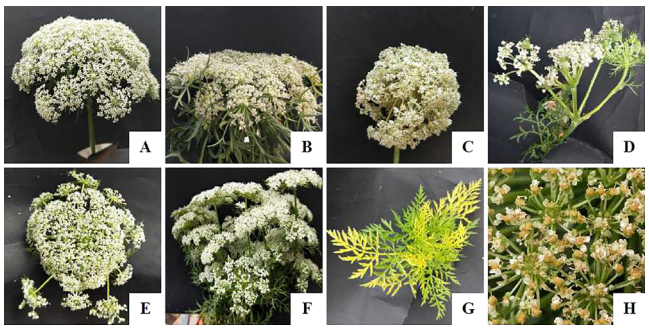

Fig. 7. Phenotypes of flower mutants in M_2 population. A: Wild type umbel (semi-bell shape); B: Flat umbel mutant; C: Spherical umbel mutant; D–F: Irregular umbel mutant; G: Umbel like leaf mutant; H: Orange stylopodium mutant.

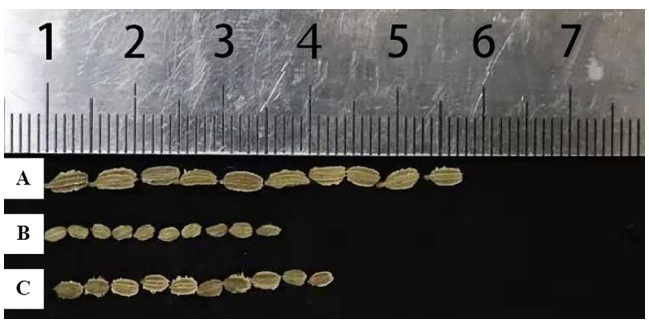

Fig. 8. Phenotypes of seed mutants in M_2 population. A: Wild type; B: Small seed; C: Medium seed.

time, branch number, flower shape and seed size in reproductive phase. However, a few mutations like mosaic leaf, yellow-green mosaic stalk, branches and umbels like leaf, irregular umbel and orange stylopodium were not inheritable.

Inheritance of yellow taproot and purple-red epidermis taproot mutations

The F_1 constructed by crossing yellow taproot mutant with wild type line "17005" showed an orange taproot that was the same as the wild type. Among 194 F_2 taproots, 58 and 136 were yellow taproots and orange taproots, respectively. Similarly, we crossed the purple-red epidermis taproot mutant with the wild type plant. The epidermis color of F_1 taproot was the same as that of wild type. In F_2, 38 purple-red epidermis taproots and 142 orange taproots were observed. Chi square test showed that segregation were consistent with a single recessive gene underlying yellow

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Wild type            ATGACTGCTTCATCCTCTTCTGCCGCTTCATATATCCATA  40
Yellow taproot mutant ATGACTGCTTCATCCTCTTCTGCCGCTTCATATATCCATA  40

Wild type            TGGTGCAACACTTGATAGAGGAGTGCATATTATTTAACAT  80
Yellow taproot mutant TGGTGCAACACTTGATAGAGGAGTGCATATTATTTAACAT  80

Wild type            GAGCAAGGAAGAATGCATGGATGCGCTTGCCAAACATGCC 120
Yellow taproot mutant GAGCAAGGAAGAATGCATGGATGCGCTTGCCAAACATGCC 120

Wild type            AACATCTTACCTGTCGTTACTTCCACAGTGTGGAAGGAGC 160
Yellow taproot mutant AACATCTTACCTGTCGTTACTTCCACAGTGTGGAAGGAGC 160

Wild type            TGGAGAAAGAGAATAAACAGTTCTTTGAGGCTTACTCCAT 200
Yellow taproot mutant TGGAGAAAGAGAATAAACAGTTCTTTGAGGCTTACTCCAT 200

Wild type            GAATACAAGAGAAATGAGAAGTTTGGTTAAGGAGAAATCA 240
Yellow taproot mutant GAATACAAGAGAAATGAGAAGTTTGGTTAAGGAGAAATCA 240

Wild type            GCGGTTACTGATTATGAGTTGGAAACTATGAAGCAAAGAA 280
Yellow taproot mutant GCGGTTACTGATTATGAGTTGGAAACTATGAAGCAAAGAA 280

Wild type            TCCACAAGATGCTAAGCGATCTTAGATCTTCTGCATCTGA 320
Yellow taproot mutant TCCACAAGATGCTAAGCGATCTTAGATCTTCTGCATCTGA 320

Wild type            TGGGTCAACTTGTTGA                         336
Yellow taproot mutant TGGGTCAACTTGTTGA                         336
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Fig. 9. Sequence alignment of CDS of DCAR_032551 between wild type and yellow taproot mutant.

and purple-red epidermis taproot mutations, respectively (**Table 3**). Sequence alignment of CDS of DCAR_032551 showed that there was no SNP mutation between wild type and yellow taproot mutant (**Fig. 9**). Two CAPs markers didn't show polymorphisms between wild type and yellow taproot mutant (**Fig. 10**).

Discussion

The optimal treatment for carrot EMS mutagenesis

Mutant library construction with EMS mutation has been successfully applied in many kinds of vegetables (Chen *et al.* 2018, Galpaz *et al.* 2013, Hou *et al.* 2016, Lu *et al.* 2014, Matsukura *et al.* 2007, Yang *et al.* 2016), which has provided important resources for gene mining and functional study of important traits of vegetables (Li 2014). Generally, the suitable treatments for EMS mutagenesis were screened according to LD_{50}. Some were based on LD_{50} of germination (Zhang 2012), and some were based on LD_{50} of emergence (Lu *et al.* 2015). However, the response of different crops to the combination of EMS concentration and treatment time were significantly different. It is necessary to ensure obtaining enough heritable progenies and seeds for further research when we try to increase

Table 3. Inheritance of yellow taproot and purple-red epidermis taproot mutation

Item	Yellow taproot mutants			Purple-red epidermis taproot mutants		
	Actual number	Theoretical number	χ^2 ($\chi^2_{0.05,1}$)	Actual number	Theoretical number	χ^2 ($\chi^2_{0.05,1}$)
Wild type	136	145.5		142	135	
Mutant	58	48.5		38	45	
Total	194	194	2.23 (3.84)	180	180	1.24 (3.84)

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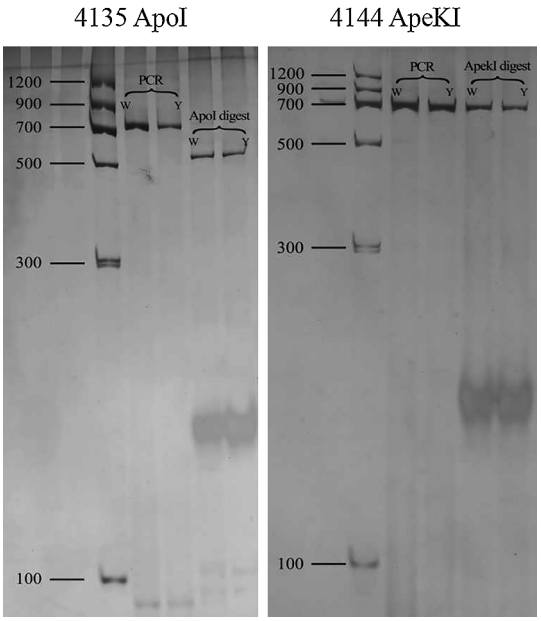

Fig. 10. Gel electrophoresis image of the two CAPs markers linked to Y_2. W indicates wild type, and *Y* indicates yellow taproot mutant.

mutation frequency by increasing mutation concentration. Therefore, it is very important to determine the optimal treatment of EMS mutagenesis for certain crops to construct a mutant library efficiently. In our study, the lethal rate of germination and emergence were both close to LD_{50} after carrot seeds were treated with 0.5% EMS for 6 h. Although lethal rate of germination for carrot seeds treated with 1.0% EMS for 6 h was not significantly different from that which were treated with 0.5% EMS for 6 h, the lethal rate of emergence increased to 67.2%. So both LD_{50} for germination and emergence could be used as the criterion for identification of the optimal EMS combination for carrot mutagenesis.

Seeding methods and perspective of carrot mutants

Carrot is suitable for the direct seeding in production and transplanting would lead to the occurrence of fork root and loss of commercial value. However, the emergence rate of M_1 seeds treated by EMS significantly decreased if they were directly sowed in the field. Comparatively, emergence rate increased when M_1 seeds were sowed in nursery trays with a peat: vermiculite ratio of 3:1. Although most of taproots of M_1 showed forking after being transplanted, more M_2 families could be obtained. M_2 families with many seeds were directly seeded in order to phenotype accurately while those with a dozen seeds were still transplanted to keep the resource. We also found that pollen fertilities of some M_2 families were still low, and the emergence rates of some M_2 and M_3 were low as well. Therefore, these mutants should be seeded more, and taproots of them for seed stalks should be transplanted as much as possible.

Taproot color is an important quality trait for carrot because it is associated with carotenoid and anthocyanin contents which play critical roles in human nutrition and health. In this study, we found that some mutations showed different root colors, such as purple, purple-red, red, dark orange, light orange, yellow and white. These mutants could be used as important materials for gene mining of root color. Some of them with comprehensive characters would be deemed as important breeding materials. In addition, a variety of mutant materials closely related to carrot growth and development were obtained by EMS mutagenesis from this study, including mutations in cotyledon, leaf color and shape, growth point, bolting time, dwarfing stalks, purple-green primary stems, secondary branch number, umbel shape, small seed size, etc. These mutants provide important resources for carrot's functional genomic research.

Inheritance of some mutant traits

We crossed the yellow taproot (**Fig. 5J**) and purple-red epidermis taproot (**Fig. 5F**) mutants with the wild type to develop the F_2 population, respectively and proved that both mutations were related to single recessive gene mutations. However, previous research identified *Y* and Y_2 loci on chromosomes 5 and 7 respectively, which control taproot color such as $Y\_Y_2\_$ (white), $yyY_2\_$ (yellow), $Y\_y_2y_2$ (pale orange), and yyy_2y_2 (dark orange) (Ellison *et al.* 2017). DCAR_032551, the candidate gene of *Y* locus, conditioned carotenoid accumulation in carrot taproot due to two insertions in its second exon that created two frameshift mutations. However, sequence alignment showed no SNP mutation within the coding sequence region between wild type and yellow taproot in this study. Two CAPs markers linked to Y_2 didn't show polymorphism between them either (Iorizzo *et al.* 2016). It seemed that these yellow mutants were controlled by a new gene. The molecular mechanisms of these phenotypic changes are still need to be figured out at the DNA level. In this carrot mutant library, for some mutated traits such as purple taproots and vernalization, the genes for them have been cloned or identified (Alessandro *et al.* 2013, Xu *et al.* 2017, 2019, 2020). For some traits of M_3 which were still separating like taproot size and leaf color, we would continue to self-pollinate.

Author Contribution Statement

WZ designed this study and screened the optimal EMS treatment. WZ and LZZ constructed the carrot mutant library and two segregating populations. LZZ, CSF, ZYX evaluated the mutant traits. WZ analyzed the inheritance of yellow and purple-red epidermis taproot mutant.

Acknowledgments

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