



Creation of cotton mutant library based on linear electron accelerator radiation mutation

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ABSTRACT

Cotton (*Gossypium* spp.) is one of the most important cash crops worldwide. At present, new cotton varieties are mainly produced through conventional cross breeding, which is limited by available germplasm. Although the genome of cotton has been fully sequenced, research on the function of specific genes lags behind due to the lack of sufficient genetic material. Therefore, it is very important to create a cotton mutant library to create new, higher-quality varieties and identify genes associated with the regulation of key traits. Traditional mutagenic strategies, such as physical, chemical, and site-directed mutagenesis, are relatively costly, inefficient, and difficult to perform. In this study, we used a radiation mutation method based on linear electron acceleration to mutate cotton variety 'TM-1', for which a whole-genome sequence has previously been performed, to create a high throughput cotton mutant library. Abundant phenotypic variation was observed in the progeny population for three consecutive generations, including cotton fiber color variation, plant dwarfing, significant improvement of yield traits, and increased sensitivity to *Verticillium* wilt. These results show that radiation mutagenesis is an effective and feasible method to create plant mutant libraries.

1. Introduction

Cotton is one of the most important cash crops worldwide. China's cotton production and consumption account for 25% and 32% of the global whole, respectively. At present, improving fiber quality while increasing yield is the main goal of cotton genetics and breeding [1]. Fiber quality is a comprehensive index including fiber length, strength, and fineness. Cotton yield is determined by the number of bolls per plant, number of plants per unit area, lint percentage, and single boll weight [2]. Unfortunately, there is a negative correlation between fiber quality and yield, and it is difficult to achieve synchronous improvement of these traits through conventional breeding techniques.

In genetics, mutant materials are the basis for understanding the functions and relationships between genes. Early genetic research generally adopts positive methods to determine the genes controlling specific traits through the analysis of gene mutations [3]. Although the large-scale genome sequencing of a large number of species has

produced a great quantity of nucleic acid sequence information, only relying on natural variation to obtain mutants for function-based research is far from meeting the needs of current molecular biology research [4]. In order to parse the biological information represented by the obtained gene sequence, one must find the corresponding mutant materials [5]. Using the obtained whole-genome sequence information and the reverse genetics method, large-scale high-throughput screening is carried out on the mutant library, and finally a mutant resource library covering all genes is obtained to analyze the functions of all genes in the genome [6]. For example, many genes were cloned and functionally analyzed using mutant materials in maize [7]. At present, high throughput mutant libraries have been established in *Arabidopsis*, rice, and maize. However, there is no large-scale mutant library in cotton. Therefore, it is necessary to construct a cotton mutant library by mutation breeding techniques.

Current mutagenic strategies mainly include physical, chemical, and site-directed mutagenesis. Physical mutagenesis refers to inducing

Abbreviations: TM-1, *Gossypium hirsutum* acc. T exas Marker-1.

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genetic mutation by using physical factors such as radiation. Physical mutagenesis methods include X-ray, gamma (γ)-ray, ultraviolet, ion radiation (neutron and charged ion radiation), among others including ion beam, electron beam, and laser, as well as space-based mutation with the development of aerospace technology [8]. In cotton, the most common physical mutagens are γ -ray, laser, electron beam, ion beam, and space-based mutagenesis. Chemical mutagenesis refers to single nucleotide variation by altering nucleotide components such as phosphate, purine, and pyrimidine. Common chemical mutagens include nitroso compounds, azides, and antibiotics [9]. In cotton, ethyl methanesulfonate (EMS) is commonly used [10,11]. Chemical mutagenesis benefits include easy application, less damage to the genome, and high mutation rate. EMS had been used to produce mutants that show significantly improved yield and fiber quality [11], changes in oil content and composition of seeds [12], and increased resistance to *Fusarium wilt* and viral infection [13]. Site-directed mutagenesis uses the CRISPR/cas9, based on an adaptive immune defense formed in the long-term evolution of bacteria and archaea. CRISPR/cas9 gene editing technology can be used for specific DNA modification of targeted genes. Recently, this technique has flourished in plants and has been widely used in cotton breeding [14]. However, these conventional mutation methods include certain limitations such as operation difficulties, long treatment periods, or low efficiency, which limits practical application. In addition, nanotechnology is also considered to have a broad prospect in the field of mutant construction, such as Nano-Co treatment can cause double-strand DNA breakage [15]. Nano-Ni treatment may cause DNA mutations, such as base substitution and base insertion/deletion [16]. However, at present, nanotechnology is widely used in biomedical systems, such as drug research and development and drug targeted therapy, but this technology is rarely used in the construction of plant mutants.

Radiation mutagenesis has been widely used in cotton breeding. For example, the cotton seeds irradiated by γ -rays have been used to obtain heat-resistant and early-maturing mutants [17]. The cotton variety 'Lumian 1', with high and stable yield, was successfully created through X-ray radiation mutagenesis of the hybrid progeny of 'Zhongmian 2' and '1195' lines [18]. In recent years, a series of mutants have also been obtained by space-based mutagenesis [19]. For instance, Song et al. obtained and characterized the leaf-yellowing mutant by space-based mutation [20]. Wang et al. isolated and characterized one dwarf mutant through atomic energy mutation [21]. Tong et al. screened cotton mutants for glyphosate resistance through ^{60}Co - γ -ray mutation [22]. Additionally, Mu et al. identified cotton mutants with chicken-foot leaves or gossypol-free glands through ^{60}Co - γ -ray radiation [23]. Ion beam implantation technology has also been used to create new germplasm for improving the quality of cotton [24]. Chen et al. found that laser treatment of cotton can promote growth, increase yield, and improve fiber quality of cotton [25].

It should be noted that excessive radiation can be toxic to organisms. Ionizing radiation can damage DNA, reduce reproductive capacity, reduce somatic cell growth, inhibit bone marrow stromal cells and increase the dose rate-related genotoxicity [26–28]. When *Drosophila melanogaster* was treated with high dose rate gamma rays radiation, it would cause *Drosophila melanogaster* death [29]. The cause of this death may be that when ionizing radiation is absorbed by cells, it may lead to the direct breaking of chemical bonds of biological macromolecules, and ionizing radiation may also affect proteins, nucleic acids and complex lipids, which is the result of reactive oxygen species (ROS) produced by radiation decomposing water or changes in the function of mitochondria [30]. And a large number of studies have shown that the amount of gene damage caused by radiation is directly related to dose rate [31,32], for example, in rat fibroblasts exposed to 0.0, 3.9, 7.4 and 11.3 Gy for 4 or 67 h, the rate of chromosome aberration increases with the increase of dose and dose rate [33]. Ishizaki et al. measured the changes of DNA damage after human fibroblasts were irradiated with different doses (0–5 Gy), 0.3 or 137 Cs source of 1.8 Gy/min [34]. In another study, C57BL6/FYDR/FYDR strain mice were exposed to a

cumulative dose of 400 times the background level (about 10.5 cGy) under low dose rate of 0.00017 cGy/min, and no significant DNA damage was detected, but the number of oxidized bases, micronucleus formation, homologous recombination and gene expression increased in 7.1 cGy/min dose rate [35]. It should also be noted that inappropriate radiation dose will lead to changes in oxidative stress response and apoptosis pathway, protein synthesis, heat shock, immune response, DNA repair, cell cycle control and other related genes [36,37]. To sum up, as long as we control the radiation dose, we may obtain the desired genetic variation, and it is feasible to obtain the cotton mutant library based on the linear electron accelerator technology used in this study.

In this study, we established a large-scale cotton mutant library based on the *Gossypium hirsutum* L. TM-1 (Texas Marker-1) variety using linear electron acceleration-based radiation mutagenesis. TM-1 is a *Gossypium hirsutum* L. inbred line bred by Texas Agricultural Experimental Station in the United States. TM-1 is often used as the standard reference cotton of *Gossypium hirsutum* L. for genetic and cytogenetic research [38]. Long-term study proved that TM-1 is a stable cotton inbred line. Moreover, Li et al. have completed the whole genome sequencing of TM-1, which is conducive to the construction of mutant library of cotton [39]. Through field screening, we found many mutants with a multitude of phenotypes compared with the control, such as colored cotton, dwarfing of plants, chicken-foot leaf morphology, zero fruit branch, and more. The establishment of the mutant library provides a powerful germplasm resource for accelerating the research of cotton gene function and the creation of new high-quality cotton varieties.

2. Materials and methods

2.1. Linear electron acceleration radiation mutagenesis

The cotton variety used in this study was allotetraploid *Gossypium hirsutum* 'TM-1', which was maintained by Henan University. Plump seeds, uniform size and clean epidermis seeds were used in this experiment. Seeds were exposed to a radiation dose of 250 Gy for 1 s in a 10 MeV/20 kW reverse wave electron accelerator (prestige: IS1020). This instrument is operated automatically and can be turned automatically. It can mutagenize 10–20 tons of seeds in a single radiation. The best radiation procedure is obtained from our experiment, and the results were shown in Table 1.

2.2. The propagation and phenotypic identification of mutant library

The M0 generation seeds produced by mutation were self-bred for three generations in the field. The relevant mutants were screened in M2 generation.

Methods for phenotypic identification of mutants: we planted control TM-1 in the mutant library and compared the mutant phenotype with the control phenotype. The cotton in the control group was white, and the colored cotton identified by the mutant library was brown and green. In the control group, the plant was strong, the leaf surface was free of disease spot, the leaf surface of the susceptible cotton mutant was attached to disease spot, the leaf was yellow and wrinkled, and the plant was dwarfed. Compared with the control group, the dwarf cotton mutants was obviously dwarfed, and the phenotypic dwarfing was up to 2–3 times. The leaf type of cotton in the control group was normal round palm leaf, the mutants with chicken-foot leaves was similar to that of chicken foot, and the leaf cracking was serious. The cotton leaf in the

Table 1
Mutation of cotton seeds treated with different mutagenic parameters.

Mutagenic dose	Mutagenic time	Fatality rate/Yellowing rate
150Gy	1s	< 1%
250Gy	1s	10%–20%
350Gy	1s	> 30%

control group was normal dark green, and leaf yellowing mutants was obviously yellowish. The fruit branch of cotton in the control group elongated outward, and the nulliplex fruit branch mutants was obviously shortened.

3. Results and analysis

After being irradiated in a linear electron accelerator, the seeds were self-bred for three generations, and the phenotype was observed and analyzed in the third generation. The mutant library of M2 generation has reached 300,000 seeds. Then we counted the number and mutation frequency of various mutants in M2 generation. The statistical results are shown in Table 2.

3.1. The screening and morphological analysis of colored cotton mutants

Colored cotton can reduce the bleaching and dyeing components of the textile process and potentially reduce environmental pollution. Therefore, since the colored cotton mutant was discovered, it has become the research focus [40]. At present, natural colors are limited to brown, green, and red [41]. Although the genetic basis is unknown, naturally-colored cotton has poor fiber quality and uneven color distribution, and so it is currently not feasible to be used by the textile industry. In order to analyze the relationship between cotton color and quality, we obtained a series of brown and green colored cotton lines, each consisting of approximately 50 individuals. These new brown and green cotton mutants were similar to control materials in relation to yield and fiber quality. Some representative colored cotton mutants are shown in Fig. 1.

3.2. Screening and morphological analysis of susceptible cotton mutants

Both *Verticillium* wilt (*Verticillium dahliae* [42]) and *Fusarium* wilt (*Fusarium oxysporum* [43]) are soil-borne fungal diseases. Both diseases endanger cotton by infecting the vascular tissue. After infection, the vascular bundles of cotton are blocked and cannot transport nutrients to the leaves, resulting in yellowing and wilting of cotton leaves, epiphytic disease spots, and death. This has led to a decrease in yield and quality of cotton. Screening susceptible cotton mutants can use reverse genetics to identify disease resistance genes, study the disease resistance mechanism, and cultivate new cotton varieties with strong disease resistance. We screened hundreds of mutants that were severely infected by the pathogen. These mostly dwarf mutants showed yellow and wrinkled leaves and epiphytic spots, which are in line with the characteristics of *Verticillium* and *Fusarium* wilts. The growth phenotypes of some representative susceptible mutants are shown in Fig. 2.

3.3. Screening and morphological analysis of cotton mutants with chicken-foot leaves

Cotton leaf types are mainly divided into normal leaf, sub-chicken-foot leaf, chicken-foot leaf, and super chicken-foot leaf [44]. The chicken-foot leaf type is named for its deep lobes, resulting in a shape

Table 2
Statistical table of mutation frequency and phylogeny of M2 generation.

Mutant type	Mutant number	Mutation frequency (%)
Brown colored cotton	64	0.021%
Green colored cotton	36	0.012%
Susceptible cotton mutants	734	0.245%
Dwarf cotton mutants	1013	0.340%
Cotton mutants with chicken-foot leaves	46	0.015%
Leaf yellowing mutants	212	0.071%
Nulliplex fruit branch mutants	45	0.015%

similar to a chicken foot. Chicken-foot leaf cotton has many advantages unrelated to leaf shape, including early maturity, insect resistance, higher overall utilization of light energy due to its stronger light transmittance, higher yield, and higher fiber strength [45]. Through phenotypic observation, we screened dozens of chicken-foot-like mutants. The phenotype of some representative mutants is shown in Fig. 3.

3.4. Screening and morphological analysis of leaf yellowing mutants

The yellowing of cotton leaves may be caused by a reduction in chlorophyll content. Chlorophyll is key to plant photosynthesis and environmental adaptability. By screening cotton mutants with yellow leaves, genes regulating chlorophyll can be determined by reverse genetics. Through phenotypic observation, we identified more than 200 mutants with yellow leaves. Fig. 4 shows the growth phenotype of some representative yellowing mutants.

3.5. Screening and morphological analysis of dwarf cotton mutants

Under normal growth conditions, cotton stalks can grow quite tall, which can result in lodging. In production, it is necessary to control cotton growth by applying chemical agents such as ketamine or manually top-cutting. Therefore, screening dwarf cotton varieties is very beneficial for dense planting and mechanized harvesting of cotton [46]. We screened hundreds of mutants with dwarf plant phenotypes. These cotton mutants have the characteristics of short growth and short stem nodes. The phenotypes of some representative dwarf mutants are shown in Fig. S1.

3.6. Screening and morphological analysis of shortened fruit branch mutants

Normal cotton is an indeterminate fruit branch type containing multiple fruit nodes with indefinite growth. Shorten branch cotton mutants show limited node growth and flower buds differentiation in the axils of main stems and leaves [47]. Shorten branch cotton has practical significance for studying fruit branch development, regulating plant structure and architecture, making cotton plants more compact, and making cotton more suitable for high density planting and machine picking. Through phenotypic observation, we found dozens of cotton mutants with shortened type nulliplex fruit branches. The fruit branch development morphology of some representative mutants is shown in Fig. S2.

4. Discussion

The creation of cotton mutants is of great significance for cotton genetic improvement and the creation of new varieties. Conventional breeding methods have made great contributions to the cultivation of cotton varieties. However, the increasingly biased manipulation of available genetic variability in cotton germplasm resources has led to the great loss of genetic potential and increased susceptibility to pests [48]. This selection pressure reduces allelic diversity, hinders the improvement of cotton agronomic traits, and limits our research on the molecular mechanisms underlying plant response to environmental stress and pathogens [49]. Broadening the genetic background through mutation can diversify functional genes, produce new traits, and create more germplasm resources [50]. In addition, the mutant library is of great importance to the study of gene function. For example, acquired traits such as fiber length, stem height, yield quantity, leaf morphology, fiber color, and other mutants have made a great contribution to the genetic breeding and basic research of cotton. Various mutation techniques have been used to study cotton gene function, such as T-DNA insertion [51], gene silencing [52], physical and chemical mutation, among others. These physical, chemical and site-specific mutation techniques have made great contributions to cotton breeding [53] by

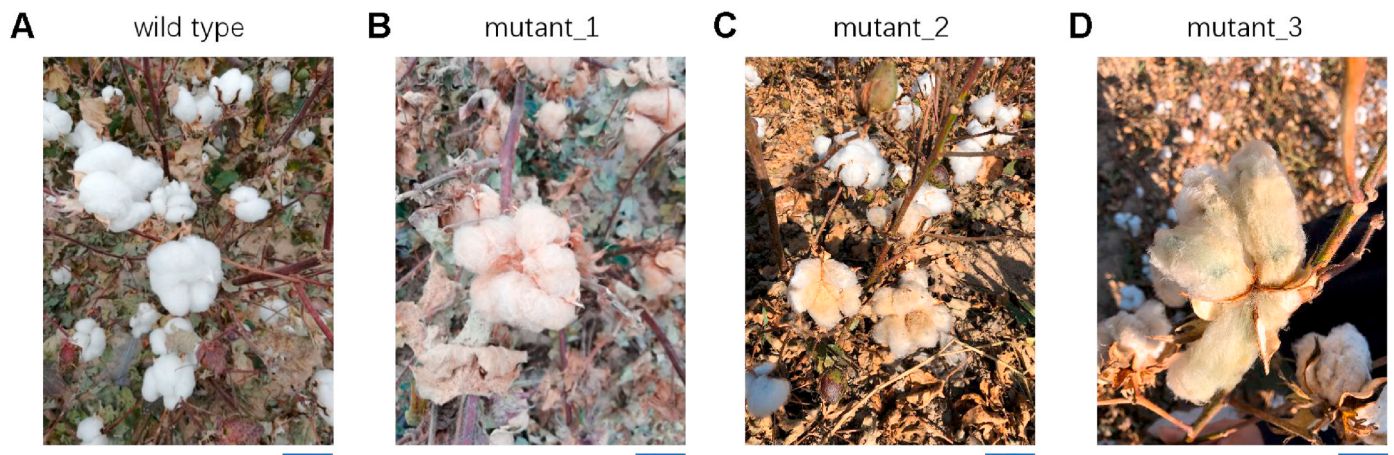


Fig. 1. Brown and green colored cotton mutants. The phenotype of wild type cotton. (B) The phenotype of representative brown cotton mutant. (C)–(D) The phenotype of representative green cotton mutants. Scale bar:5 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

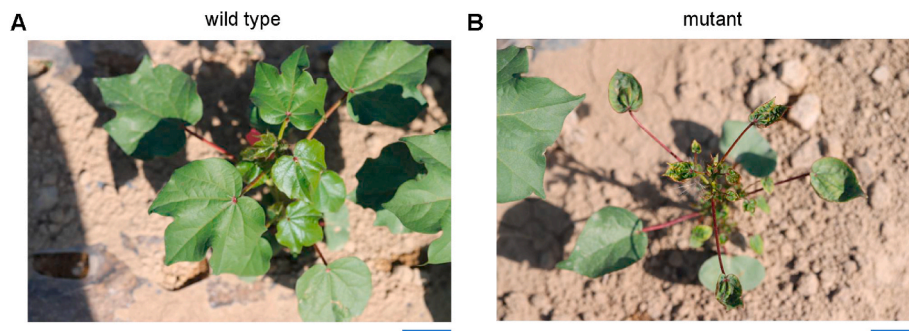


Fig. 2. The phenotype of mutant that show increased sensitivity to *Verticillium* wilt and *Fusarium* wilt. The leaf phenotype of wild type cotton when grown in soil. (B) The representative mutant showing shrinkage and yellowing leaves when grown in soil. Scale bar:5 cm.

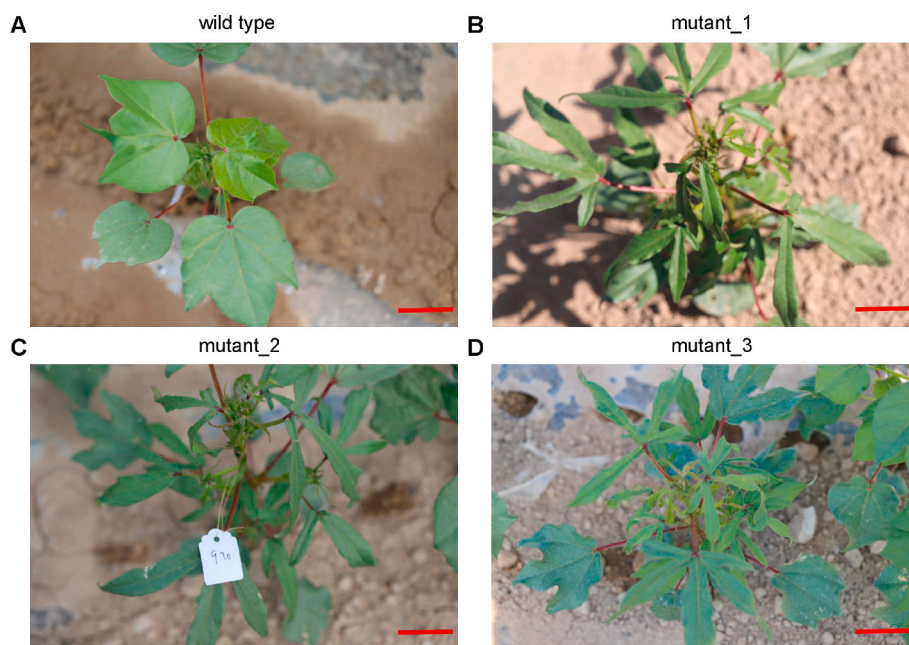


Fig. 3. The phenotype of cotton mutant with okra leaf. (A) The leaf phenotype of wild type cotton seedling. (B)–(D) The leaf phenotype of some representative okra leaf cotton mutants. Scale bar:5 cm.

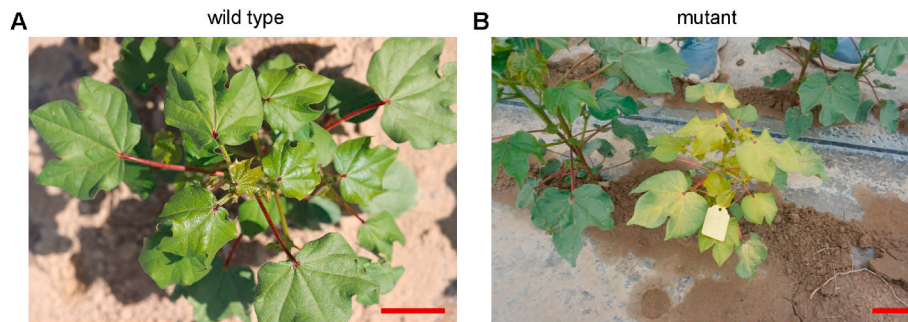


Fig. 4. The phenotype of cotton mutant with yellow leaves.

(A) The leaf phenotype of wild type cotton seedling. (B) The leaf phenotype of representative cotton mutant with yellow leaves. Scale bar:5 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increasing available genetic variation. However, these techniques have practical limitations due to difficult operations, high cost, and low seed treatment capacity. In this study, we developed a new method of radiation mutagenesis using a linear electron accelerator. The cotton mutant library created in this study provides valuable germplasm resources for cotton genetic improvement and functional genomics.

Author contributions

Conceptualization of the project: X.S. Experimental design: X.S and Z.L. Performance of some specific experiments: Z.Z., Z.L. J.W., Y.Z., Y.Z., A.Q., X.Y., R.W. X.Y., and C.G. Data analysis: Z.L. J.W., Y.Z., and Y.Z. Manuscript drafting: S.X. Contribution to the editing and proofreading of the manuscript draft: G.B., and X.S. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2022.101228>.

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