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Specific Gibberellin 2-Oxidase 3 (SbGA2ox3) Mutants Promote Yield and Stress Tolerance in *Sorghum bicolor*

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Abstract: Sorghum (*Sorghum bicolor* (L.) Moench) is a raw material that can be used as food, feed, bioenergy, and wine; it is also a gramineous crop with drought, salt, waterlogging, and high temperature resistance. However, liquor-making sorghum faces the disadvantages of having a narrow genetic basis, poor resources, and few high-quality varieties. Ethyl methane sulfonate (EMS) is a common alkylating agent that can react with one or more bases to alkylate and cause changes in the molecular structure of DNA, thereby causing mutations. It has a minimum effect on organisms and the highest efficiency. The obtained mutant populations are of great significance for cultivating new plant varieties and enriching plant germplasm resources. Therefore, in this study, 'Hongyingzi' a liquor-making sorghum variety, was studied using seeds treated with 0.5% EMS and 415 M3 generation plants were obtained. (1) Investigation and statistical analysis of agronomic traits in mutant libraries showed that in the M3 generation, nine important phenotypic mutant lines were obtained, including plant type, leaf blade, spike, glume, growth period, fertility, plant height, and drought resistance. The variation frequency from high to low was as follows: glume color (75.42%) > spike type (54.70%) > spike shape (47.23%) > chaff coating degree (28%) > plant growth period (23.86%) > plant height (23.61%) > absorption degree (16.14%) > branchiness (10.84%) > leaf color (4.58%) > tillering (2.16%). (2) The PCR sequencing of *SbGA2ox3* from 415 sorghum M3 plants revealed that the mutation frequency of *SbGA2ox3* was 1/99.02 kb. Eight plants underwent mutations, but only one line experienced missense mutations of different amino acid types, changing Ser/Ala/Val/Leu/Gln/Ser/Pro/Ala to Asn/Thr/Gly/Val/Gln/Ala/Ser. The mutant line also had shorter plant height, reduced glume coating degree, and enhanced drought resistance. The constructions of the sorghum mutant library widened the sorghum germplasm library and provided a method for sorghum breeding with a molecular basis for the functional verification of related genes and the analysis of related regulatory networks.

Keywords: *Sorghum bicolor*; EMS mutagenesis; agronomic traits; *SbGA2ox3*; drought stress

Citation: Weng, W.; Tang, Y.; Xiong, R.; Bai, Q.; Gao, A.; Yao, X.; Wu, W.; Ma, C.; Cheng, J.; Ruan, J. Specific Gibberellin 2-Oxidase 3 (SbGA2ox3) Mutants Promote Yield and Stress Tolerance in *Sorghum bicolor*. *Agronomy* **2023**, *13*, 908. https://doi.org/10.3390/agronomy13030908

Academic Editors: Jeong-Dong Lee, Ivan Kreft and Hyun Jo

Received: 21 February 2023
Revised: 13 March 2023
Accepted: 15 March 2023
Published: 18 March 2023

1. Introduction

In recent years, global food production and supply faced enormous threats and challenges because of extreme changes in the global environment, climate, and the COVID-19 epidemic (The State of Food Security and Nutrition in the World, FAO 2020). Sorghum (*Sorghum bicolor* (L.) Moench) is a cereal crop following corn, rice, wheat, and barley in terms of production and acreage; therefore, sorghum has considerable potential to address food security [1,2]. It is highly tolerant to arid, semi-arid, and saline soils, and is adapted to worldwide cultivation [3,4]. Sorghum is widely used for harvesting grains (grain sorghum), syrup production (sweet sorghum), grazing (feed sorghum), and biomass production (bioenergy and cellulose sorghum) [5]. It is noteworthy that sorghum plays an increasingly important role in liquor making and energy production, which is also an important direction for the future development of sorghum breeding and can greatly

improve economic benefits for farmers. Sorghum attracted the attention of many scholars because of its multiple uses and economic benefits in the wine industry. However, sorghum breeding still has the disadvantages of poor plant type, poor stress resistance, and low yield. Therefore, creating and screening high-quality sorghum resources, and cultivating high-quality varieties suitable for production, are key tasks in sorghum development.

As an effective means for germplasm resource innovation, ethyl methane sulfonate (EMS)-induced mutations were widely applied for variety improvements and as the basis of functional gene analysis [6]. EMS is an alkylating agent, and it induces mutations through a direct reaction with purine and pyrimidine molecules in nucleotides [6,7]. Mutagenesis is non-directional; therefore, its impact on plants involves almost all tissues and organs [7,8]. From the perspective of appearance, the characteristics of plants from inside to outside and from top to bottom are likely to change, including yield, quality, and resistance [8,9]. EMS was successfully applied to several plants through multiple generations of screening and identification [10]. Through mutation of agronomic and yield traits, new high-quality materials can be screened from the mutant offspring. Excellent traits, such as dwarfing, early maturity, large grain size, and more tillers, were selected from wheat by EMS mutations [11]. EMS mutagenesis can create new traits that are difficult to obtain using by conventional breeding technology and is widely used in crop germplasm resource innovation, construction of EMS mutant libraries, and mining of new genes [12]. Therefore, EMS-induced mutation is one of methods for sorghum germplasm innovation and key gene mining.

Gibberellin (GA) is a plant hormone closely related to plant growth, development, and stress resistance, such as seed germination, stem elongation, leaf expansion, pollen maturation, flowering, fruit and seed development, as well as biological and non-biological stress resistance [13–17]. Bioactive gibberellins synthesized by higher plants include GA1, GA3, GA4, and GA7 [18], which are mainly catalyzed by three types of oxidases: gibberellin 20-oxidase (GA20ox), gibberellin 3-oxidase (GA3ox), and gibberellin 2-oxidase (GA2ox). Among these, GA20ox and GA3ox are enzymes that catalyze active gibberellin [19,20]. GA2ox is an oxidase with a regulatory function in the third stage of gibberellin synthesis. GA2ox converts biologically active GA1 and GA4 to non-biologically active GA8 and GA34, mainly through hydroxylation of C-2 [21]. GA2oxs mainly function in two ways. The first is through the inactivation of GA via a dioxygenase pathway that relies on 2-oxoglutarate; the second is through the decomposition of the precursor of the precursor of active GA through 2β-hydroxylation [22]. The *GA2ox* gene was cloned and identified in various plants, including *Oryza sativa* L [23], *Arabidopsis thaliana* [24], and *Populus alba* [25]. In rice, site-directed mutagenesis was performed in three conserved domains of *OsGA2ox6*, which reduced GA levels in the plant and increased grain yield by 10–30%, resulting in reduced plant height, increased tillering, increased photosynthetic rate, and improved biological and non-biological tolerance of the plant [26]. The overexpression of *GhGA2ox1* in upland cotton resulted in increased proline accumulation and enhanced tolerance to salt and drought stress [27]. Overexpression of *AtGA2ox1* in Bahia grass resulted in decreased endogenous bioactive GA and malondialdehyde contents; higher chlorophyll, proline, and soluble sugar contents; and a higher growth rate [28]. These results indicate that *GA2oxs* are related to endogenous gibberellin activity, plant dwarfing, germination, flowering, tillering, stress tolerance, and other growth and development processes. Therefore, *GA2oxs* can be considered as key candidate genes for studying plant growth, development, and stress regulatory mechanisms.

'Hongyingzi' is characterized by easy threshing, wide adaptability, drought resistance, barren resistance, strong stress resistance, and high yield and can meet the requirements of the Maotai-flavor liquor brewing process. Therefore, it is an ideal material for EMS treatment, and plants with good mutant phenotypic traits can be used for basic research and germplasm innovation. Due to the toxicity of EMS, treated plants should have good performance of comprehensive traits; especially stress resistance, to be considered successful. Even if a plant with a genetic mutation phenotype is identified, its inability to adapt to

the local environment is fatal because it cannot overcome the biological damage caused by EMS, resulting in the loss of mutant resources. This study focused on problems such as low yield, single germplasm, and lack of functional genes in sorghum. An effective mutant bank was established by mutagenizing sorghum 'Hongyingzi' seeds with EMS to screen germplasms with good plant type, leaf shape, spike development, growth period, fertility, plant height, and drought resistance. The *GA2ox3* mutant was screened by PCR product sequencing, and its agronomic traits and stress resistance were identified. We found that point mutations in some amino acids of *GA2ox3* conferred beneficial traits in sorghum, such as semi-dwarfing, increased biological yield, and enhanced abiotic and biotic stress tolerance. This study is of great significance for innovations in sorghum germplasm, new variety cultivation, and functional gene mining.

2. Materials and Methods

2.1. Construction of the EMS Sorghum Mutant Population

In this study, the special liquor-making sorghum variety 'Hongyingzi' for Kweichow Moutai was selected by the Fengyuan Organic Sorghum Breeding Center of Renhuai City, Zunyi City, Guizhou Province, China. The variety has one spike per plant, no tillering, and no branching. The length of the second stem node is approximately 9.1 cm, whereas the thickness of the second stem node is approximately 0.7 cm, and number of main stem nodes is nine. The plant height is approximately 197.4 cm, spike type is laterally dispersed, the spike shape is in a broom shape, the glume color is red, the glume coating degree is 3/4, no abortion occurs, the field drought resistance index is grade 3, and the whole growth period lasts approximately 136 days.

Treatment of sorghum 'Hongyingzi'with EMS solution: The EMS stock solution was diluted to a concentration of 50 mM/L with 10% phosphoric acid solution and 1000 g of neat and plump sorghum 'Hongyingzi' seeds were selected. Seeds were placed in a conical flask and covered with 50 mM/L EMS mutagenesis solution. The seeds were shaken on a shaking table at 20 °C and 220 rpm for 8 h and then washed with running water for 30 min. The treated seeds were germinated in an artificial climate box (27 °C) and then sown in a seedling raising tray and grown in a greenhouse. When the seedlings grew two leaves and one heart, they were transplanted into a field (106°27′18″–106°52′30″ E and 26°11′10″–26°34′00″ N). This area has a subtropical monsoon humid climate with obvious plateau climate characteristics, warm winters and cool summers, and changeable spring and autumn climates. Water resources are rich; light energy resources are scare; light, heat, and water are available during the same season; and the vertical climate difference is obvious. The average annual temperature is 14.9 °C and the rainfall is 1178. 1 mm. Bagging was conducted after heading and harvesting M1 seeds per plant. The remaining patients were managed according to conventional field management methods.

Sub-propagation: Seeds M1 and M2 were sown in a seedling tray, and seedlings were raised in a greenhouse. Seeds were planted and harvested according to the line; the greenhouse environment was maintained at 70–75% relative humidity, 16 h light/8 h dark–light cycle, and 22 °C. The seedlings were transplanted to the field after they grew two leaves and one heart. This management mode was consistent with that of the M0 generation.

2.2. Investigation of the Agronomic Character of Plants in the Field

Agronomic traits of the M3 generation plants were investigated and recorded, including the number of panicles per plant, tillering ability, branching ability, spike type, spike shape, glume color, glume coating degree, abortion degree, plant height, stem length in the second section, stem diameter in the second section, number of main stem nodes, flag leaf length, and width. After strict bagging self-crossing of mutant plants, the leaf color traits of M3 plants were investigated at seedling stage. The number of panicles per plant, tillering ability, branching ability, plant height, stem length in the second section, stem diameter in the second section, number of main stem nodes, flag leaf length, and width of M3 were investigated at the mature harvest stage. The spike type, spike shape, glume color, glume

coating degree, and abortion degree were investigated for the harvested spike. The test criteria for sorghum agronomic traits were determined according to the standards provided by the National Germplasm Resources Platform and National Crop Scientific Data Center (Supplementary Table S1).

2.3. EMS Library Preparation and Sequencing

The young sorghum leaves at the jointing stage were placed in a freeze-drying machine (Ningbo SHUANGJIA instrument Co., Ltd., Ningbo, Zhejiang Province, China) and freeze-dried for 72 h. Then, the leaves were ground into powder in a ball mill, and the total DNA of the plants was extracted by using the CTAB method. The quality of the extracted DNA was detected by agarose gel electrophoresis with a concentration of 1%. All DNA will be stored in a refrigerator at −20 °C for subsequent use. Total DNA sorghum DNA was extracted using by the CTAB method, and DNA from all 415 M3 families was used to generate the corresponding DNA pools. *SbGA2ox3* is an oxidase that functions to convert bioactive GA1 and GA4 into non-bioactive GA8 and GA34 [20]. Three pairs of specific primers for *SbGA2ox3* (Supplementary Table S2) were designed using Primer 5.0 for PCR amplification of 415 DNA samples from the M3 generation, and the PCR products were sent to Anhui General Biology Co., Ltd. for sequencing. The PCR procedure conditions were as follows: 94 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; then 72 °C for 5 min and 16 °C for ∞. Sequencing results were compared with the original *SbGA2ox3* gene sequence using SnapGene5.2, and the mutation sites and types were counted. Sequencing results with front and tail ends of 140 bp did not consider whether they were mutated. Plants with a base mutation in the *SbGA2ox3* gene were screened.

2.4. Mutation Density Evaluation

PCR sequencing of *SbGA2ox3* in 415 M3 generation plant DNA was performed; the numbers and types of all mutation sites, except the part with front and tail 140 bp, were counted, and the mutation density was calculated as follows:

Mutation density was estimated as the total number of mutations divided by the total number of base pairs (amplicon size × individuals screened), as described previously [29].

Mutation density = 1/(gene length × mutation population size/number of mutation sites).

Mutation frequency refers to the proportion of a specific mutation form in the whole mutagenic population. The equation was as follows:

M3 phenotypic mutation frequency (%) = number of M3 phenotypic mutations/total M3 population × 100.

2.5. SbGA2ox3 Sequences and Phylogenetic Analysis

Online searches for *SbGA2ox3* homologs were performed through NCBI using Blast P and Blast X. Performing multiple sequence alignment and constructing a phylogenetic tree by using MEGA7, the phylogenetic tree was constructed using the N-J method (1000 times). The physicochemical properties of *SbGA2o3* were analyzed by online software, such as ProtParam (http://web.expasy.org/protparam/2021, accessed on 12 September 2022).

2.6. Determination of Soluble Sugar and Malondialdehyde Content

After grain filling of sorghum, watering of the plants in the flowerpots was stopped, and the physiological indexes were detected after continuous drought for 5 days, respectively, weighing 0.1 g of the sorghum leaves after the drought. The soluble sugar and malondialdehyde contents in sorghum leaves subjected to drought treatment were detected according to the kit instructions produced by Beijing Bosch Biotechnology Co., Ltd. Each line was repeated three times, with wild-type as control and three biological replicates per line.

2.7. Gene Expression Analysis of SbGA2ox3 in Sorghum

The non-mutagenized 'Hongyingzi' was taken as a material. The mature leaf (M-leaf), young leaf (Y-leaf), stem, flower, and root in the pustulation period are shown; during the fruit maturity, pre-fruit stage (F-Fr-E), mid-fruit period (F-Fr-M), late fruit stage (F-Fr-L), pre-peel (F-P-E), peel metaphase (F-P-M), and late peel stage (F-P-L) were sampled and placed at −80 °C for subsequent use.

Abiotic stress-induced expression of *SbGA2ox3* sorghum seeds were planted in a greenhouse. After the plant grew two leaves and one heart, the seedlings were treated with six abiotic stresses: PEG (30%), NaCl (5%), darkness, water flooding, cold damage (4 °C), and heat (40 °C). The rhizome and leaf tissues were taken out at 0, 3, 12, and 24 h after treatment and stored at −80 °C.

Sorghum tissues at different developmental stages and under different abiotic stress treatments were harvested, and the total RNA was isolated using the RNA Preparation Pure Plant Plus kit (TIANGEN BIOTECH Co., Ltd., Beijing, China). The integrity of the extracted RNA was checked using 1% agarose gel electrophoresis and the RNA purity and concentration were measured using a spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China); cDNA synthesis was performed using the HiScript®III first strand cDNA synthesis kit (+gDNA wipers). Quantitative real-time PCR (RT-qPCR) was performed using the Cham Q Universal SYBR qPCR Master Mix. 1.0 μL of template cDNA, 10.0 μL of 2 × SYBR mix, 0.4 μL of each primer, and ddH_2O to top off the reaction volume to 20 μL. The qPCR procedure was conducted as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 15 s; then 60 °C for 60 s and 95 °C for 15 s (Bio-Rad, Hercules, CA, USA). All procedures were performed according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). *SbUBQ10* from sorghum was used as an internal reference. The relative expression level of the gene was calculated using the $2^{-\Delta\Delta ct}$ method [21]. The primers used are listed in Supplementary Table S2.

2.8. Statistical Analysis

The test data were analyzed using Microsoft Excel. All numerical data were presented as the mean ± SD (error bars indicate the standard error of the mean). Values were considered significant at * $p < 0.05$; and ** $p < 0.01$.

3. Results

3.1. Development of the Sorghum bicolor Mutant Population

In this study, 1207 individual plants from the M1 generation were harvested, and 3–5 plants were planted from each plant line for the next generation. Owing to force majeure, 994 individual plants of the M2 generation and 415 individual plants of the M3 generation were obtained. During the population growth of the M2 and M3 generations, abundant mutant phenotypes were found at different growth and developmental stages from seedling to mature harvest.

The phenotypic traits of the M3 generation plants were investigated during the entire growth period, with untreated sorghum ('Hongyingzi') used as the control. As shown in Table 1 the mutation types of sorghum ('Hongyingzi') induced by EMS mainly included ten types: plant type (tillering and branching), leaf color, spike shape and spike type, degree of glume coating, glume color, growth period, fertility, and plant height. Among them, the frequency of the glumes' color variation was the highest at 75.42%; the glumes of 'Hongyingzi' were red in color. The colors of the glumes in M3 were also black (45.06%), brown (27.47%), and grey (2.89%) (Table 1, Figure 1). The remaining mutation types in sequence of frequency were spike type, spike shape, glume coating degree, growth period, plant height, fertility, branching, and leaf color with a variable frequency of 2.16–54.70% (Table 1). Among them, the variation frequency of spike type from high to low was: around dispersion (24.34%), medium dispersion (6.27%), and medium tight (14.70%), and tight (9.40%) (Table 1, Figure 1). The variation frequencies of spike shape were: umbrella shape (26.75%), spindle shape (8.19%), and rod shape (12.29%) (Table 1, Figure 1). The variation

frequency of glume coating was 1/2 coating (0.96%) and full coating (26.99%) (Table 1; Figure 1), and 100% coating of glume seriously affected seed threshing and germination, which was one of the key traits for sorghum production. Therefore, selecting germplasm with a low degree of coating is a goal of sorghum breeding.

Table 1. Statistical table of plant mutation types and mutation frequency.

Agronomic Traits	Type	Number of Mutant (Line)	Variation Frequency	Total Number of Mutant Plants (Line)	Total Variation Frequency
Tillering	Weak tillering	4	0.96%	9	2.16%
	Strong tillering	5	1.20%		
Branchiness	Weak branching	36	8.67%	45	10.84%
	Medium branching	6	1.45%		
	Strong branching	3	0.72%		
Leaf color	Albino seedling	10	2.41%	19	4.58%
	Yellow seedling	6	1.45%		
	Purple leaf	3	0.72%		
Spike type	Around dispersion	101	24.34%	227	54.70%
	Medium dispersion	26	6.27%		
	Medium tight	61	14.70%		
	Tight	39	9.40%		
Spike shape	Umbrella-type	111	26.75%	196	47.23%
	Spindle-shaped	34	8.19%		
	Bar-shaped	51	12.29%		
Chaff coating degree	Coating 1/2	4	0.96%	116	28%
	All coated	112	26.99%		
Glume color	Brown	114	27.47%	313	75.42%
	Black	187	45.06%		
	Grey	12	2.89%		
Plant growth period	Mature early	85	20.48%	99	23.86%
	Mature late	14	3.37%		
Abortion degree	Unbeaten	1	0.24%	67	16.14%
	Minority abortion	59	14.22%		
	Majority abortion	7	1.69%		
Plant height	Height	41	9.88%	98	23.61%
	Low	57	13.73%		

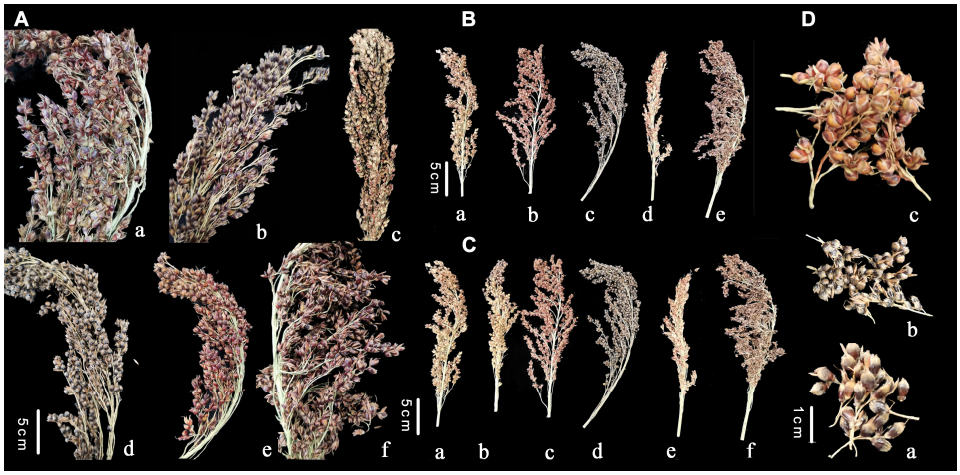

Figure 1. Spike mutation of plants. (**A**) **a**, Red glume; **b**, brown glume; **c**, black glume; **d**, gray glume; **e**, purple glume; **f**, wild-type. (**B**) **a**, spindle; **b**, umbrella; **c**, broom; **d**, bar; **e**, wild-type plants. (**C**) **a**, releasing; **b**, medium tight; **c**, around dispersion; **d**, lateral spread; **e**, tight; **f**, wild-type plants. (**D**) **a**, glume fully encapsulated; **b**, glume covered by 3/4; and **c**, glume covered by 1/2.

Tillering ability, branching ability, and plant height were also evaluated in the mutant library. The results show that the tillering ability of nine plants increased, with a total variation frequency of 2.16% (Table 1 and Figure 2B). The 'Hongyingzi' material itself was unbranched, but some plants were unexpectedly found to have branches in the test site, and the weak branching was dominant, with a variable frequency of 8.67%, medium branching (1.45%), and strong branching (0.72%) (Table 1, Figure 2C). Compared with the non-mutant plants (±20 cm), 98 plants had a change in height (Figure 2E), and 41 plants had increased height, with a maximum of 292.6 cm and a variable frequency of 9.88%. There were 57 dwarf plants, the shortest measuring approximately 84.8 cm, with a variation frequency of 13.73% (Table 1 and Figure 2D). Increased tillering ability, increased branching, and semi-dwarfing plant height are also key traits with the potential to increase crop yield. The early or late maturity of crops has a serious impact on their yield and quality. In this study, the plants that matured 15 days earlier or later were categorized under early maturity or late maturity and had variation frequencies of 20.48% and 3.37%, respectively (Table 1). The maturity time and plant structure of crops are closely related to the implementation of field mechanization. Agricultural mechanization became the main farming mode. Therefore, mutants suitable for mechanized operations deserve more attention.

Figure 2. Plant type mutation. (**A**) Wild-type plant; (**B**) strong tillering plant; (**C**) strongly branched plant; (**D**) plant dwarfing; (**E**) plant height increased; and wild-type plants were the control.

EMS treatment can cause varying degrees of biological damage to plants. In this study, we found plants with different degrees of abortion, including complete abortion (0.24%), most abortions (1.69%), and few abortions (14.22%). Among them, 20 individual plants were observed to undergo leaf color variation with a total variation frequency of 4.5%, among which, 10 plants converted into albino seedlings with an available frequency of 2.4%, six plants mutated into yellow seedlings with a variable frequency of 1.4%, and three plants changed to purple-red leaves with a variation frequency of 0.7% (Figure 3). Plant abortion and leaf color mutations have little significance in traditional breeding, and both are unfavorable traits. However, plants with leaf color mutations and different degrees of abortion are also important for studying crop photosynthesis, chlorophyll synthesis pathways, and flowering and pollination pathways.

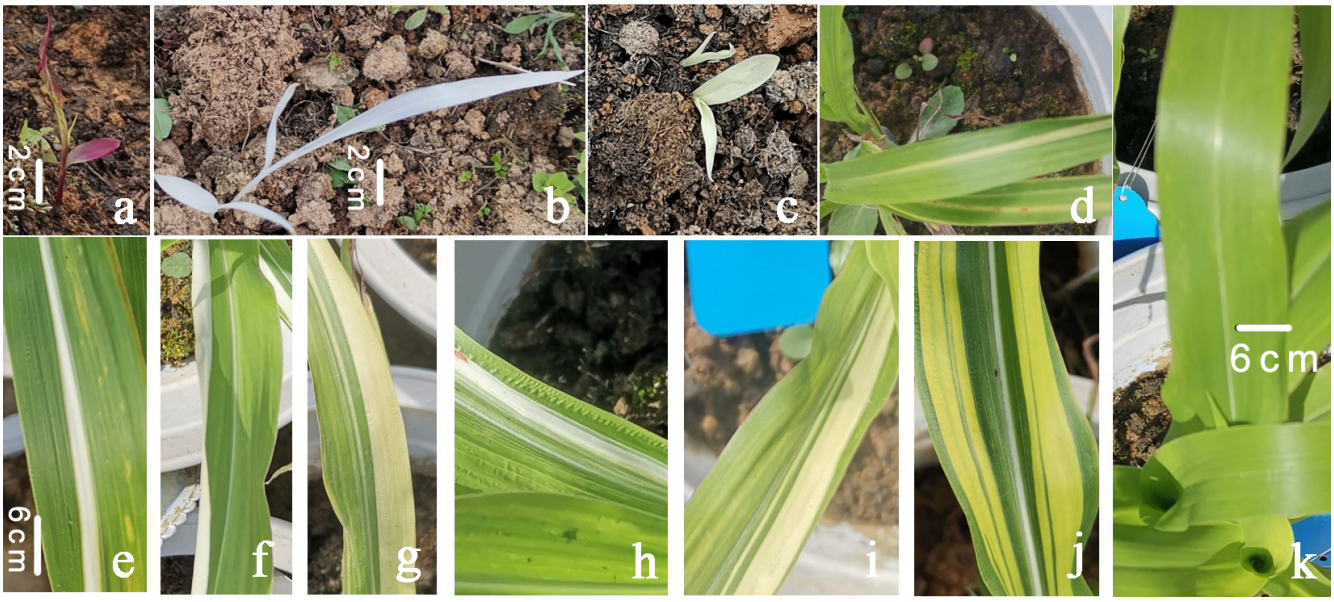

Figure 3. Types of leaf mutants. (**a**) Purple leaves; (**b**) albino seedlings; (**c**) etiolated seedlings; (**d**) yellow veins; (**e**) white veins; (**f**) leaf margin white; (**g**) leaf margin yellow; (**h**) white beside veins; (**i**) yellow beside veins; (**j**) stripe on leaf; and (**k**) wild-type plants.

In summary, the method of EMS mutagenesis adopted in this study produced a wealth of mutant traits, indicating that EMS mutagenesis was one of the effective ways to create mutants. The identification of excellent mutants will provide effective materials for broadening the sorghum germplasm resources and forward and reverse genetic research.

3.2. Prediction and Structural Analysis of the Sorghum SbGA2ox3 Gene

In the present study, *SbGA2ox3*, which plays an important role in plant growth, development, and stress tolerance, was selected as the target gene for PCR product sequencing. First, the gene was preliminarily analyzed according to the phylogenetic tree and bioinformatics analyses. Blast was performed in NCBI using the amino acid sequence of the gene (LOC8055462), and an NJ evolutionary tree was constructed using MEGA-X to encode the amino acid sequence of the gene in the 20 plants. As shown in Figure 4, the amino acid sequence of the protein is highly homologous to the amino acid sequence of corn (NP_001348171.1) by up to 98%, and it is a GA2 oxidase. The *SbGA2ox3* gene was 3579 bp in length, and the CDS was 917 bp in length, encoding 330 amino acids. Protein domain analysis of *SbGA2ox3* using InterProScan revealed that the protein belongs to the 2OG-Fe (II) oxygenase superfamily, which contains a conserved 2OG-Fe (II)-Oxy domain.

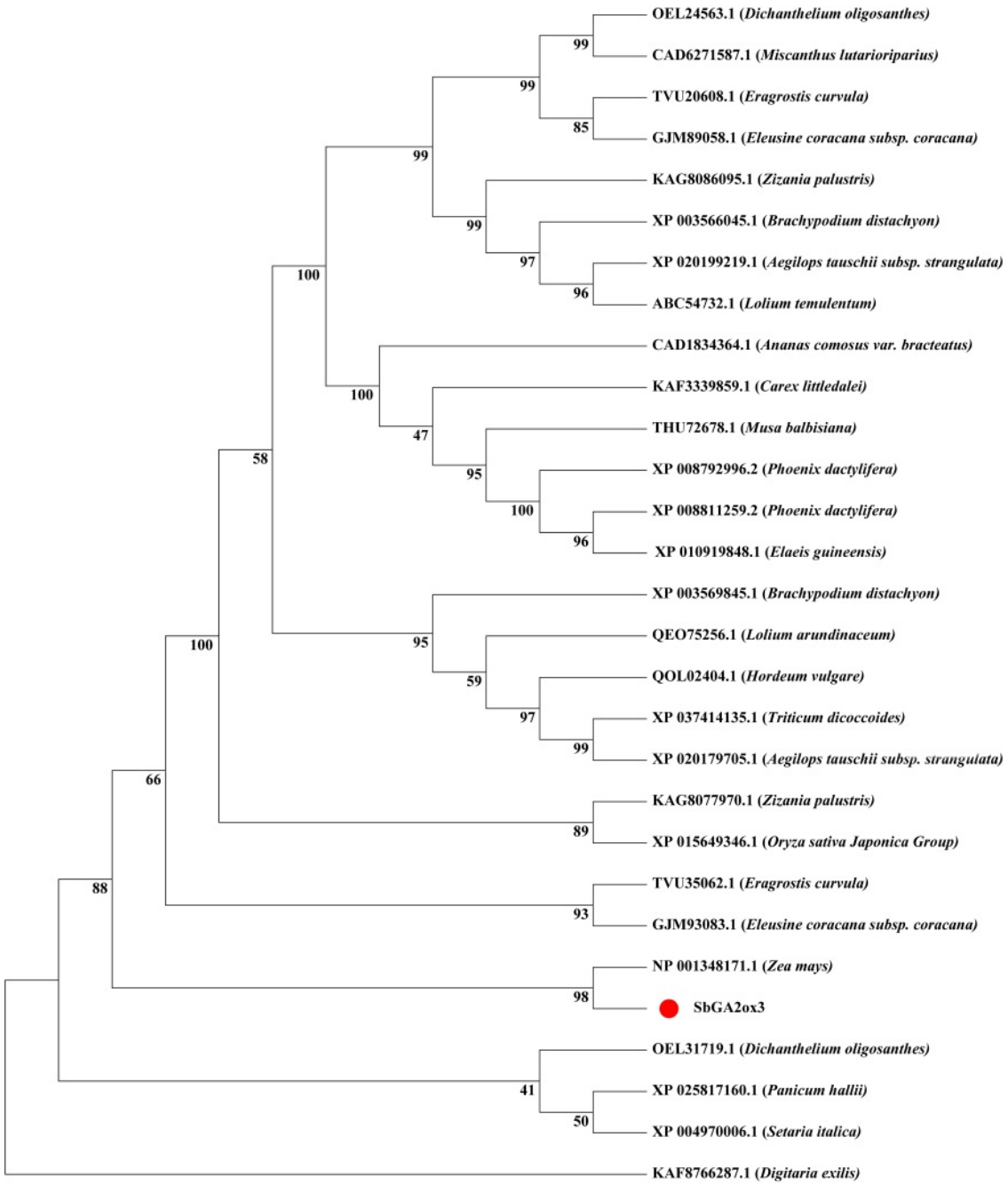

Figure 4. The phylogenetic tree of sorghum *SbGA2ox3*. The *SbGA2ox3* protein in sorghum is labeled with a red circle. A phylogenetic tree was constructed using Mega-X software using the NJ method with 1000 leads.

3.3. Expression Analysis of the Sorghum SbGA2ox3 Gene

The tissue specificity of the *SbGA2ox3* gene was analyzed at 11 stages of sorghum 'Hongyingzi' growth, including the pustulation period (Figure 5A) and fruit development stage (Figure 5B). The results show that during the pustulation period (Figure 5A), the relative expression levels of the *SbGA2ox3* gene ranged from high to low as follows: roots > flowers > stems > young leaves > mature leaves. At the fruit development stage (Figure 5B): the fruits showed no significant differences during the early, middle, and late stages of fruit development; however, with fruit maturation, the expression level of the *SbGA2ox3* gene in the peel increased, and the relative expression level at the late stage of the peel reached more than 200. The results show that the expression of the *SbGA2ox3*

gene was significantly different in different tissues and at different developmental stages in sorghum.

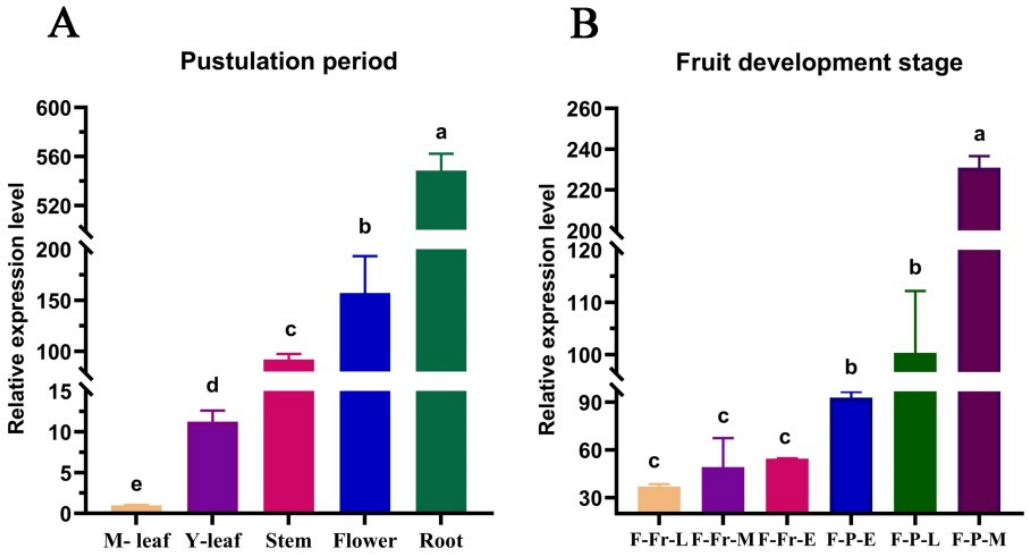

Figure 5. Tissue specificity analysis of *SbGA2ox3.* (**A**) The expression levels of *SbGA2ox3* in mature leaf (M-leaf), young leaves (Y-leaf), stems, flowers, and roots at the pustulation period; and (**B**) in early fruit (F-Fr-E), mid-fruit (F-Fr-M), late fruit (F-Fr-L), early peel (F-P-E), mid-peel (F-P-M), and late peel (F-P-L) at the fruit development stage. The young leaf was used as the control. Values are expressed as means ± SD ($p < 0.05$); each data value is from three repetitions. Significant differences are indicated between the different letters ($p < 0.05$).

The *SbGA2ox3* gene was expressed in the roots, stems, and leaves of sorghum and induced by PEG, NaCl, water flooding, heat (40 °C), cold (4 °C), and dark stress (Figure 6). The overall trend of gene expression first increased, and then decreased, and the highest value appeared at 3 or 12 h, with the expression level dozens or even 100 times higher than that in the control. After treatment, expression in the leaves was higher than that in the stems, and the lowest expression was in the roots. This might indicate that *SbGA2ox3* is highly related to abiotic stress in plants and responds quickly in the leaves. Studies showed that *GA2ox3* can change GA levels in plants and the internal antioxidant system and maintain the expression of multiple genes related to cell osmotic potential, thereby improving plant tolerance and promoting plant growth and development [19]. Therefore, this gene was selected as the target candidate and subjected to ordinary PCR sequencing to detect mutations.

3.4. Mutant Screening of SbGA2ox3

In total, 415 sorghum mutant lines from the M3 generation were subjected to PCR amplification and sequencing to detect *SbGA2ox3* mutations (Table 2). Based on the number of SNPs, the mutation frequency of *SbGA2ox3* in the mutant population was calculated as 1/(1/(415 × 3.579 kb/15) = 1/99.02 kb. Eight amino acid mutations were identified, of which, three showed silent mutations and four showed insertions in the intron region, which were not considered in the present study. Only two isolates showed potent missense mutations. Among them, *SbGA2ox3* in 3013 plants was mutated from Val to Met, and both amino acids were hydrophobic. Sequence analysis of *SbGA2ox3* in 3614 plants revealed eight different mutation sites in the CDS region, changing Ser/Ala/Val/Leu/Gln/Ser/Pro/Ala to Asn/Thr/Gly/Val/Gln/Ala/Ser, in which three sites were mutated into different amino acids.

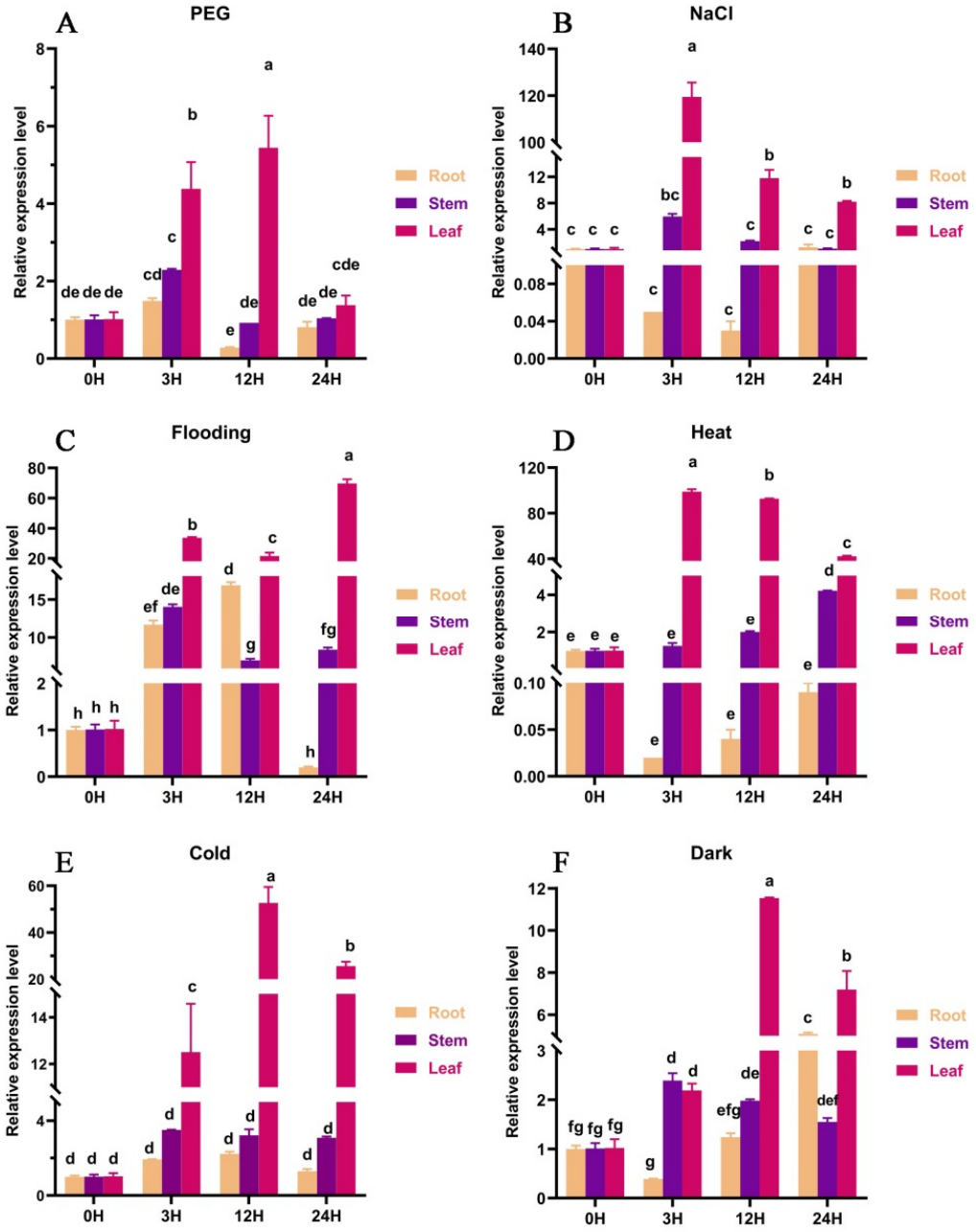

Figure 6. Induced expression of *SbGA2ox3* under abiotic stress. (**A**)The relative expression levels of *SbGA2ox3* in response to 30% PEG, (**B**) 5% NaCl, (**C**) flooding, (**D**) heat (40 °C), (**E**) cold (4 °C) and dark (**F**) in the root, stem, and leaf. The material at 0 h was used as the control. Values are expressed as means ± SD ($p < 0.05$); each data value is from three repetitions. Significant differences are indicated between the different letters ($p < 0.05$).

3.5. Drought Resistance and Phenotypic Analysis of Mutants

In the M3 generation of the mutant, the sorghum seedling stage was treated with drought for five days and the malondialdehyde and soluble sugar contents were detected, and drought resistance was evaluated. The agronomic traits of the 3614 mutant were analyzed. The results show that the plants became shorter (Figure 7A), their leaves shortened (Figure 7B), the spike type became scattered and umbrella-shaped, and the degree of chaff coating was 1/2. A shorter plant height and a lower degree of chaff coating are all favorable phenotypes for mechanized applications. Abiotic stress damages the plant cell membrane

lipids. Simultaneously, the plant produces osmotic substances (soluble sugars) that protect it from damage.

Table 2. Mutation sites and types of the *SbGA2o3* gene.

Plant Number	Normal Base	Mutant Base	Normal Amino Acid	Mutant Amino Acid	Mutation Type
3614	G/G/T/C/ G/T/C/G	A/A/G/G/ A/G/G/T	Ser/Ala/Val/Leu/ Gln/Ser/Pro/Ala	Asn/Thr/Gly/Val/ Gln/Ala/Ala/Ser	Missense mutation
3408	G	A	Ser	Ser	Silent mutation
3266	C	T	Pro	Pro	Silent mutation
3034	C	G			Silent mutation
3013	G	A	Val	Met	Missense mutation
3425	-	T			Insertion mutation (intron)
3540	-	T			insertion mutation (intron)
3510	-	G			insertion mutation (intron)

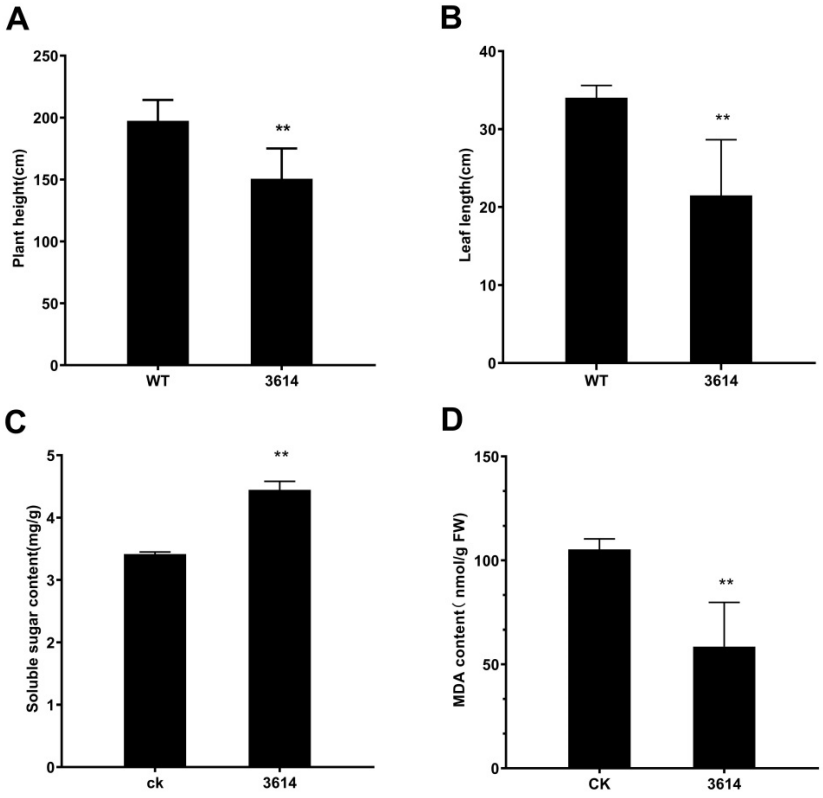

Figure 7. Phenotypic analysis of mutant 3614. Plant height (**A**) and (**B**) leaf length; soluble sugar (**C**) and MDA content (**D**) after drought treatment. Values are expressed as means ± SD; ** $p < 0.01$.

In this study, the soluble sugar and malondialdehyde (MDA) contents of 3614 sorghum plants were determined after continuous drought for 5 days. As shown in Figure 7C,D, under drought stress, the soluble sugar content of the mutant strain 3614 was significantly higher than that of the control. The MDA content was significantly lower than that of the control. These results indicate that in this study, a mutant material 3614 with breeding value was found through EMS induction of sorghum 'Hongyingzi'. It was not only semi-dwarf, with a reduced degree of chaff coating, but also had drought tolerance. Therefore, this material was considered very important in this study.

4. Discussion

Brewing sorghum is challenged by a narrow genetic basis, poor resources, main agronomic traits to be improved, resistance breeding and quality breeding to be strengthened,

and a lack of varieties suitable for mechanized production [30]. In the process of breeding research, new breeding materials are usually obtained from the genetic variation of the source; therefore, the key task and goal of genetic improvement is to purposefully establish and utilize valuable specific or variable traits. EMS is a stable and effective chemical mutagen that causes genome-wide mutations. Material background characteristics, EMS mutagenesis concentration, and population size were key factors for obtaining the target mutation.

Sorghum has a small genome of approximately 730 M bp, providing the basic conditions for EMS induction [31]. In this study, based on the preliminary work in the laboratory, only 0.5% EMS was used, which is close to the concentration of the LD50. In this study, we constructed a population of about 992 M2 plants and screened mutants with one or more agronomic traits coexisting in seven types, including plant type, leaf blade, and spike type, spikelet coating degree, growth period, fertility, and plant height from a population of 415 M3 plants (Figures 1–3 and Table 1). Many studies showed that sorghum mutants with favorable leaf shape, fertility, and growth stage can be produced by EMS mutagenesis [32,33]. Beneficial mutants with shorter plant height, enhanced tillering ability, enhanced branching, and low spikelet coating degree covered in this study are rarely reported. Through field agronomic trait statistics, screening, identification, and selection of single mutation and multivariate enrichment mutants that meet the breeding objectives are still the key bases for our germplasm innovation.

In this study, a large-scale sorghum EMS mutant bank was constructed to lay the foundation for gene function research, sorghum-related reaction mechanism analysis, sorghum germplasm applications, and variety improvement. Tillering and branching are two important plant traits. Sorghum tillering can result in poor consistency in plant height, spike length, leaf number, and mechanized harvesting efficiency. Tillering results in greater changes in biological yields, such as fresh stem weight and fresh spike weight, whereas ineffective tillering disperses nutrients and affects dry matter accumulation in stems and spikes, leading to an increase in empty sorghum grains and a decrease in 1000-grain weight, thereby affecting yield [34]. Increasing effective tillering in the vegetative stage usually increases the number of ears in the reproductive stage, which is a key agronomic trait related to grain yield [35]. The branching pattern of a plant can have an important impact on many aspects, such as plant type, yield, light energy utilization rate, and environmental adaptability [36]. Therefore, it is important to study the genes that control branching and tillering to increase crop yield.

Currently, the objectives of agricultural production include dwarf breeding, high-yield breeding, and adaptation to mechanized production. In M3, the spike-type mutation into tight or medium-tight was beneficial for mechanized production. Mutation of the rod shape into the spike shape can be used as a breeding mechanized production germplasm material, and some dwarf lines were found in the plant height mutation; semi-dwarfing plants adapted to mechanization are one of the key objectives of current wine sorghum breeding. These mutations provide excellent germplasm material for sorghum breeding. Plant germination, plant height, tillering, root growth, flowering, and seed yield are related to GA content [36]. GA2ox is an important enzyme for degrading active GAs, and it is a key enzyme in maintaining the balance of bioactive GAs and intermediates in plants [21]. In the present study, we identified the *SbGA2ox3* gene, which is closely related to the *GA2ox* gene in maize. Through tissue-specific analysis, it was found that the *SbGA2ox3* gene was expressed throughout the whole plant breeding period, but *SbGA2ox3* was enriched and expressed in the roots and flowers at the grain-filling stage, similar to *AaGA2ox4* found in breadfruit trees [20]. *AaGA2ox4* expression in breadfruit trees was reported to be induced by drought and high salt stress, which was seen in this study [20] (Figure 6). These results indicate that *SbGA2ox3* plays a role in plant growth, development, and the abiotic stress response. Therefore, *SbGA2ox3* is of great significance for plant survival and can be used as a candidate gene for molecular breeding.

The first condition for efficient screening using reverse genetics is the presence of mutagenized population with a high mutation density. To efficiently screen the mutants of genes related to important agronomic traits, our research group continuously self-crossed and phenotypically screened the mutants M1 to M3. However, the increase in the generation of mutagenized populations and phenotypic screening may reduce the mutation frequency of the population. The mutation frequency of the *SbGA2ox3* gene obtained by PCR sequencing was 1/99.02 kb, which was higher than that of rice 1/294 kb [37], barley 1/520 kb [38], corn 1/485 kb [39], tomato 1/737 kb, and other species with complex genomes [40]. Therefore, in sorghum, the ideal point mutation can be screened from the small mutagenized population, which reduces the workload of constructing the mutagenized library and is beneficial for the application of TILLING technology. As expected, a functionally deficient mutant of a single gene does not immediately confer any apparent phenotype. This might be because there are many homologous genes with similar functions in plants or because the traits are often controlled by multiple genes. Therefore, this condition was also observed during the screening of mutants in this study. Alternatively, in a mutant with an ideal phenotype, the gene was not mutated, which was potentially due to mutations in other genes, as the EMS-induced mutations were random. Based on the plant mutation rate and mutation frequency of the *SbGA2ox3* gene, the EMS mutant constructed in this study was very effective and lays an important material basis for sorghum breeding and future mechanistic research.

Sorghum plant height can greatly affect the total biomass and yield of the plant and is correlated with lodging resistance and mechanical harvesting of sorghum [41]. Therefore, sorghum plant height is always a key trait studied by researchers. Studies showed that increased expression of *GA2oxs* in plants reduces the content of active GAs, thereby dwarfing the plants [42]. Based on the latest research on the special effects of gibberellin on plants under abiotic stress, detecting the expression changes of *SbGA2ox* in the sorghum drought stress will provide a new theoretical basis for analyzing the drought-resistant response mechanism and genetic improvement of drought-resistant traits of sorghum. A significant upregulation of the *GA2ox3* gene under drought stress was also found during the analysis of leaf transcriptional spectra at the seedling stage in maize [43]. This result is consistent with the results of the present study, showing that *SbGA2ox3* could be induced under abiotic stress (including drought and salt stress) in sorghum. In this study, an important mutant line, 3614, was obtained using the *SbGA2ox3* gene and drought resistance screening. The plant height was decreased and the spike shape shifted to an umbrella shape. The panicle-type mutation was around dispersion, the degree of glume coating was reduced to 1/2, and most importantly, the line showed strong drought resistance. In rice, the insertion mutants of *OsGA2ox5*, *OsGA2ox6*, and *OsGA2ox9* showed upregulated gene expression, remarkably dwarfed phenotype, and remarkably shortened leaf length compared with the control [44]. Therefore, the acquisition of the mutant line provides key experimental material for research on sorghum plant types and the response mechanism under drought stress of the *SbGA2ox* gene.

5. Conclusions

Conclusions and Prospects

In this study, a mutant population of sorghum 'Hongyingzi' with 415 individual plants was obtained by EMS mutagenesis, and seven mutant types, including plant type, leaf blade, spike type, spikelet coating degree, growth period, fertility, and plant height, were obtained, among which the beneficial mutation types included: shorter plant height, decreased glume coating degree, increased branching, and enhanced drought resistance. However, it is difficult to obtain beneficial mutant plants using other methods. The mutation density of the *SbGA2ox3* gene in this population was 1/99.02 kb by PCR sequencing. In addition, there were two missense mutations out of the 15-point mutations in the *SbGA2ox3* gene obtained. Thus, a drought-resistant mutant was obtained. The sorghum mutant population constructed in this study not only laid a foundation for the functional analysis

of important sorghum genes and research on their regulatory mechanisms, but also greatly enriched the existing sorghum germplasm resources and accelerated the sorghum molecular breeding process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030908/s1, Table S1: The main field biological traits names of Sorghum and its statistical criteria; Table S2: Primer information for the *SbGA2o3* gene.

Author Contributions: Experimental design, Y.T. and J.R.; data analysis, Y.T., W.W. (Wenfeng Weng) and R.X.; validation, Q.B., C.M. and J.C.; charting, W.W. (Wenfeng Weng), Y.T., X.Y. and W.W. (Weijiao Wu); first draft, W.W. (Wenfeng Weng), Y.T. and J.R.; edited and revised the paper, J.R. and A.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Science Foundation of China (32161143005, 32160669) and the Provincial Science and Technology Plan Project (Qiankehe Support [2022] key026).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Acknowledgments: We acknowledge the College of Agronomy, Guizhou University, Guiyang, China, for providing the experimental facilities and other materials necessary for this study. We also thank the Fengyuan Organic Sorghum Breeding Center of Renhuai City for providing the sorghum seeds.

Conflicts of Interest: The authors declare no conflict of interest.

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