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Identification and Genetic Analysis of a Novel Allelic Variation of *Brittle-1* with Endosperm Mutant in Maize

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Abstract: Endosperm mutants are critical to the studies on both starch synthesis and metabolism and genetic improvement of starch quality in maize. In the present study, a novel maize endosperm mutant A0178 of natural variation was used as the experimental material and identified and then characterized. Through phenotypic identification, genetic analysis, main ingredients measurement and embryo rescue, development of genetic mapping population from A0178, the endosperm mutant gene was located. The results showed that the mutant exhibited extremely low germination ability as attributed to the inhibited embryo development, and amounts of sugars were accumulated in the mutant seeds and more sugars content was detected at 23 days after pollination (DAP) in A0178 than B73. Employing genetic linkage analysis, the mutant trait was mapped in the bin 5.04 on chromosome 5. Sequence analysis showed that two sites of base transversion and insertion presented in the protein coding region and non-coding region of the mutant *brittle-1* (*bt1*), the adenylate translocator encoding gene involved in the starch synthesis. The single base insertion in the coding region cause frameshift mutation, early termination and lose of function of *Brittle-1* (*BT1*). All results suggested that *bt1* is a novel allelic gene and the causal gene of this endosperm mutant, providing insights on the mechanism of endosperm formation in maize.

Keywords: Maize; endosperm mutant; *brittle-1*; gene mapping; allelic variation

1 Introduction

As one of the critical crops corn are world widely grown as food for humans and livestock [1]. In China, the planting area of corn has exceeded those of rice and wheat, becoming the veritable largest crop since 2017. Fresh sweet corn and waxy corn are special and high-efficiency crops in the eastern provinces of China, which are being increasingly favored and purchased by consumers [2–4].

Sweet corn refers to an endosperm mutant resulting from recessive mutations in a single gene or multi-genes that regulate the transformation of endosperm carbohydrate in corn kernels [5]. Thus far, multiple recessive endosperm mutant genes related to sweet corn have been identified (e.g., *su1, su2, sh1, sh2, bt1, bt2* and *se1*) [6–9]. The mentioned mutant genes are capable of blocking starch synthesis in the endosperm at the stages of corn grain filling and milky maturity; as a result, considerably soluble sugar is accumulated in the grains, and starch synthesis is significantly hindered [10–12].

Since the endosperm mutant genes *su1* and *sh2* have been extensively employed in modern sweet corn breeding [13–17], they exhibit a relatively narrow genetic basis. Other types of endosperm mutant genes requires in-depth studies and applications. In the present study, the phenotypic assessment, genetic analysis and gene identification of an endosperm mutant were performed. This mutant was confirmed to result from a novel allelic gene of maize *Brittle-1* gene, *bt1*, and the formation mechanism of mutation was revealed according to gene sequence variation.

2 Materials and Methods

2.1 Plant Materials

The experimental materials consisted of three maize inbred lines, namely, B73, Ye478 and A0178. A0178 with the endosperm mutant gene was independently discovered in the winter of 2015 in Hainan Breeding Station of Jiangsu Academy of Agricultural Sciences. Because of the poor germination of mutant seeds, it has been maintained in heterozygosis. B73 and Ye478 with normal endosperm are the foundation inbred lines in China's maize breeding; they have acted as parents to breed a series of novel hybrids extensively cultivated in China. Two sets of F_2 populations deriving from the cross of A0178 with B73 or Ye478 in the winter of 2016 in Hainan were adopted respectively to achieve subsequent genetic analysis and verification in the present study. Moreover, F_1 deriving from the cross of A0178 and B73 were backcrossed with A0178 in the winter of 2016 in Hainan to yield BC_1F_1 population, consisting of 273 individuals, which were used for genetic linkage analysis and gene mapping.

2.2 Measurement of Sugar Content

In this study, the fresh samples of ears were harvested from mutant A0178 at approximately 23 DAP. Ten intact corn kernels of relatively uniform size were peeled from the central region of the ears containing the mutant type kernels, which was achieved by phenotypic identification with tweezers. Subsequently, 100 μl of the fully mixed inclusions of the kernels was transferred for the measurement of the sugar content measured with a rapid digital sugar meter (PAL-1, made in Japan). The sugar content was measured by three biological replications.

2.3 Embryo Rescue

The ears of mutant A0178 at 13 to 15 DAP were rinsed with sterile water, soaked in a 0.1% sodium hypochlorite solution for 30 min, and then rinsed with sterile water twice. The immature embryos of mutant kernels were peeled off on an aseptic bench, cultivated with scutellum facing down on standard Murashige and Skoog medium for 15 days [18]. Subsequently, the seedlings were transplanted into the field.

2.4 Differences in Development between the Embryos of Mutant and Normal Kernels at Different Developmental Stages

The fresh samples of ears were harvested from corn inbred line A0178 at 13, 15, 17, 19, 21, 23, 26 and 29 DAP. Ten intact corn kernels were peeled with the method as described above. The immature embryos of mutant kernels were peeled off with tweezers, of which the length and width were recognizated and calculated with the default parameter values of SmartGrain 1.1 [19].

2.5 DNA Extraction and SSR Genotyping

Genomic DNA were extracted from young leaves or seed embyo with the CTAB method [20]. More than five hundred SSR markers were taken based on the coverage of maize chromosomes according to the published mapping information from the Maize Genetics and Genomics Database (MaizeGDB, http://www.maizeGDB.org) and were employed to survey the parental polymorphism. Polymorphic markers were further used for genotyping of BC_1F_1 population.

SSR-PCR amplifications were performed with slightly modified method [21] in a total volume of 20 μl with 50 ng genomic DNA, 1× PCR buffer, 0.5 μM of forward and reverse primers, 0.2 mM deoxyribonucleotide triphosphate (dNTP), as well as 1 unit Taq Polymerase. The PCR profile consisted of an initial denaturation period at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, an annealing process at 55°C for 45 sec, and an extension process at 72°C for 45 sec. Next, a final extension was performed at 72°C for 10 min. 2 μl of the final reaction product was loaded onto a 6% acrylamide bisacrylamide gel (29:1) in 0.5× Tris/borate/EDTA (TBE) buffer and electrophoresed at 200 volts for 120 min. Subsequently, the separated fragments were visualized with a silver-staining method.

2.6 Genetic Analysis and Gene Mapping of Endosperm Mutant

Two sets of F_2 populations developed from the cross of mutant A0178 with B73, or Ye478, were employed to conduct subsequent genetic analysis and verification in the present study. Given the phenotypic observation results, the kernels from the two F_2 populations were grouped into mutant and normal types, respectively. Chi-square analysis was conducted at a significance threshold of 5% to identify deviations from the expected Mendelian segregation ratios between the number of mutant and normal kernels from the two F_2 populations, respectively.

Furthermore, F_1 deriving from the cross of A0178 and B73 was further crossed with A0178 (an embryo rescue homozygous recessive plant) to generate a set of BC_1F_1 population, consisting of 273 individuals; it was employed to conduct genetic linkage analysis. Twenty-four normal-type embryos and twenty-two mutant-type embryos selected randomly to form a sub-population for mapping. The DNA of each sample in this sub-population were extracted to conduct SSR genetic linkage analysis, respectively. The polymorphic molecular markers between the parents were adopted to screen out these samples to identify the markers tightly correlated with the target trait. To further confirm the locus of the mutant gene, SSR markers near this polymorphic marker from the identical chromosome were exploited to amplify the BC_1F_1 mapping population. The molecular linkage map was plotted with the method proposed by Li et al. [22]. Recombination frequency was converted to linkage distances (centimorgan, cM) using the Kosambi mapping function.

2.7 Cloning and Sequence Analysis of bt1

A pair of specific primers (5′-TGT TGC CCT TCC AAA CCT TT-3′, 5′- GAC CGA ACA CAG ACA TGC TT-3′) with complete open reading frames were developed with Primer3 [23] based on the *Brittle-1* gene sequence [24]. With genomic DNA as the template, the PCR product was cloned and then sent to Sangon Biotech (Shanghai) Co., Ltd., for sequencing.

2.8 Co-Segregation Analysis of the Insertion Mutation of bt1 and Mutant Phenotype

According to the sequences around 3-base insertion mutation of *bt1* gene we designed a pair of primers (5′-CGT GCA AGA AGA TCC TCG TC -3′, 5′-GAG ACA AAC ACA CGA TCC GG-3′) for genotyping the BC_1F_1 mapping population mentioned above [21].

3 Results

3.1 Phenotypic Analysis of the Endosperm Mutant

The mature kernel of the mutant was severely shrunken, with only peel, embryo and a small amount of endosperm remained (Fig. 1). The mutant exhibited significantly low seed germination rate, and only less than one percent of mutant seeds could germinate. During the observation of the seed development per 2 days since 10 days after pollination (DAP), the size of the mutant kernels were larger than that of the normal kernels and appeared translucent shape from 14 DAP. At 22 DAP, the mutant kernels were filled with milky liquid, while the sclerotic endosperm began to appear in the wild type. At the late stage of milk ripening, the normal kernels exhibited a slightly decreased volume, the mutant kernels began to shrivel and turn grey, while the embryo slowly turned brown. Moreover, almost no endosperm was found in the mature mutant seeds though there existed a small amount of endosperm around the seed coat. Sugar content analysis revealed that the sugar content in mutant kernels and normal kernels at 23 DAP was 17.5 ± 0.3% and 8.3 ± 0.5%, respectively. Extremely significant differences were identified in the sugar content between mutant and normal type ($p < 0.01$).

Figure 1: The phenotypes of the ear and kernels with the endosperm mutant gene. (A) Mature ear of A0178. Black arrows show the kernels of mutant type; (B) the kernels of mutant type of A0178 mature ear

In this study, we found that the germination rate of this mutant type was extremely lower than that of other endosperm mutants (e.g., the mutant carrying *su1* or *sh2*). To exclude the possibility that the genetic background might affect the seed germination rate, two BC_1F_1 populations deriving from the cross of A0178 and B73, A0178 and Ye478, respectively, were developed and used to assess the germination rate. As suggested from the results, the germination rate of the individuals exhibiting the mutant phenotype reached 0.0–1.5% in both populations, demonstrating that the genetic background showed no relationship to the low germination rate.

The developmental dynamics of normal and mutant kernels shown in Figs. 2–4 suggest no significant difference in the length and width of the mutant embryos at 13 DAP. However, the mutant embryos exhibited significantly smaller length and width than normal ones from 15 to 29 DAP, suggesting that the development of mutant embryos in the middle and late stages of kernel development was considerably inhibited.

The phenotype of the mutant with endosperm deficiency in this study is similar to that of sweet endosperm mutation genes (e.g., *su1, su2, sh2*) used extensively in modern sweet corn breeding. However, the mutant is also characterized as the delay of embryo development and hence extremely low germination ability which was differed greatly to that of widely used endosperm mutant genes. Therefore, it may be a novel endosperm mutant. Here we named this mutant gene as *shrunken*-1501* (*sh*-1501*).

3.2 Genetic Analysis of Endosperm Mutant Gene

To explore the genetic basis of this endosperm mutant, two sets of F_2 populations deriving from the cross of A0178 with B73 and Ye478, respectively, were adopted to conduct the genetic analysis here (Tab. 1). The

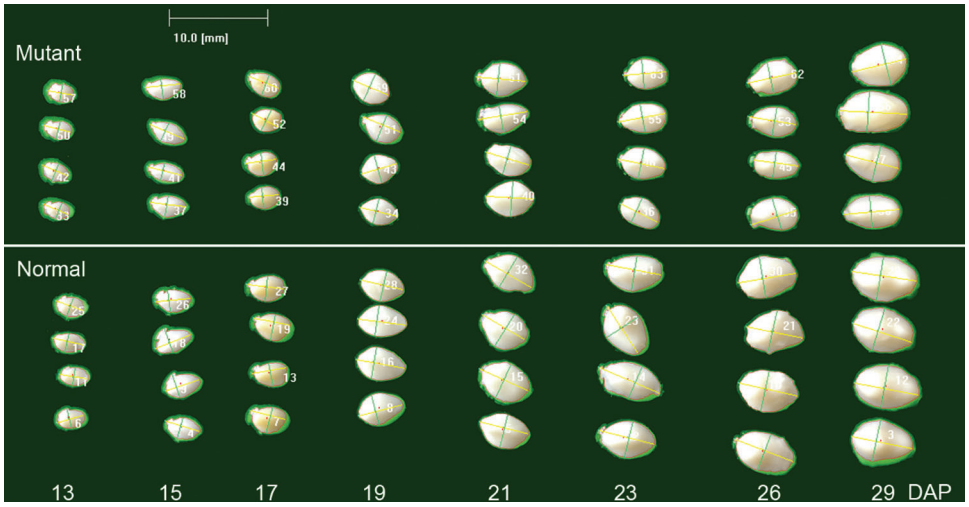

Figure 2: Developmental dynamics of embryo between normal and mutant

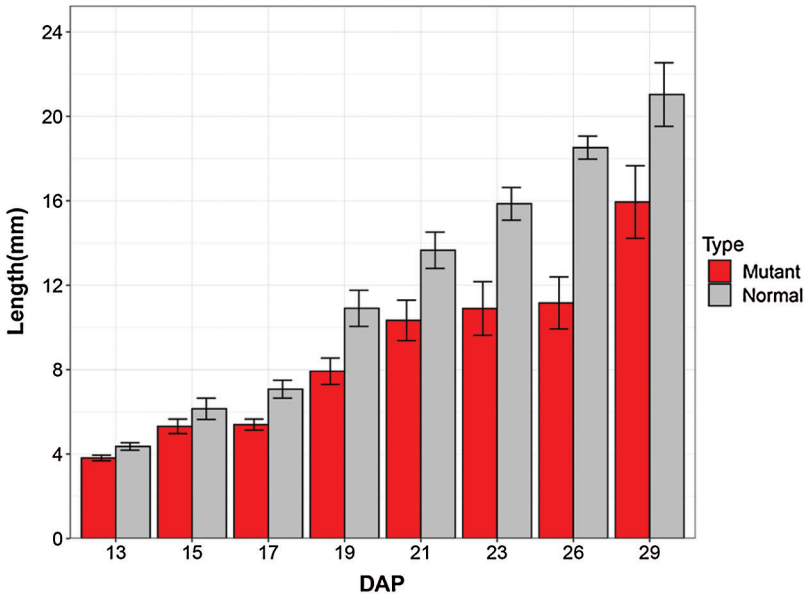

Figure 3: Comparison of developmental dynamics of embryo length between normal and mutant

segregation ratio between the number of mutant and normal kernels in both F_2 populations could fit with the expected Mendelian segregation ratio of 3:1 at a 5% significance threshold, suggesting that the sweet endosperm mutant trait in the present study was controlled by a single recessive gene. This result was also further verified in the BC_1F_1 genetic population deriving from the cross between A0178 and B73.

3.3 Gene Mapping of Endosperm Mutant

Among 542 SSR markers used to survey the DNA polymorphism between the parents, 135 polymorphic markers were adopted to amplify DNA of each individuals in the sub-population for genotyping and genetic linkage analysis. The marker umc1680 located on chromosome 5 was obviously related to the mutant phenotype, demonstrating that the mutant gene in A0178 might be located on chromosome 5 and near the marker umc1680.

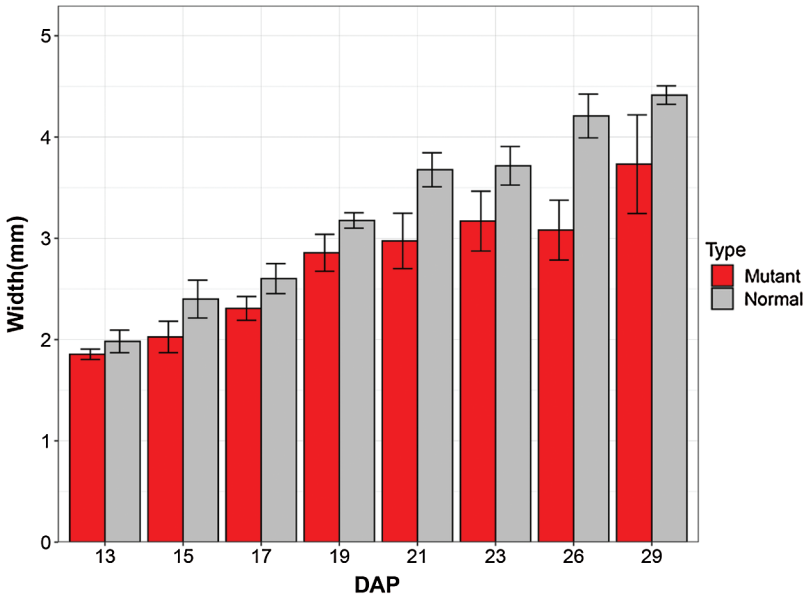

Figure 4: Comparison of developmental dynamics of embryo width between normal and mutant

Table 1: Chi-square testing of segregation of mutant in F_2 populations

Populations	Experimental value		Theoretical value		χ^2 value	*p* value
	Kernels number of mutant type	Kernels number of normal type	Kernels number of mutant type	Kernels number of normal type		
(B73 × A0178)F_2	76	207	70.75	212.25	0.519	0.471
(Ye478 × A0178)F_2	54	154	52.00	156.00	0.103	0.749

To further ascertain the locus of the mutant gene, total of 21 polymorphic SSR markers near umc1680 on chromosome 5 were used to analyze the BC_1F_1 mapping population. As indicated from the results, the mutant gene (*sh*-1501*) in A0178 was located in bin 5.04, flanked by umc1686 and bnlg2323 on chromosome 5 (Fig. 5).

3.4 Allelic Variation Analysis of BT1 Gene

According to MaizeGDB database (www.maizegdb.org), it was reported that the *brittle endosperm 1* gene from B73, *Brittle-1* (*BT1*) (UniProt accession: GRMZM2G144081), was located in this interval as well. To verify whether *bt1* was the causal gene of the mutant, the genomic DNA sequences in the parent B73 and A0178 were amplified with *BT1* gene-specific primers. Alignment of sequences removing introns showed that compared with the *BT1*, two sites of base transversion and insertion were found in the *bt1* gene sequence. The first site was a G→CC mutation at 1051 of *BT1*, and the second was a AGG insertion at 1257–1258 (Fig. 6). The G→CC mutation caused frameshift mutation, early termination of *bt1*-A1078, which is different with M79333 (GenBank accession number). Therefore, *bt1* is a novel allelic variation of *Brittle-1* with endosperm mutant in maize. To further verify the allelic variation, a pair of primers were designed based on the AGG insertion site and employed to amplify the BC_1F_1 mapping population. The result showed the co-segregation of the individual phenotype and the marker, and the reliability of the allelic locus was further verified. Base on the linkage analysis and sequence analysis the causal gene of the endosperm mutant A0178 was renamed as *bt1-1501*.

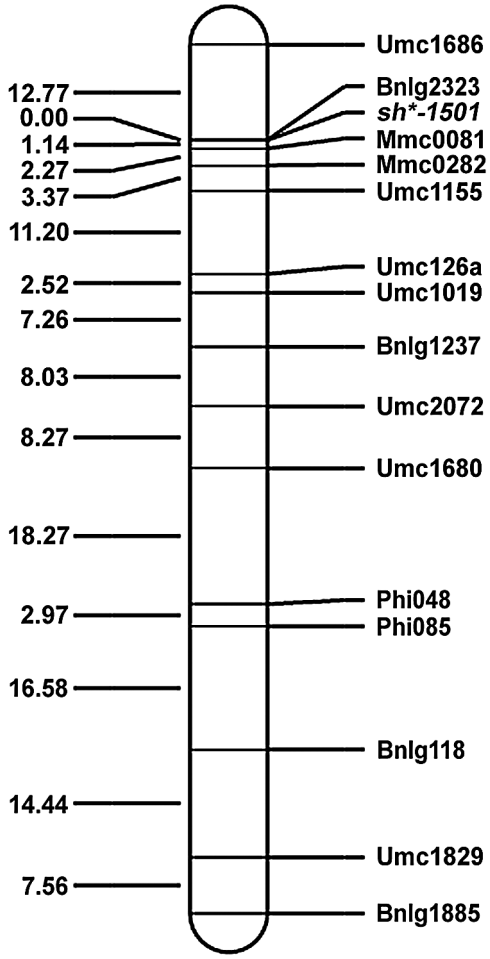

Figure 5: Genetic mapping of the endosperm mutant gene. *sh*-1501* represents the endosperm mutant gene

4 Discussion

Endosperm development in maize results from a series of enzymatic reactions related to starch synthesis. At present, seven relevant genes have been cloned [25]. Loss of function of any of these genes are likely to cause the differences in endosperm accumulation, and to affect the quantity and quality of starch, sugars or proteins in the endosperm, thereby to induce mutations of different phenotypes. Though every endosperm mutant exerts respective specific allele effects, non-allele interactions (i.e., interactions between genes) also impact the content and proportion of various nutrients in the endosperm [26,27].

This study initially speculated the known endosperm mutant genes as the casual candidate gene for the mutation of this study. However, as influenced by the complexity of the maize genome and for the problems related to homologous genes, and the existence of mutations in the regulatory regions of relevant genes, the mutation trait is difficult to identified through the candidate gene strategy. Primarily mapping of the mutant trait and then identification of candidate gene based on the linkage analysis of classical genetics is considered an accurate and easy approach.

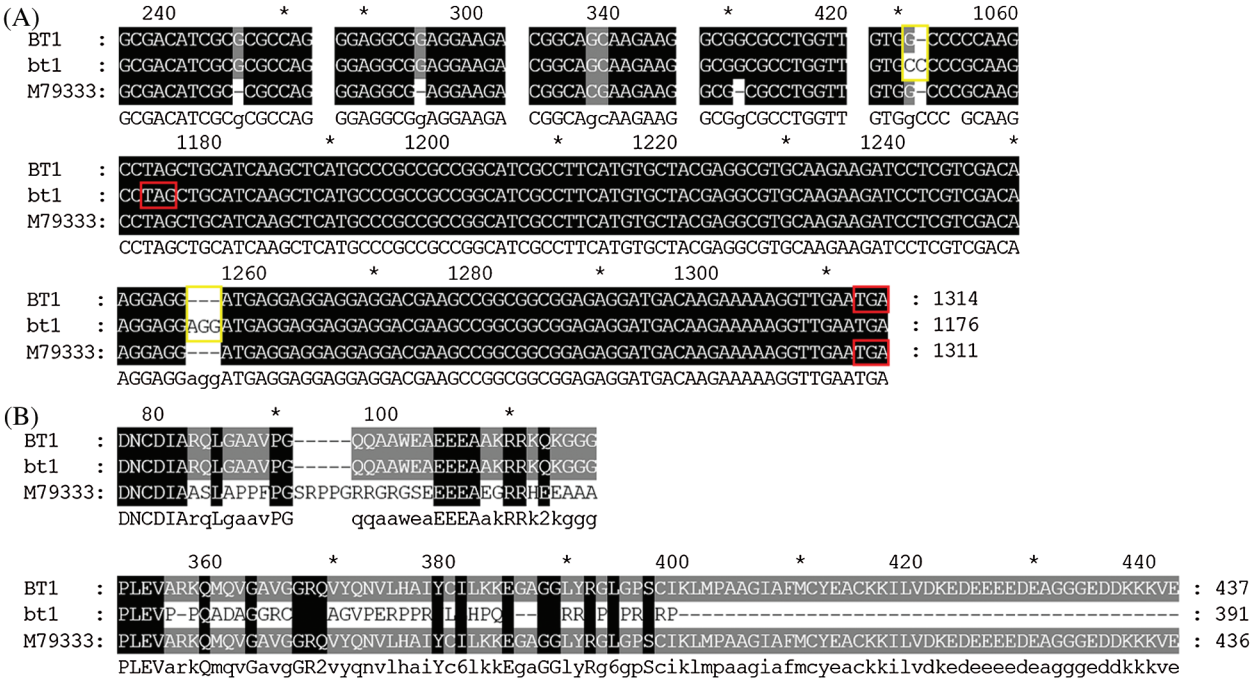

Figure 6: Sequence analysis of *BT1* genes. Alignment of nucleotide sequences (A) and amino acid sequences (B). Yellow boxes showed the two insertion sites, and red boxes showed the terminal codons. *BT1* represents the *brittle endosperm 1* gene from B73 (UniProt accession: GRMZM2G144081); *bt1* represents the endosperm mutant gene from A0178; M79333 is GenBank accession number, represents *Zea mays* brittle-1 protein (bt1) from R802

bt1 (*brittle-1*) and *bt2* (*brittle-2*) are two sweet types of maize endosperm mutants first reported by Cameron et al. [28]. Sullivan et al. [29] identified and cloned a Spm-hybridizing restriction enzyme fragment, co-segregating with the *bt1*-m allele and disappearing from wild-type revertants of *bt1*-m. Non-Spm portions of it acted as probes to identify wild-type (*Bt1*) cDNAs in an endosperm library. The deduced translation product from a 1.7-kb *Bt1* cDNA clone displays an apparent plastid transit peptide at its amino terminus and sequence similarity to several mitochondrial inner-envelope translocator proteins, suggesting a potential effect on amyloplast membrane transport. As suggested by Liu et al. [30], ADP-glucose absorption and conversion into starch in the immature embryos of *bt1* mutant exhibited only 25% activity of that in wild type. Sullivan et al. [31] reported that the *Bt1* locus encoded amyloplast membrane proteins and could be a powdery membrane-specific adenylate transporter by biochemistry and immunolocalization studies at the electron- and light-microscopic levels. The major function is to transport ADP-glucose from the plastids of endosperm cells to the amyloplasts. Shannon et al. [24] also evidenced that the brittle-1 protein (BT1), an adenylate translocator with a KTGGL motif common to the ADP-glucose-binding site of starch synthases and bacterial glycogen synthases, as well as the functions in the transfer of ADP-glucose into the amyloplast stroma.

BT1 homologous genes from different plants participated primarily in starch biochemical synthesis [29,32,33]. Li et al. [34] found that the rice *Brittle1* (*OsBT1*) gene was expressed specifically in the developing endosperm. The osbt1 mutant exhibited a white-core endosperm and achieved a significantly lower grain weight than the wild-type; it did not form sweet endosperm. However, Inan et al. [35] reported that *BT1* homologous gene (*shs1-1*) in the Arabidopsis also participated in multiple

stress responses with *SOS* gene. For instance, this mutant exhibited altered sensitive responses to salt as well as to cold stress.

The *bt1* mutant (A0178) identified in the present study was mapped in the bin 5.04 on chromosome 5, and had endosperm mutation. Sequence analysis showed that the sequence of mutant gene in the present study was not consistent with reported genes related to endosperm development and formation in maize. So *bt1* is a novel allelic gene and the causal gene of this endosperm mutant. Sugar content analysis revealed that amounts of sugars were accumulated in the mutant seeds and more sugars content was detected at 23 DAP in A0178 than B73. It is speculated that the mutant gene may be involved in starch anabolism. In addition, the *bt1* mutant (A0178) had also low germination rate. According to a previous study [36], the Arabidopsis genome contains a gene (*Atbt1*) encoding a highly hydrophobic membrane protein of the mitochondrial carrier family, with six predicted transmembrane domains, as well as exhibiting substantial structural similarity to Brittle1 proteins from maize and potato. Survival of homozygous *Atbt1::T-DNA* mutants is noticeably limited, and those that do survive produce non fertile seeds. This suggests that different allelic variants of *BT1* may have different mutant phenotypes. Over the past few years, recent advances in genome engineering technologies based on the CRISPR-associated RNA-guided endonuclease Cas9 have enabled the systematic interrogation of genome function. To verify the correlation between sequence variation and phenotype and elucidate the gene function, we can attempt to target and edit the different conserved regions of this gene with the CRISPR-Cas9 system and create the materials exhibiting single sequence variation. The mentioned studies will provide insights on genetic and physiological mechanism of maize endosperm development.

5 Conclusions

In this study, our observations indicate that the maize endosperm mutant A0178 of natural variation exhibited extremely low germination ability as attributed to the inhibited embryo development, and amounts of sugars were accumulated in the mutant seeds at 23 DAP, and the mutant trait was mapped in the bin 5.04 on chromosome 5. Sequence analysis showed that two sites of base transversion and insertion presented in the protein coding region and non-coding region of the mutant *bt1*. The single base insertion in the coding region cause frameshift mutation, early termination and lose of function of *BT1*. All results suggested that *bt1-1501* is a novel allelic gene and the causal gene of this endosperm mutant. This study will provide insights on genetic and physiological mechanism of maize endosperm development.

Author Contribution Statement: QCM and JHY proposed the original idea. QCM provided the experiment methods and supervised the experiment data collection. JHY provided the materials. FZ and JT participated in the fieldwork and the evaluation of the phenotype. SW, MJZ and YPC performed laboratory experiments including DNA extraction, PCR, gene mapping and sequencing, etc. SW performed data analysis and drafted the manuscript. QCM and JHY revised the manuscript. All authors have read and approved the final version of manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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