A CRISPR-Cas9-Derived Male Sterility System for Tomato Breeding

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Abstract: Male sterility can reduce cost and enable high seed purity during hybrid seed production. However, the commercial application of male sterility in hybrid seed production has not been widely used in tomatoes. CRISPR/Cas9-mediated gene editing can facilitate acceleration for the practical application of male sterility in hybrid seed production. Here, by using the CRISPR-Cas9 system, two genes DYSFUNCTIONAL TAPETUM1 (SlDYT1) and Glutathione S-transferase (SlGSTAA), which underly the two closely linked loci Male sterile 10 (Ms10) and Anthocyanin absent (AA), were knocked out simultaneously in two tomato parental lines. The generated dyt1gstaa double mutants developed green hypocotyl owing to anthocyanin deficiency and exhibited stable male sterility. Up to 92% effectiveness in selecting male sterility was achieved using green hypocotyl as a morphological marker, and thereafter an efficient and stable propagation strategy of male sterility with the aid of the morphological marker selection was developed. Furthermore, dyt1gstaa-derived hybrid seeds were produced and found to have comparable yield, weight, and germination rate with the corresponding WT-derived F1 seeds. The dyt1gstaa system not only increased hybrid seed purity to 100% but also facilitated its rapid and cost-effective determination. Moreover, this system was discovered to have no evident side effects on important agronomic traits. This study suggested that our CRISPR/Cas9-created dyt1gstaa system can be deployed in tomato hybrid seed production.

Keywords: male sterility; visible marker-based propagation strategy; feasible application

1. Introduction

Hybrid breeding has been extensively utilized in tomato production [1]. However, hybrid seed production relies on laborious emasculation and pollination. Using male sterility (ms) to produce hybrids is proven to be a promising way to reduce labor and ensure high varietal purity, thus receiving great attention [2,3].

In tomatoes, ms was first reported in 1915, and until now, more than 50 different tomato ms mutants have been found, and several corresponding genes have been identified [4]. Sterility mutant positional sterility-2 (ps-2) produced normal pollen grains but had non-dehiscent anthers due to single-nucleotide polymorphism (SNP) in a polygalacturonase gene [5,6]. Male sterile 10(ms10) was characterized by failed pollen production and exerted stigma. ms32 showed completely impaired pollen and tapetum...
development. It is caused by a mutation of a bHLH transcription factor [7]. Tomato *ms15* bears flowers with deformed anthers and exerted stigmas, for which one B-class MADS-box *TM6* was identified as a candidate gene [8]. The mutation of another B-class MADS-box gene, *SIGLO2*, resulted in a photoperiod-sensitive male-sterile mutant 7B-1, which displayed complete sterility in long days but recovered fertility in short days [9].

All the *ms* mutants identified in tomatoes belong to recessive genic male sterility. However, the commercial application has been unsuccessful due to two main limitations. One is the difficulty of propagating large quantities of male-sterile seeds [10]. In general, male-sterile plants are propagated by cross-pollinating male-sterile plants (*ms/ms*) with heterozygote male-fertile plants (*Ms/ms*). Progeny will segregate as 50% male-sterile and 50% male-fertile genotypes (*Ms/ms*), thus only 50% male-sterile plants can be used for producing hybrid seeds, and the other 50% male-fertile plants should be removed [11]. Using linked morphological markers to identify male sterility is an efficient way to address this problem. For example, the linkage of male sterility (e.g., *ms10* and *ms15*) with green hypocotyl provided an easy way to identify male sterility at the seedling stage [8,12,13]. The other limitation is the time-consuming and laborious efforts to introgressing the *ms* gene into an elite line. Usually, at least six generations of backcrossing are needed. Moreover, this approach has been facing challenges, such as false selection, linkage drags, and so on.

The CRISPR-Cas9 system has been proven to be a powerful tool to exploit heritable variations in tomatoes [14-17] and thus provides an efficient way to solve the above-mentioned limitations. Male sterility mutants have been successfully created via CRISPR-Cas9-mediated gene editing in several studies [18-21]. However, these studies are still far from practical applications in breeding. On the one hand, they only knocked out male sterility genes. Thus, male sterility selection and propagation is still a big problem to be solved. On the other hand, male fertility was not achieved in elite inbred lines, which made it hard to evaluate its breeding application, such as side effects on important agricultural traits. Significant progress was achieved in our previous and recently published work [22,23], which both created a male-sterility system with linked markers to assist people with male sterility identification. But convincing data in terms of the propagation method and application of male sterility in hybrid seed production are still lacking.

*Anthocyanin absent* (*aa*) mutant develops green hypocotyl and has been successfully used to select *ms10* at the seedling stage because the two loci are closely linked [24]. It was found that *ms10* encodes a basic helix–loop–helix transcription factor to regulate anther development which corresponds to the Arabidopsis *DYSFUNCTIONALTAPEETUM1(DYT1)* and rice *UNDEVELOPEDTAPEETUM1(UDT1)* homologs [12,25,26]. And *aa* encodes glutathione S-transferase, which is required for anthocyanin sequestration and transport [13]. In this study, two genes, *SIDYTI* and *SIGSTAA*, which underly the *Ms10* and *AA* locus, respectively [12,13], were knocked out using the CRISPR-Cas9 system. The generated *dyt1gstaa* double mutants exhibited green hypocotyl and 100% male sterility under different genetic backgrounds and various environments. Using green hypocotyl as the morphological marker is effective in selecting and propagating male sterility plants. When using this green hypocotyl male sterility system to produce hybrid seeds, seed purity increased obviously, suggesting the promising practical potential of this system for hybrid seed production.

### 2. Results

#### 2.1. CRISPR-Derived *Dyt1Gstaa* Mutants Were Generated in Elite Inbred Lines

CRISPR-Cas9-mediated targeted mutagenesis of *SIDYTI*, which is responsible for pollen development and its linked gene *SIGSTAA*, which controls anthocyanin accumulation in hypocotyls, was conducted in tomato elite bred lines (Figure 1a). A construct with two synthetic gRNAs targeted to the first exon of two genes, respectively,
was produced and introduced into one elite pink-fruited tomato inbred line, TB0993, and one elite red-fruited tomato inbred line, TB0249. For either of the two lines, genome-editing events analysis by sequencing indicated that ≥50% of transgenic plants carried homozygous or biallelic double mutations with small indels (≤12 bp) at both target sites (Figure 1b–e) without off-target editing, which suggested the high editing efficiency and specificity of the CRISPR/Cas9 system we used.

Figure 1. Knocking out *SIDY1* and *SIGSTAA* in tomato using the CRISPR/Cas9 system. (a) Schematic diagram of chromosomal location of *SIDY1* and *SIGSTAA*. The male fertility gene *DYT1* is located ~1.10 Mb upstream of *SIGSTAA* on Chr02, which is responsible for anthocyanin accumulation. (b,c) PCR and sequence-based genotyping of T0 plants. Two gRNAs were designed to target the first exon (E1) of *SIDY1* (b) and *SIGSTAA* (c), and the target sites are indicated by red arrows. Mutations in T0 regeneration plants in the background of elite inbred lines TB0993 and TB0249 are shown below the target sequences, respectively, with deletions and insertions indicated by red dashes and blue letters. Number of base pairs (bp) inserted (+) and deleted (−) is indicated on the right-hand side. The letters on the left-hand side represent different genotypes. Ho, homozygote; Bi, biallelic; He, heterozygote. (d,e): Summary of mutagenesis of *SIDY1* (d) and *SIGSTAA* (e) in the two elite inbred lines TB0993 and TB0249.

2.2. CRISPR-Derived Dyt1Gstaa Mutants Exhibited Complete and Stable Male Sterility

In T2 generation, two transgene-free homozygous double-mutated lines *dyt1gstaa* TB0993 #1 and *dyt1gstaa* TB0249#1 were screened out for phenotypic analysis, among which mutations in *SIDY1* and *SIGSTAA* were both predicted to introduce premature stop condon (Figure S1). The two lines were both found to produce obviously shrunken, thinner, and shorter anther cones with occasionally protruded stigma at anthesis (Figures 2a,b and S2a,b). No visible pollen grains were produced in the two mutated lines, which was supported by fluorescein diacetate (FDA) staining assay and pollen germination assay (Figures 2c,d and S2c,d). Accordingly, the two mutated lines failed to set any fruit except a few tiny seedless “nuts” fruitlets after self-pollination (Figures 2e and S2e) but produced seeded fruits after cross-pollination with WT pollens (Figures 2f and S2f).

For the stability assessment of male sterility, three F2 segregating populations, SP1, SP2, and SP3, were generated. The male sterility of each plant from the three populations was examined at the anthesis stage in terms of pollen activity and fruit set (Figure 2g,h). When grown in Tongzhou farmland, where tomato flowers developed continuously under near-optimal temperatures and humidity, all the homozygous *dyt1* plants displayed male sterility. When planted in Sanya farmland, which is characterized by high temperature and humidity, *dyt1* plants exhibited similar male sterility. These results
highlight the valuable and extensive application potential of the CRISPR-derived dyt1gstaa system in tomato hybrid seed production.

Figure 2. Phenotyping for male sterility of CRISPR/Cas9-derived dyt1 mutants. (a,b) dyt1gstaa/TB0993 produced thinner and shorter anther cones (a) with stigmas protruding out (b) occasionally. (c,d) No viable pollen grains of dyt1gstaa/TB0993 were detected via the FDA assay (c) and pollen germination assay (d). (e) dyt1gstaa/TB0993 failed to set seeded fruits indicating its male sterility. (f) dyt1gstaa/TB0993 gave rise to normal fruits successfully when pollinated with WT pollen. (g,h) Male sterility stability analysis. Three dyt1gstaa-derived F2 populations, SP1, SP2 and SP3, were generated following crosses dyt1gstaa/TB0993 × TB0993 (WT), dyt1gstaa/TB0249 × TB0249 (WT) and dyt1gstaa/TB0993 × TB0748, which represented multiple genetic backgrounds. F2 populations were grown in Tongzhou farm in Beijing (g) and Sanya farm in Hainan province (h), which represented contrasting environmental conditions for stability assessment of male sterility.
Fruit-set rate and pollen vitality frequency of the first three flower clusters from three groups of genotyped plants (mm, Mm, and MM) were examined. Pollen vitality frequency was determined using the FDA method. Fruit set was determined upon self-pollination assisted via a “shaking plants” method. The data represent the mean ± SD from thirty biological replicates. and: not detected.

2.3. Propagation of the Male Sterility Lines

Anthocyanin synthesis in the dyt1gstaa/TB0993 #1and dyt1gstaa/TB0249#1 was completely blocked owing to SIGSTAA disruption, which gave rise to the green color in contrast to the purple color of WT (Figure 3a–c). This allows for the visual screening of male-sterile plants using this morphological marker. Screening efficiency was assessed via linkage analysis of dyt1 and gsta in two dyt1gstaa-derived BC1F1 populations, BC1F1-TB0993 and BC1F1-TB0249 (Figure 3d), and was calculated to be approximately 97.3% and 96.9%, respectively, which suggested high efficiency of this method.

Next, we explored a practical protocol for male sterility propagation based on the generated dyt1gstaa system. Homozygous dyt1gstaa plants (mmaa) were cross-pollinated with WT pollen to generate a hemizygous maintainer line (MmAa). The cross between mmaa and MmAa produced the generation 1 (G1) progeny population (hereafter named “the propagation population”), which was predicted to segregate half green (consisting of 97% mmaa and 3% Mmaa) and half purple plants (consisting of 97% MmAa and 3% mmAa). Purple and green G1 plants were grown separately. At the flowering stage, the first flower clusters or flowers on the first lateral branches of the green plants were allowed to be self-pollinated, and the male fertile plants with self-pollinating fruits (the 3% Mmaa) were removed. The remaining green plants (97% mmaa) were then cross-pollinated with pollen from the purple plants (please note that only the MmAa purple plants can produce viable pollen), which gave rise to the new generation of the propagation population. We performed the propagation procedure for four successive generations and genotyped the individuals in G1 and G4. As expected, the composition of genotypes in G4 resembled that in G1 (Figure 3e).

Ovary size differences were found as early as in stage 12 between dyt1 mutants and their corresponding wild type (Figure S3). Considering that the dyt1gstaa lines developed smaller ovaries (owing to impairment of DYTI), we wondered whether dyt1gstaa mutations would have an adverse influence on seed development and thus affect yield and quality of dyt1gstaa seed during propagation. The seed yield, 1000-seed weight, and germination rate determined in the G4 propagation population (containing about 50% dyt1gstaa seeds) were comparable with those of the self-propagated wild type (Figure 3f).

Overall, the above results indicated high stability and efficiency of the established strategy for propagating the dyt1gstaa male sterility.
Figure 3. Efficient and stable male sterility propagation based on the dyt1 gstaa system. (a,b) dyt1 gstaa/TB0993 developed green hypocotyls and cotyledons owing to anthocyanin deficiency during the seedling stage, which could be served as a reliable morphological marker. (c) Obvious color differences between dyt1 gstaa/TB0993 and WT plants were observed in young leaves, petioles, peduncles, sepals, and axillary buds during the adult stage. (d) Recombination analysis between the CRIPSR-derived dyt1 and gstaa mutation in two BC1F1 populations. “m” represents dyt1 mutation, and “a” represents gstaa mutation. Selection efficiency was expressed as the percentage of mm plants in all the visually selected green (aa) seedlings. (e) Genotype compositions of the G1 and G4 propagation populations. For each population, 768 plants were genotyped using kompetitive allele-specific PCR (KASP) markers for dyt1 and gstaa. (f) Seed yield and seed quality of propagation population. The seed number per fruit, seed number per plant, 1000-seed weight, and germination rate of two G4 propagation populations (referred to as G4/TB0993 and G4/TB0249) were shown to be comparable with those of their corresponding self-propagated WT. Values are the means of different biological replicates ± SD.
2.4. The Created Dyt1Gstaa System Had No Side Effects on Main Agronomic Traits When Used for Hybrid Production

To further assess the practical application of the dyt1gstaa system in hybrid production at a commercial scale, F1 seeds were produced using dyt1gstaa/TB0993 #1 and dyt1gstaa/TB0249#1 as female parental lines, which were propagated as described above. Seed yield and quality were determined, and it was found that no visible differences were found in yield, weight, and germination rate compared to that of WT-derived seeds. dyt1gstaa-derived F1 seeds had an obviously higher seed purity (up to 100%) as expected (Figures 4a and S4a). Fruit ripening time (days from anthesis to the breaker stage), single fruit weight, fruit number per inflorescence, as well as yield of dyt1gstaa-derived F1 hybrids, were comparable to WT-derived F1 hybrids (Figures 4b,c and S4b,c). Moreover, the main quality indexes of fruit at harvest, including total soluble solids content (°Brix), levels of three major antioxidants (lycopene, β-carotene, and ascorbic acid), as well as fruit firmness did not show any significant difference between dyt1gstaa- and WT-derived F1 hybrids (Figures 4b and S4b).

These results suggested that the dyt1gstaa system can be well applied to the commercial production of hybrid seeds.

Figure 4. Performance of the dyt1gstaa and WT-derived elite F1 hybrids. (a) Seed yield and seed quality of the dyt1gstaa-derived F1 hybrid JF101-S. Seed number per fruit, 1000-seed weight, and seed germination of JF101-S were comparable to those of the WT-derived hybrid JF101. But seed
3. Discussion

Male sterility enables reduced cost and high seed purity during hybrid seed production and thus has become a powerful tool in breeding [22]. All discovered male sterility in tomatoes is under genic recessive control. However, none has been widely used in hybrid breeding in practice [22]. One limitation lies in the fact that introgressing a male sterility gene into an elite inbred line is quite time consuming and laborious, and linked drag are usually irradicable. Knocking out male sterility genes using the CRISPR-Cas9 system to create mutants has been described in tomatoes [18-21]. However, these studies did not use elite inbred lines as experimental materials, which made it hard to evaluate the breeding application. In this study, we knocked out gene SlDYT1 which is responsible for pollen development in two elite inbred lines, big-pink-fruited TB0993 and big-red-fruited TB0249, and the high editing efficiency and specificity suggested the success of our used CRISPR/Cas9 system in targeted mutagenesis.

CRISPR-derived dyt1 mutants displayed complete male sterility as described previously [12]. Moreover, we also found that the male sterility caused by the dyt1 mutation is very stable under various genetic backgrounds and environmental conditions. Previous studies indicated that SIDYT1 encodes a basic helix–loop–helix transcription factor to regulate anther development [12]. The SIDYT1 protein sequence had high similarity to AtDYT1 in Arabidopsis and OsUDT1 in rice, both of which are involved in meiosis and tapetum development [12,25,26]. Considering the conserved function of SIDYT1, AtDYT1, and OsUDT1, it is reasonable to predict that the SIDYT1 homologs in other vegetable crops can also be used to introduce male sterility via CRISPR/Cas9 technology.

A strong correlation between parthenocarpy and aberrant stamen development or the disruption of male gametogenesis has been extensively described in tomato plants [27-30]. In our study, dyt1/TB0993 and dyt1/TB0249 both developed seedless “nuts” fruits. Interestingly, under the same environmental cultivation conditions, dyt1/TB0249 displayed a high rate of parthenocarpy than the wild type, while the parthenocarpy rate of dyt1/TB0993 was shown to be comparable with TB0993. These results may indicate that aberrant stamen development resulting from impairment of SIDYT1 induces parthenocarpy in a genetic background-dependent way.

Interestingly, dyt1 gstaa lines developed smaller flowers (including thinner stamens and smaller ovaries). The differences in the size of the ovary could even be seen as early as stage12. And it was found to be a consequence of impairment of DYT1 but not GSTAA because the size differences were only discovered between dyt1 mutants and its corresponding wild types but not between gstaa mutants and wild types. Thus, SIDYT1s not only involved in stamen development but may also be responsible for ovary development. A previous study showed that SIDYT1 was mainly expressed in anthers [12]. Thus, it is interesting to explore the molecular mechanisms underlying the inhibited ovary development in further research.

Considering dyt1 male sterility can be identified using aa as a morphological marker. SIGSTAA, which underlies aa locus [13], was knocked out simultaneously with SIDYT1, and the homozygous gstaa mutants exhibited all-round anthocyanin-deficiency green color, which can be easily distinguished via visual method from wild type. Further screening efficiency assessment via linkage analysis showed up to 92% effectiveness in selecting male sterility using this morphological marker. Notably, the dyt1
A system-based propagation strategy was designed in practice, which allows for efficient and stable multiplication of male sterility seeds.

Side effects of the CRISPR-derived dyt1 gsta system on agronomic traits were carefully examined. As expected, it had no visible negative influence on key agronomic traits, such as fruit shape, color, yield, and so on. Moreover, we found that the dyt1 gsta system did not influence seed yield and seed quality. However, higher seed purity (100%) was achieved.

It was worth noting that Liu et al. (2021) also reported the generated ms10 male sterile lines with two different seedling markers using a similar approach. However, they did not provide convincing data for practical breeding applications in terms of male sterility stability, propagation strategy, and so on. In contrast, we analyzed the male sterility stability under different genetic backgrounds as well as environmental conditions. And we further proposed a strategy to propagate dyt1 gsta seeds without costly genotyping. Moreover, a careful evaluation of the side effects of this system was also performed. Our data demonstrated that our created dyt1 gsta system and the proposed propagation method for breeding are stable and efficient without visible adverse effects, which supported its feasible application in the tomato breeding program.

In summary, the results from this work pave the way for the rapid and feasible application of the CRISPR-derived male sterility system in tomato hybrid seed production. It is reasonable to predict that a similar strategy can also be used for hybrid seed production in other vegetable crops.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

All of the tomato inbred lines used were originally developed by our lab. Gene edition targeting SlDYT1 and SlGSTAA was conducted in pink-fruited inbred line TB0993 and red-fruited inbred line TB0249 and gave rise to dyt1gsta double mutants in the T0 generation. Crossing dyt1 gsta mutants with corresponding WT plants to obtain T1 and further self-pollination of the T1 plants allowed the generation of T2 offspring. In the T2 population, genotyping for foreign fragments and dyt1, as well as the gsta mutations, was performed to identify transgene-free homozygous dyt1 gsta lines.

Three F2 populations, SP1, SP2 and SP3, were generated following crosses dyt1 gsta/TB0993 × TB0993 (WT), dyt1 gsta/TB0249 × TB0249 (WT), and dyt1 gsta/TB0993 × TB0748 (pink-cherry fruited inbred line). For male sterility stability assessment, the three populations were grown in Tongzhou farm in Beijing (March to May 2021) and Sanya farm in Hainan province (October to December 2021), where temperature and relative humidity were automatically measured using recorders at an interval of 30 min. During the flowering stage, plant self-pollination was assisted via a “shaking plants” method.

Two BC1F1 populations, BC1F1/TB0993 and BC1F1/TB0249, were generated by crossing a homozygous dyt1 gsta line (green, mmaa) with a hemizygous maintainer line (purple, MmAa). The plants were subjected to linkage analysis of the “green hypocotyl” and male sterility, together with the above three F2 populations (SP1, SP2, and SP3) via individual genotyping for dyt1 and gsta.

The dyt1 gsta line (green, mmaa) was maintained via backcross with heterozygous plants (purple, MmAa). To assess the stability and efficiency of the dyt1 gsta propagation system, dyt1 gsta lines were propagated at Tongzhou farm in Beijing (March 2020 to August 2021) and Sanya farm in Hainan province (September 2019 to February 2021) for four successive generations. During every round of crossing, green and purple plants were visually sorted out at the seedling stage, and recombinants (fertile green plants) were removed based on fruit set after spontaneous self-pollination on first flower cluster or one retained lateral branch. The first generation (G1) and the fourth generation (G4) were subjected to analysis in terms of genotype composition, seed yield, and seed germination.
Two \textit{dyt1 gstaa}-derived F1 hybrids, JF101-S and JF501-S, were generated following crosses \textit{dyt1 gstaa/TB0993} × \textit{TB0994} and \textit{dyt1 gstaa/TB0249} × \textit{TB0244} to assess the side effects of \textit{dyt1 gstaa} lines on hybrid production. Moreover, the WT-derived F1 hybrids JF101 and JF501 were also generated following crosses \textit{TB0993} × \textit{TB0994} and \textit{TB0249} × \textit{TB0244} and used as the controls. For each cross, 200 female plants were used. Evaluation of side effects on agronomic traits of \textit{dyt1 gstaa} system was performed in tunnel greenhouses at Tongzhou farm (March to August 2021) and Sanya farm in Hainan province (September 2021 to February 2022). Female parents for cross and hybrids for side effects evaluation were decapitated when the sixth inflorescence developed, and flower and fruit thinning was performed with 4–5 fruits per inflorescence.

4.2. CRISPR/Cas9-Mediated Mutagenesis

The knockout vector was constructed using the binary vector PTX041, as described previously [31]. Two synthesized sgRNAs targeting the first exon of \textit{SIDYT1} and \textit{SIGSTAA} were cloned into the PTX041 vector and then introduced into \textit{Agrobacterium tumefaciens} EHA105 for subsequent transformation into tomato inbred lines \textit{TB0993} and \textit{TB0249}, respectively. PCR was performed for generated T0 plants using primers designed based on the flanking sequence of on-targets as well as predicted off-targets, and the amplicon(s) from each line was then cloned into a high-copy vector for sequencing (Tianyi Huiyuan Bioscience & Technology, Inc. (Beijing, China)).

The primers used are listed in Table S1.

4.3. Pollen Vitality Analysis

Pollen vitality was determined using the FDA staining method [32] combined with the germination in vitro method [33]. For the FDA staining method, fresh stamens taken from flowers at the anthesis stage were squashed to release pollen into 20 µL FDA solution (2 µg mL$^{-1}$) on a glass slide. A Zeiss AX10 fluorescence microscope was used for microscopic observations under fluorescent light conditions. For the germination in vitro method, fresh pollen harvested at the anthesis stage was spread on germination medium consisting of 1% agar, 13% sucrose, and 15 mg/L boric acid and then observed using a Zeiss AX10 microscope in a bright field. All experiments were performed with three biological replicates for CRISPR/Cas9-derived mutants (\textit{dyt1gstaa/TB0993} and \textit{dyt1gstaa/TB0249}) and with thirty biological replicates for three alternative genotypes of \textit{dyt1} (MM, Mm, mm) from SP1, SP2, and SP3 populations.

4.4. Linkage Analysis

Individuals from two BC1F1 populations (BC1F1/TB0993 and BC1F1/TB0249) were genotyped for \textit{dyt1} and \textit{gstaa}, and the recombination fraction was calculated as the percentage of recombinants in all of the plants.

4.5. Assessment of Side Effects on Seed Yield, Seed Quality, and Agronomic Traits

At the flowering stage, flowers at anthesis in the second inflorescences were tagged. Seeds were collected from fruits at the red stage and were subjected to yield and quality analysis. Seed yield was calculated as the average seed number per fruit, which was determined by collecting seeds from 10 fruits of the second inflorescence with 5 replicates. Seed weight was calculated as the average weight per 1000 seeds represented by 5 replicates. Seed germination was determined after 3 days of cultivation in an artificial climate chamber at 30 °C. For each assay, 200 seeds were used with three replicates. Seed purity was determined using a male parent-specific molecular marker \textit{Ty1} (Table S1) for four F1 hybrids (JF101, JF101-S, JF501, and JF501-S) or using the color marker (green hypocotyls) for JF101-S and JF501-S. For seed germination and seed purity assay, 384 seeds were used, and three biological replicates were performed.
Hybrid fruits were collected at the breaker stage and weighed. Their longitudinal diameters (LD) and transverse diameters (TD) were determined using a Vernier caliper, and fruit shape index was calculated as the ratio of TD to LD. At the B + 7 stage (7 days after the breaker stage), fruits were harvested to measure fruit firmness, soluble solid content (SSC), lycopene content, and β-carotene content. Flesh firmness was determined using a hardness tester HPE II FF (Bareiss, Germany), with the results recorded in N. Then, each fruit was cut in half. One half was used to determine the SSC of the juice, and the other half was subjected to lycopene and β-carotene content measurements via HPLC with the method described previously [34]. All experiments above were performed with fifteen biological replicates.

4.6. Statistical Analyses

All data represent the mean value ± standard deviation (SD) of biological replicates. Statistical significance was determined using Student’s t test at the 0.05 (*) level.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071785/s1, Figure S1: Predicted truncated dyt1 (a) and gstaα (b) protein sequences; Figure S2: Phenotyping for male sterility and anthocyanin deficiency of CRISPR-Cas9 derived mutants; Figure S3: CRISPR-derived dyt1 mutants developed smaller ovaries; Figure S4: Performance of the dyt1 gstaα- and WT-derived elite F1 hybrids; Table S1: Primers used in this study.

Author Contributions: C.L. (Changbao Li) and C.L. (Chuanyou Li) conceived and supervised the project. M.Z. performed the experiments. W.Z. helped perform the experiments. L.D., M.D., G.Y., M.M. and C.S. analyzed the data. M.Z. and M.D. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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