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USING GENETIC AND CRISPR/CAS9 METHODS TO ACHIEVE QUADRUPLE MUTANT IN TRICHOME TRIMERIC COMPLEX FOR FUTURE STUDY IN FIBER INITIATION MECHANISM

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Abstract

Despite many similarities between the molecular machinery for initiation in cotton fiber and Arabidopsis trichome, there are significant distinctions in how fiber and trichome pattern. While trichome initiation are clearly specified, there is a randomness in fiber initiation in seed coat. While characterizing the cotton fiber genes, the corresponding Arabidopsis gene is replaced by the cotton homolog, and all the complemented lines showed a defined trichome pattern similar to wild type *Arabidopsis*. Owing to the lack of appropriate tools, the fundamental dissimilarities in the patterning process remain unresolved. To completely understand the cotton fiber initiation mechanism and the difference in pattern formation between cotton fiber and Arabidopsis trichome, we aimed to create a cotton trimeric complex MYB2-DEL65/DEL61-TTG3 in Arabidopsis to study the fiber initiation mechanism. To achieve this ultimate objective, the first step is to generate Arabidopsis quadruple mutant (*gl1gl3egl3ttg1*). In this paper we successfully applied genetic and CRISPR/Cas9 approaches to obtain the quadruple mutant. Further, we also introduced the cotton MYB2-A gene into the quadruple mutant. The material generated by this paper will subsequently be used to decipher the molecular basis for cotton fiber initiation.

Key words: Fiber initiation; Trimeric complex; Quadruple mutant; CRISPR/Cas9; *gl1gl3egl3ttg1;* MYB2-DEL65; DEL61-TTG3.

Introduction

The trichome in Arabidopsis leaves has become an important model system to elucidate how individual cells are established (Pesch & Hülskamp, 2009). The trichome is a large, three-branched single cell that promotes and progresses in a well-defined pattern. It has been documented that trichome formation is governed by a MYB- basic helix- loop- helix (bHLH)- WD40 complex in which the R2R3 MYB protein is GLABRA1 (GL1) (Oppenheimer *et al*., 1991; Sato *et al*., 2019), the bHLH protein is GLABRA3 (GL3) or its redundant partner ENHANCER OF GLABRA3 (EGL3) (Payne *et al*., 2000; Wang *et al*., 2019; Zhang *et al*., 2003), and the WD40 repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Glover, 2000; Ramsay & Glover, 2005; Schiefelbein, 2003; Serna & Martin, 2006; Walker *et al*., 1999). Upon assembly of these proteins, the active GL1-GL3-TTG1 complex regulates trichome formation by directly triggering expression of homeodomain-leucine zipper (HD-ZIP) class IV gene GLABRA2 (GL2) (Fambrini & Pugliesi, 2019; Hulskamp, 2004; Larkin *et al*., 2003; Serna, 2004).

Mutations affecting positive regulators of trichome, and patterning have been extensively studied. The recessive *gl1* and *ttg1* mutants have the most severe effect on trichome initiation. Strong loss-of-function mutations in either gene produce glabrous phenotypes on most aerial organs (Koornneef, 1981; Koornneeff *et al*., 1982) (Fambrini & Pugliesi, 2019; Sato *et al*., 2019). While the *gl1* mutation only appears to suppress trichome formation, several other developmental defects could be observed in the *ttg1* mutant, including the production of ectopic root hair, the reduction of anthocyanin pigmentation, and the lack of seed coat mucilage (Doroshkov *et al*., 2019; Galway *et al*., 1994). To date, the most severe mutant allele in the third activator GL3 only shows a subtle reduction in trichome number (Koornneeff *et al*., 1982). Moreover, the trichomes that develop have fewer branches and are slenderer than normal. Combination of *gl3* and *egl3* single mutants results in the completely glabrous phenotype, although trichome initiation in the *egl3* mutant plant depicts a modest reduction of trichome number compared to the wild type (Cox & Smith, 2019; Morohashi *et al*., 2007; Zhang *et al*., 2003).

Cotton fiber is a crucial component in fabric generation, and there is currently an enormous attentiveness in illustrating in how fiber initiate and develop. Because of the newly released cotton genome sequences, more molecular data can be used and translated to understanding of cotton fiber initiation and development (Li *et al*., 2015; Li *et al*., 2014; Wang *et al*., 2012). Cotton fibers are unicellular trichomes that grow from epidermal cells in the seed coat. While the Arabidopsis trichomes follow strictly regulated patterns due to a parallel inhibition mechanism, cotton fibers are randomly initiated, indicating a fundamental difference in the mechanism of trimeric complex function in these two species.

The current use of the Arabidopsis trichome model system is based on replacing one mutant defective Arabidopsis gene with one cotton fiber initiation homolog. In other words, only one protein component of the trimeric complex will be substituted by a cotton homolog protein while the other proteins in the Arabidopsis trimeric complex will be maintained. As a result, the trimeric complex predominantly comprised of Arabidopsis proteins with one cotton fiber initiation protein will subsequently initiate the trichome formation (Humphries *et al*., 2005; Liu *et al*., 2017; Wang *et al*., 2013; Wang *et al*., 2004). Although this method was documented to be useful and

explanatory in elucidating the functions of individual genes, it still represents and retained most of the Arabidopsis trimeric complex components. As a result, signaling pathway includes protein-protein interactions, or activation of downstream genes, will be affected by from the specified pattern of trichomes in the Arabidopsis lines complemented with one cotton genes.

Consequently, characterization of individual genes will not provide information on lack of fiber pattern formation. To fully discern the molecular mechanism of cotton fiber initiation mechanism, our goal was to design a cotton trimeric complex in Arabidopsis. To achieve this, we will initially disrupt the trimeric complex genes (GL1, EGL3, GL3, and TTG1) in Arabidopsis by creating a quadruple mutant by genetic and CRISPR/Cas9 approaches.

The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has been widely utilized in various genome editing that uses precisely target and modify DNA sequence with high fidelity (Hussain *et al*., 2018). This technology requires the expression of Cas9 protein, and the production of a guide RNA that corresponds to the DNA sequences of the genes of interest. Upon the base pairing process of guide RNA to the target DNA, Cas9 protein will be directed to target site. With its helicase properties, Cas9 protein will cut the double strand DNA. As a result, mutations will be introduced by error-prone nonhomologous end-joining DNA repair (Tang *et al*., 2018). Because of its simplicity and high efficiency, CRISPR/Cas9 system has been considered a highly valuable tool in gene targeting strategy.

In this paper, we used a genetic and genome editing (CRISPR-Cas9) approach to obtain the quadruple mutant of positive regulators of trichome formation. By generating quadruple mutants and replacing with cotton trimeric complex, we will shed light on the cotton fiber initiation mechanism and will generate information to design novel strategies for fiber yield improvement.

Material and Methods

Plant materials and growth conditions: The trichomeless Arabidopsis *gl3-1 egl3-77439* (Kanamycin resistant) double mutant (CS6516), and two single EMS mutants *ttg1-1* (CS89) and *gl1-1* (CS1644) were previously described by Esch *et al*., (2003), Humphries *et al*., (2005) and Guan *et al*., (2014), respectively (Esch *et al*., 2003; Guan *et al*., 2014; Humphries *et al*., 2005). All the seeds were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). Seeds were surface- sterilized, vernalized and transferred to a growth chamber with the following environmental conditions: 22ºC, light intensity of 130-150 $Em^{-2}s^{-1}$, 16:8h, light: dark photoperiod and relative humidity of 80% (Clough & Bent, 1998; Junker *et al*., 2015). Seven days after germination, seedlings were transferred to soil in the same temperature and light conditions. To screen for transgene plants, antibiotic was supplemented in MS medium with following concentration: Kanamycin (50mg.ml^{-1}) or Hygromycin (50mg.ml^{-1}) or Basta (50mg.ml^{-1}).

Generating and genotyping the quadruple mutant by genetic approach: The first step in creating a cotton trimeric complex is to deplete the Arabidopsis trimeric complex, which was achieved by generating a quadruple mutant (*ttg1-1 gl3-1 egl3-77439 gl1-1*). Arabidopsis homozygous double mutant (*gl3-1 egl3-77439*) was crossed with *ttg1-1* mutant to generate the triple mutant (*gl3-1 egl3-77439 ttg1-1*). The triple mutant was crossed with *gl1-1* mutant with transgene MYB2 from cotton diploid genome A (unpublished data), and we have generated two independent quadruple mutants *gl1-1 gl3-1 egl3-77439 ttg1-1*/MYB2-A. Subsequently, two plants *gl1-1 gl3-1 egl3-77439 ttg1-1*/MYB2-A were self-fertilized to obtain the quadruple mutants *gl1-1 gl3-1 egl3-77439 ttg1-1*.

Genotypes of progeny were confirmed as follows. Genotyping of the *gl1-1* mutant (CS1644), *ttg1-1* (CS89) and *gl3-1* mutant in the *gl3-1 egl3-77439* double mutant (CS6516) were carried out on base-transition mutations at position 1305 of the GL1 locus, at position 1116 of the TTG1 locus, and at position 1137 of the GL3 locus, respectively. The PCR primer pairs designed and used for Cleaved Amplified Polymorphic Sequences (CAPS) analysis of *gl1-1* mutant allele, *gl3-1* mutant allele, and *ttg1-1* mutant allele were GL1 DdeI-F/ GL1-DdeI-R (Guan *et al*., 2014); GL3-EMS-F/ GL3-EMS-R; TTG1-EMS-F/ TTG1-EMS-R, respectively. All the primer sequences are listed in Table 1. The size of resulting product was 234-bp and produced 201bp and 33bp fragments by *Dde*I in the wild type, while the *gl1-1* mutant allele was resistant to digestion. The *ttg1-1* mutant allele containing a PCR fragment of 218 generated two fragments of 184bp and 34bp after *MluC*I digestion, whereas the *gl3-1* mutant allele was resistant to *Aci*I digestion. The PCR-based genotyping for *egl3-77439* mutant allele was conducted by using primer combinations of EGL3-77439ASCI-R/LB-R and EGL3-77439ASCI-F/ EGL3-77439ASCI-R to amplify the T-DNA-tagged and the wild-type alleles, respectively. Genotyping MYB2 was conducted by using primers *Xho*I-*GhMYB2*-F/ *No*tI-*GhMYB2*-R.

Generating and genotyping the quadruple mutant by using the CRISPR system: We aimed at developing a single vector for expression of the Cas9 system and the trans-activated crRNA cassette. The vectors pMDC43-2NLSCas9 and pGEM-3xU6gRNA were previously constructed in our lab (unpublished data) containing the Arabidopsis U6 promoter and U6 terminator together with trans-activated crRNA cassette and Cas9 system (Fig. 1). The target sequence for disrupting *GL1* was created by annealing the two sets of primers: Forward 1-*Xba*I/ GL1_CRISPR_Reverse and Reverse 1-*Xba*I/ GL1_CRISPR_Forward from the template PGEM-3xU6gRNA. Two resulting fragments harboring *Xba*I sites at 5' and 3' ends will be subsequently joined together by second PCR with primers Forward 1-*Xba*I / Reverse 1-*Xba*I. The amplified products were fused to pMDC43-2NLSCas9 by ligation with restriction enzyme *Spe*I and sequenced (Fig. 1). The constructs were sequenced for verification.

PMDC43-2NLSCas9 containing the target sequence for disrupting *GL1* genes was electroporated in *Agrobacterium tumefaciens* GV3101 and transformed by floral dip method to homozygous triple mutant for *gl3-1 egl3-77439 ttg1-1A* (Clough & Bent, 1998). To screen for transgenic seeds, Hygromycin (50mg.ml^{-1}) and Cefotaxime (50mg.ml^{-1}) were supplemented on MS plates. The resistant seedlings were transferred to soil and subjected to genotyping process. Primers GL1-CRISPR1-1 genotype F/ GL1-CRISPR1-1 genotypeR were designed to amplify the target region of *GL1* (Table 1). The amplified PCR products were subsequently subjected to hybridization steps with conditions of the manufacturer (New England Biolabs).

After purification, the hybridized products were digested with T7 Endonuclease I and visualized on 3% agarose gel.

Phenotypic analysis and microscopy: At 15-day rosette stage, Arabidopsis wild type and transgenic leaves were collected and examined under an Olympus SZ61 industrial microscope. Images were taken by 5-megapixel digital color camera Olympus UC50 (Japan).

Results and Discussion

Construction and confirmation of the *gl3-1 egl3-77439 ttg1-1 gl1-1* mutant from a genetic approach: Here we report on generating the triple mutant *gl3-1 egl3-77439 ttg1-1* and the quadruple mutant *gl3-1 egl3-77439 ttg1-1 gl1-1*. The double mutant *gl3-1 egl3-77439* was initially crossed with *ttg1-1* to generate the triple mutant *gl3-1 egl3-77439 ttg1-1*. By creating a Punnett square in the F_2 generation, we calculated that the chance we obtained the homozygous triple mutant was one out of sixty-four plants. Hence, we created a strategy to reduce the painstaking process in genotyping. After harvesting the F_2 seeds from the cross *gl3-1 egl3-77439* with *ttg1-1*, we selected them on MS media with antibiotic Kanamycin as the T-DNA insertion in *egl3-77439* mutant allele contains the Kanamycin resistance gene. Surviving plants were transplanted to soil and observed for the trichome initiation phenotype. We expected that the homozygous *gl3-1 egl3-77439 ttg1-1* mutant should mimic the *gl3-1 egl3-77439* glabrous phenotype; thus only trichomeless F2 plants were subjected to genotyping process.

After obtaining the homozygous triple mutant *gl3-1 egl3-77439 ttg1-1*, we proceeded with the crossing *gl1-1* mutant with MYB2 gene from cotton diploid genome A to generate with quadruple mutant *gl3-1 egl3-77439 ttg1-1 gl1-1*. Since the lethality of F2 generation from the cross *gl3-1 egl3-77439 ttg1-1* with *gl1-1* was uncertain, we used the transgenic line *gl1-1* transformed with MYB2 from the diploid cotton genome A as one parent. We reasoned that if the homozygous quadruple mutant *gl3-1 egl3-77439 ttg1-1 gl1-1* was lethal, establishing quadruple *gl3-1 egl3-77439 ttg1-1 gl1-1* lines with cotton homologs, MYB2 would mimic the triple mutant phenotype, thus retaining its vitality. It was indicated from the Punnett square that one out of two hundred fifty-six plants from F_2 generation would be the homozygous quadruple mutant. The process of obtaining the triple mutant *gl3-1 egl3-77439 ttg1-1* was repeated to reduce the lengthy genotyping procedure. After harvesting the F_2 seeds from the cross *gl3-1 egl3-77439* with *ttg1-1*, we selected them on MS media with antibiotic Kanamycin. As the 35S:*MYB2*-A harboring the Basta resistance gene, plants were transplanted to soil and sprayed with Basta solution (50mg.ml^{-1}) to select this gene and were observed for trichome initiation. We also expected that the homozygous *gl3-1 egl3-77439 ttg1-1 gl1-1/35S: MYB2-A* should mimic the *gl3-1 egl3-77439* glabrous phenotype. Thus only trichomeless F_2 plants were subjected to genotyping process.

Genotyping the *egl3-77439* mutant allele was performed by conducting two PCR amplification reactions, in which one reaction used gene-specific primers which flanked EGL3 from the beginning to the end and another pair that used the left border and the primer flank the end the *EGL3* (Fig. 2). Our result indicated that the T-DNA fragment was inserted in the fifth exon of the EGL3 sequence which would disrupt the HLH region of the protein.

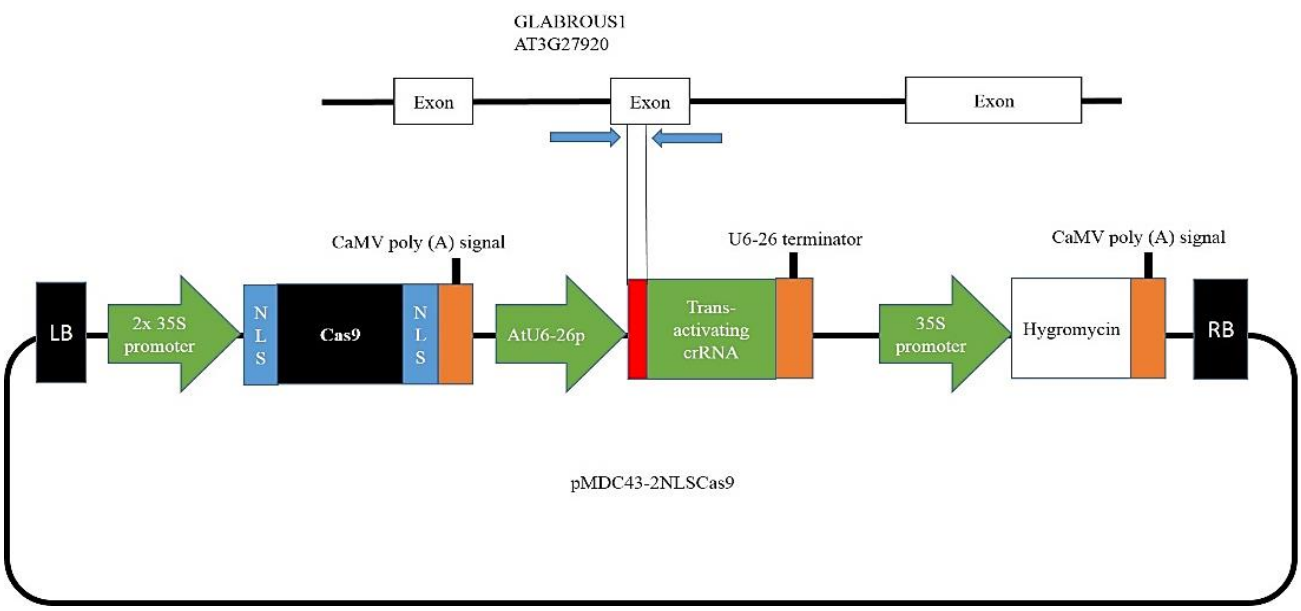

Fig. 1. Construction of pMDC43-2NLSCas9 with the target for GL1 disruption. The pMDC43-2NLSCas9 contains the sgRNA with target region (red) driven by Arabidopsis U6-26 promoter, the Cas9 gene (black) under the control of 2x35S promoter and the selection antibiotic marker hygromycin phosphotransferase. Arrows indicate left and right primers flanking the target regions in the second exon of *GL1* gene. This diagram is not drawn to scale.

Table 1. Primer sequences.

Primer	Sequences
XhoI-GhMYB2-F	ACTGGCGGCCGCATGGCTCCAAAGAAGGATGGAGT
NotI-GhMYB2-R	ACTGCTCGAGTTATACCATTGCTAATGGATCC
GL1 DdeI-F	GTTAGAAACGTTTTCCACAATTG
GL1-DdeI-R	ATCTGTTCTTCCCGGTACTCTTTTAGCTATCTAA
GL3-EMS-F	AGGGCAAATTCAAGAGCAAC
GL3-EMS-R	AGGGCAAATTCAAGAGCAAC
TTG1-EMS-F	TATGCCTGTTGCTGAGCTTG
TTG1-EMS-R	AATCAGGCTGCGAAGAAGAC
EGL3-ASCI-R	GGCCTTAATTAATTAACATATCCATGCAACCCTTTG
LB-R	CACTGGCCGTCGTTTTACAACG
EGL3-ASCI-F	GCTTGGCGCGCCATGGCAACCGGAGAAAACAG
GL1_CRISPR_Forward	GGAAAAGTTGTAGACTGAGAGTTTTAGAGCTAGAAATAGCAAGTT
GL1_CRISPR_Reverse	TCTCAGTCTACAACTTTTCCAATCACTACTTCGACTCTAGCTGTA

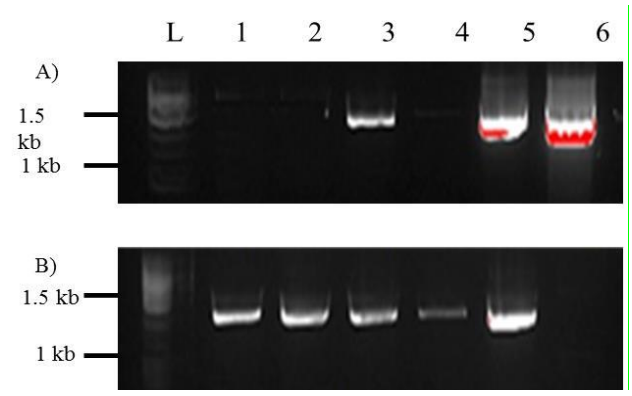

Fig. 2. Genotyping *egl3- 77439* mutant alleles. A: PCR-based with gene-specific primers, B: PCR-based with T-DNA specific primers. From left to right: lane 1 to 3: samples; lane 4: homozygous for mutant allele; lane 5: heterozygous mutant/wild type allele; lane 6: homozygous for wild type allele.

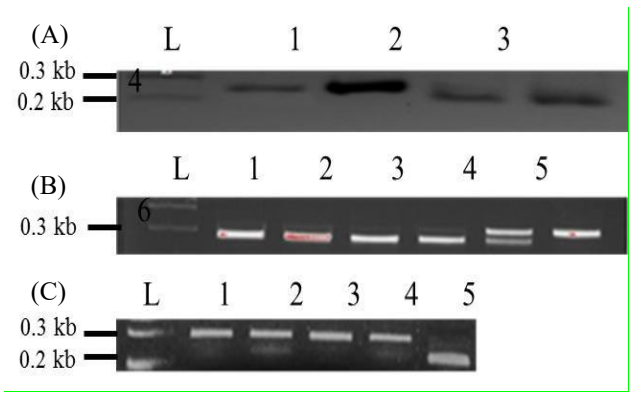

Fig. 3. A: PCR-based genotyping *gl1-1* mutant allele, lane 1: sample, lane 2: sample with homozygous for the mutant allele, lane 3: sample with heterozygous mutant/wild type allele, lane 4: sample with homozygous wild type allele. B: PCR-based genotyping *ttg1-1* mutant allele, lane 1-3: samples, lane 4: sample with homozygous for the mutant allele, lane 5: sample with heterozygous mutant/wild type allele, lane 6: sample with homozygous wild type allele. C: PCR-based genotyping *gl3-1* mutant allele, lane 1-2: samples, lane 3: sample with homozygous for the mutant allele, lane 4: sample with heterozygous mutant/wild type allele, lane 5: sample with homozygous wild type allele.

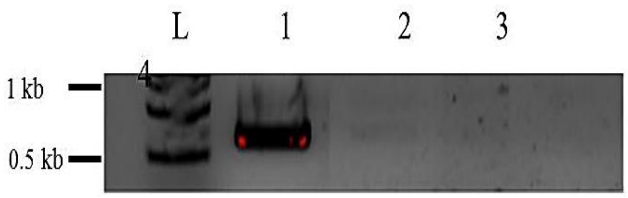

Fig. 4. Genotyping MYB2 transgene in the *gl3-1 egl3-77439 ttg1-1 gl1-1* quadruple mutant. From left to right: lane 1 to 4: samples. Lane 1 contains the MYB2 construct, lane 2: wild type.

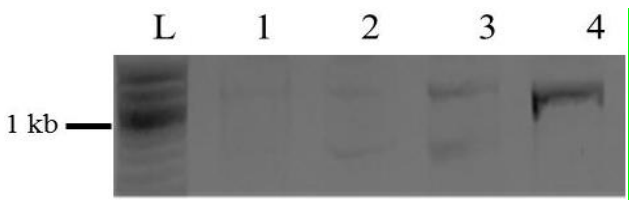

Fig. 5. Genotyping the T1 generation of triple mutant *gl1*-CRISPR/*gl3-1 egl3- ttg1-1*. From left to right: Lane 1 to 3: samples, lane 4: wild type. Lane 3 contains the *g11*-CRISPR construct.

In Cleaved Amplified Polymorphic Sequences analysis of *gl3-1*, *ttg1*-1, and *gl1-1-1* mutant alleles, PCR products were amplified which harbored the mutation sites of *gl3-1*, *ttg1-1*, and *gl1-1* (Fig. 3). A mutation in the *gl3-1* mutant allele that modified codon 378, CAG, to a stop codon TAG by a transition from cytosine to thymine, resulted in the deleted entire bHLH region of the encoded protein. A mutation identified in *ttg1-1* mutant allele that converted codon 371, from glutamine to a stop codon, generated a truncated TTG1 protein. In *gl1-1* mutant allele, single-nucleotide mutation at position 1149 of the GL1 locus led to a protein sequence modification of the SANT superfamily which is responsible for DNA binding activity.

After obtaining the F2 homozygous *gl3-1 egl3-77439 ttg1-1 gl1-1/35S: MYB2-A*, we let the quadruple mutants with MYB2 gene self-fertilized and finally get two quadruple mutant *gl3-1 egl3-77439 ttg1-1 gl1-1* plants. By carrying out PCR with primers *Xho*I-*GhMYB2*-F/ *Not*I-*GhMYB2*-R. Our results indicated that our quadruple mutants did not contain the MYB2 gene (Fig. 4).

Construction and confirmation of the *gl3-1 egl3-77439 ttg1-1 gl1-1* mutant from CRISPR approach: Targeted genome modification using CRISPR-Cas9 system has provided a means to rapidly edit the genomes in an accurate and calculable fashion. The CRISPR-associated protein 9 (Cas9) system is a newly established approach that introduces site-specific mutations on doubles- stranded DNA. With its huge potential and application, the CRISPR/Cas9 system has been utilized in many plants including *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Oryza sativa*, *Zea mays*, and *Citrus sinensis* (Bortesi & Fischer, 2015). Here, we alternatively report another method in constructing the quadruple mutant by disrupting the fourth gene GL1 in the triple mutant *gl3-1 egl3-77439 ttg1-1*. This dual approach was conducted simultaneously with the genetic cross between *gl3-1 egl3-77439 ttg1-1* and 35S:MYB2-A/*gl1-1* to ensure that we will obtain the quadruple mutant *gl3-1 egl3-77439 ttg1-1 gl1-1* by genetic approach or by CRISPR-Cas9 approach. A 20bp target disruption region in the second exon of the GL1 gene containing the *Dde*I restriction site was chosen correlating with the SANT superfamily domain, which is a putative DNA binding domain (Hahn *et al*., 2017). After harvesting the T_1 seeds from the transformation of *gl3-1 egl3-77439 ttg1*-1 with pMDC43-2NLSCas9:*GL1* construct, we selected them on MS media with antibiotic Hygromycin. As the pMDC43-2NLSCas9:*GL1* harboring the Hygromycin resistance gene, surviving plants were transplanted to soil and subjected to genotyping process (Fig. 5). Seed from triple mutant *gl3-1 egl3-77439 ttg1-1* plants putatively containing the pMDC43-2NLSCas9:*GL1* construct will be harvested and germinated MS media with antibiotic Hygromycin for the T_2 generation.

To determine the resistant plants containing the pMDC43-2NLSCas9:*GL1* construct, we employed the "Determining Genome Targeting Efficiency using T7 Endonuclease I" by New England Biolabs protocol. After amplification, the regions harboring the disrupted target for GL1, the PCR products from transgenic plants were digested while the PCR products from the wild type will be resistant to digestion (Fig. 5).

Conclusion

Our comprehensive goal is to create the cotton trimeric complex in quadruple mutant *gl3-1 egl3-77439 ttg1-1 gl1-1*. The first step in creating a cotton trimeric complex in Arabidopsis is to deplete the Arabidopsis trimeric complex which can be achieved by generating a quadruple mutant. By successfully generating quadruple mutant lines *gl3-1 egl3-77439 ttg1-1 gl1-1* with MYB2 from cotton diploid genome A, this novel tool will address the fundamental discrepancies in cotton fiber initiation pattern and Arabidopsis trichome initiation pattern. The genotyping results confirmed quadruple mutants *gl1gl3egl3ttg1* showed normal growth and development. The results from understanding the pattern formation between Arabidopsis and cotton in future studies could facilitate the cotton industry on fiber initiation mechanism.

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