CRISPR/Cas9-mediated targeted mutagenesis in upland cotton (*Gossypium hirsutum* L.) to validate gene function and for practical applications

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The CRISPR/Cas9 system, derived from prokaryotic defense mechanism, has been adapted to perform genetic alterations at specific target sites in the genome of plants and animals. CRISPR/Cas9-mediated mutagenesis, especially to knock out gene function by generating insertions and/or deletions, represents the simplest and most widely practiced version of the technology. It has become an important tool to conduct functional genomics research and to engineer useful traits in crop plants. Cotton is not only the largest source of natural fiber, but its seeds also serve as valuable byproducts in the form of food, feed and biofuel. Considering the constraints related to its production and the wide variety of products obtained from the cotton plant, it offers several targets for improvement through gene editing. My laboratory has established an efficient CRISPR/Cas9-based system to knock out gene function in cotton and used it to validate the role of two native cotton genes. It was also used to mutate and disrupt the function of an antibiotic resistance gene integrated into the genome of a deregulated, Ultra-low Gossypol Cottonseed (ULGCS), RNAi event. A Green Fluorescent Protein (GFP)-expressing cotton event, with a single integrated transgene copy, generated previously in the laboratory was used to assess the efficacy of the CRISPR system because the disruption of this gene can be easily visualized during the course of tissue culture and regeneration. Three target sites were selected within the coding sequence of *GFP* and following transformation, the timing and extent of silencing were monitored by following the loss of GFP fluorescence. Results from this study revealed the importance of selecting an appropriate target site within the gene for efficient editing. In a separate study to identify genes involved in the development of glands, we conducted RNAseq analysis on the developing embryos of Near Isogenic Lines of glanded and glandless cotton plants, followed by Virus-induced gene silencing. These studies revealed three genes as potential candidates that were designated as Cotton Gland Formation (*CGF1*, *CGF2* and *CGF3*) genes. Targeting of *CGF2* and *CGF3* with the CRISPR system resulted in abnormal gland development and complete elimination of glands, respectively. Cas9-expression cassette was segregated out in the progeny of the CGF3-knockout lines, but the glandless trait was maintained. The results demonstrated efficacy of the CRISPR/Cas9 system to mutate both sets of homoeologous alleles in an allotetraploid. On a different project, an antibiotic resistance gene (*nptII*), used for the selection of a deregulated ULGCS event (TAM66274), was targeted using the CRISPR system. The objective was to allay public concerns regarding the use of a functional antibiotic-resistance gene in a crop plant. One of the two regenerants obtained from these knockout experiments showed a 4-bp deletion close to the 5'-end of the *nptII* coding sequence resulting in a premature stop codon. Cotyledon explants from the progeny of this line failed to show any callus growth when cultured on a medium containing 200 ppm kanamycin, thus confirming the disruption of the *nptII* coding sequence and loss of its

function. PCR analysis on the T1 seedlings of this line showed absence of the Cas9 expression cassette in some of the progeny as per Mendelian segregation. Gossypol analysis on T2 seeds from one such T1 parent lacking the Cas9 cassette and a non-functional *nptII* gene showed that the ULGCS trait observed in the parental TAM66274 was maintained in the progeny of the knockout line. Overall, our results suggest that *Agrobacterium*-mediated introduction of the CRISPR reagents in cotton cells followed by regeneration of the knockout lines and elimination of the Cas9 expression cassette in the T1 progeny is a viable means to obtain desired mutations while retaining a non-GMO status for the mutated events. Advances and refinements, such as base editing, prime editing, homology-based gene targeting, viral-based delivery of CRISPR reagents, tissue culture- and transgene-free editing, are underway in many laboratories around the globe to improve and refine the CRISPR-related biotechnologies. With their ability to introduce genetic modifications in a highly precise and selective manner, these innovations offer endless possibilities to engineer desirable traits in cotton and other crops.