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Eliminating Gossypol in a Cotton Plant: RNAi-mediated Selective Gene Silencing Versus CRISPR-mediated Gene Knockout

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ABSTRACT

RNAi-mediated silencing of target native gene(s) and CRISPR-mediated, targeted knockout of a gene represent two powerful tools to study gene function and to engineer useful traits in plants. Gossypol, present in most parts of the cotton plant, serves as a defense chemical against certain pests and pathogens. Gossypol and related terpenoids are synthesized and stored in lysigenous glands present in most aboveground parts of the plant. However, gossypol's presence also limits the suitability of the seeds for human and animal nutrition. Selective, seed-specific RNAi-mediated silencing of the members of a gene family encoding δ-cadinene synthase, which catalyzes a key step in gossypol biosynthesis, reduced gossypol levels by 97% exclusively in the seeds. One such ultra-low gossypol cottonseed (ULGCS) line, TAM66274, has been deregulated in the U.S. and its seeds are considered safe for consumption as food and as feed for animals. Although CRISPR/Cas9-mediated knockout of the *CGF3* gene, a key regulator of gland development, in a cotton plant eliminated the glands and their contents including gossypol in the seeds and other aboveground parts of the plant, it could not achieve the specificity of selective RNAi silencing. Thus, although it is possible to achieve seed-specific silencing of the target gene(s) using RNAi, it is not possible to eliminate their expression. In contrast, using the CRISPR system it is possible to knock out a target gene and its function entirely, tissue-specificity is difficult to obtain. Our investigations revealed both the positive attributes and limitations of RNAi and CRISPR/Cas9 technologies.

Cotton is the largest source of natural fiber, important to the economy of several developed and developing countries. It has been cultivated for its fiber for several millennia by ancient civilizations on different continents. Presently, it is grown in more than 80 countries on five continents, with India, China, U.S., Brazil, Pakistan, Turkey, and Uzbekistan being the top seven producers (FAOSTAT, 2021). The plant not only provides spinnable fiber, but also produces large amounts of protein- and oil-rich seeds. Accounts in ancient Asian literature describe the use of cottonseed oil for lighting with the remaining meal as feed for ruminant animals (Harden, 1975; Ramanatha, 1962). That these important byproducts of the cotton crop were not used directly for human nutrition but for other purposes indicate that an awareness of the toxic nature of the seeds has existed for millennia. The cause of cottonseed toxicity is the presence of gossypol, a terpenoid that is produced and stored in lysigenous glands in the seed (Boatner et al., 1947; Lusas and Jividen, 1987; Withers and Carruth, 1918). Although the terpenoid present in the seeds and flower petals is largely gossypol, other parts of the cotton plant contain gossypol and several other terpenoids that are derived from the same basic biosynthesis pathway. Root terpenoids are present in the epidermis (including root hairs) and cortex (Bell and Stipanovic, 1978; Mace et al., 1974); however, these are synthesized and stored in the distinct lysigenous glands in the aboveground parts of the cotton plant (Pandeya et al., 2023; Stanford and Viehoever, 1918). Roots of a cotton plant contain gossypol, gossypol-6-methyl ether, gossypol-6,6-dimethyl ether, hemigossypol, desoxyhemigossypol, hemigossypol-6-methyl ether, and desoxyhemigossypol-6,6-methyl ether, whereas the green parts of the cotton plant, including leaves, stem, bracts and boll rind contain gossypol, hemigossypolon, and heliocides in their glands (Bell and Stipanovic, 1978; Scheffler, 2016; Stipanovic et al., 1988; Sunilkumar et al., 2006). In addition to their constitutive presence, terpenoids are induced in response to insect herbivory and microbial infections (Hagenbucher et al., 2019; Stipanovic et al., 1999).

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These terpenoids serve as a defensive function and provide some degree of protection against insect pests and certain diseases.

The cotton plant produces approximately 1.6 times more seeds than fiber in terms of weight. Worldwide, 25.4 million metric tons of lint and 41.6 million metric tons of cottonseed were produced in 2021 (FAO-STAT, 2021). Cottonseed has approximately 23% protein content and 21% oil content. Although gossypol is easily removed during oil processing, the remaining meal containing relatively good quality protein serves as feed for ruminants. Adult ruminants are much more tolerant of the gossypol present in the feed compared to young calves, monogastric animals, and humans in which it causes heart and liver damage (Gadelha et al., 2014; Risco and Chase, 1997). There is enough protein present in the annual global cottonseed output to meet the basic protein requirements (50 g/day/person) of approximately 500 million people if used directly for human nutrition. However, the presence of gossypol in the seed (~10,000 ppm) relegates this enormous resource as feed for ruminants that are highly inefficient in converting feed protein into edible animal protein. In contrast to beef cattle with a protein conversion ratio of 20, the protein conversion ratio for swine, chicken, tilapia, salmon, shrimp, and hen eggs is 5.7, 4.7, 5.7, 4.6, 7.7, and 2.6, respectively (Boyd et al., 2005; Rathore et al., 2020; Tilman and Clark, 2014). The glandless mutant cotton cultivated by Native Americans in the Hopi region of Arizona was discovered by a cotton breeder (McMichael, 1954, 1959). Because the plant was glandless, the aboveground parts of the plant, including the seeds, were gossypol-free. This exciting discovery provided possible means to use cottonseed protein with greater safety and efficiency. Many breeding programs were launched to exploit this seemingly useful trait and the glandless cottonseed was used to conduct nutritional studies from the 1960s through the 1980s. These trials confirmed that glandless cottonseed meal was suitable for consumption by monogastric animals and as food to improve human nutrition (Lusas and Jividen, 1987; Rathore et al., 2020). However, the absence of glands diminished the constitutive protection conferred by the terpenoids, and under field conditions, these cotton plants suffered greater damage from traditional and non-traditional cotton pests resulting in lower yields (Bottger et al., 1964; Jenkins et al., 1966; Lukefahr et al., 1966; Rathore et al., 2020). Thus, although human and animal nutrition studies confirmed the nutritive value of the cottonseed protein, commercial cultivation of the glandless cotton proved too risky, and has been largely abandoned.

RNAi FOR SEED-SPECIFIC ELIMINATION OF GOSSYPOL IN COTTON

The advent of plant biotechnology, especially genetic modification in the 1980s, brought new tools to bear upon the age-old problem of cottonseed toxicity. Beginning in 1996, researchers in my laboratory sought to use seed-specific silencing of the δ-cadinene synthase gene(s) in cotton. We isolated and characterized α-globulin B gene promoter from cotton and found it to be active only in the seed (Sunilkumar et al., 2002). Gossypol and other sesquiterpenoids are derived from δ-cadinene. δ-cadinene synthase catalyzes the first step in the cyclization of farnesyl diphosphate to δ-cadinene, and therefore the gene encoding this enzyme presented an attractive target for silencing using antisense technology. Antisense constructs with δ-cadinene synthase gene under the control of α-globulin B promoter were assembled and introduced into cotton via *Agrobacterium*-mediated transformation. Although T1 seeds from some antisense lines were found to have reduced gossypol levels, T2 seeds from most of the lines did not maintain the low levels of gossypol present in the previous generation of seeds (Rathore et al., 2012). An understanding of the mechanism of RNA-interference (RNAi, also referred to as post-transcriptional gene silencing) in the late 1990s offered a more powerful tool to silence a target gene. Although δ-cadinene synthase gene is a member of a multi-gene family, there was enough sequence homology among the members that allowed us to design a trigger sequence to assemble a hairpin RNAi construct driven by the α-globulin promoter. Forty-one fertile RNAi lines were generated and analysis of pooled T1 seeds (segregating for the transgene) from each line showed varying levels of gossypol (Sunilkumar et al., 2006). Seed-gossypol levels ranged from no reduction to as much as 90% reduction. Three lines were examined in detail over two generations. The transgene along with the low-gossypol trait cosegregated in the individually analyzed T1 generation seeds. The T2 generation seeds from a homozygous T1 parent showed approximately 94% reduction in seed-gossypol levels. Importantly,

this RNAi-mediated trait was completely seed specific; other organs of the RNAi lines showed levels of gossypol and related terpenoids similar to that of the wild-type, non-transgenic controls. Nine promising RNAi lines were grown under greenhouse conditions for five generations and their seeds were examined for gossypol levels (Rathore et al., 2012). Unlike the antisense lines, the RNAi-mediated trait was found to be stable over generations. These studies were followed by three newer RNAi lines under field conditions over three years (Palle et al., 2013). The ultra-low gossypol cottonseed (ULGCS) trait was found to be stable under field conditions. As expected, the floral bracts, floral buds, terminal portion of the axillary branch, leaves, 2-day-old bolls, and flower petals of the ULGCS lines showed no diminution in the levels of gossypol and other terpenoids. Other parameters evaluated were fiber and seed yield, seed protein and oil content, and fiber quality. No reductions in any of these parameters were observed in the ULGCS lines. Encouraged by these results, the potential sustainability of cotton production, and the promise of higher value of ULGCS positively impacting cotton farmers' incomes, Cotton Inc. funded further development and deregulation of the ULGCS trait. During the second phase, hundreds more lines were generated and screened for the ULGCS trait. Following initial screening of these lines for the seed gossypol levels, generational stability of the trait and small-scale field trials, one line (TAM66274) was selected for regulatory field trials for agronomic performance in multiple states over multiple years and was also subjected to biochemical, molecular, and genetic analyses. These studies established the equivalence of TAM66274 to the non-transgenic parental controls with the exception that the RNAi line had ultra-low levels of gossypol in the seeds (~350) ppm. This level is well below the safe limits established by the Federal Department of Agriculture (450 ppm) and World Health Organization (600 ppm) in food products (Lusas and Jividen, 1987). Regulatory approvals were granted for the TAM66274 line by the U.S. Department of Agriculture-Animal and Plant Health Inspection Service in 2018 (USDA APHIS, 2018) and the Food and Drug Administration in 2019 (FDA, 2019). Thus, creation of TAM66274 by silencing a target gene family encoding an important step in gossypol biosynthesis in a seed-specific manner also serves as a powerful example of the utility of RNAi in selective metabolic engineering.

CRISPR-MEDIATED GENE KNOCKOUT TO ELIMINATE GOSSYPOL IN THE COTTON PLANT

The CRISPR/Cas9 system adapted from a prokaryotic defense mechanism has become the most popular tool for gene editing, with knockout of a target gene being the simplest and most widely used application to obtain specific traits in plants. It is used either to introduce new traits or validate gene function. Our unpublished results from RNAseq analysis on developing cotton embryos and other tissues from near isogenic glanded and glandless cotton plants showed that several members of the δ-cadinene synthase gene family were expressed in the glanded embryos. Therefore, knocking out any one or all of them was unlikely to result in seed-specific elimination of gossypol. In a related project to identify the genes responsible for gland development, we identified 11 potential candidate genes using RNAseq analysis on developing embryos (14 d post anthesis) from glanded and glandless cotton plants (Janga et al., 2019). Virus-induced gene silencing targeting of each showed that silencing three of these genes either reduced the number of glands or resulted in their abnormal development in the newly emerging leaves. The results indicated the importance of these three genes in the development of glands in cotton and were designated cotton gland formation genes: *CGF1*, *CGF2*, and *CGF3*. We targeted *CGF2* and *CGF3* genes individually using the CRISPR system to determine the possibility of knocking out all four alleles of the target gene in tetraploid cotton. Further, we wanted to ascertain whether the knockout of all alleles of each of these genes would result in the elimination of glands and therefore gossypol. The number of glands in the leaves of *CGF2* knockout (*CGF2*-KO) lines were reduced significantly, concomitant with approximately 90% reduction in gossypol level and almost complete elimination of other leaf terpenoids (hemigossypolon and heliocides). Moreover, the glands that were present in the leaves of *CGF2* lines were smaller and appeared abnormal. *CGF3*-KO lines had no visible glands in any part of the plant and a complete absence of gossypol in the seeds (Janga et al., 2019). Thus, CRISPR-mediated gene knockout proved to be efficacious in validating gene function in an allopolyploid plant. As mentioned earlier, we did not find a member of δ-cadinene synthase gene family that was exclusively expressed in the seed,

therefore, this gene is not suitable as a CRISPR-target if seed-specific elimination of gossypol is the desired trait. Although it could be possible to use CRISPR-interference for seed-specific suppression of a target gene, such as the *CGF3* gene, it undermines the most attractive feature of CRISPR-mediated gene editing, that is, avoiding the genetically modified organism label for wider public acceptance and lesser regulatory scrutiny.

CONCLUSIONS

The development of ULGCS makes available a hitherto untapped protein resource for improving nutrition security without additional input or land use and therefore makes cultivation of cotton highly sustainable. The two examples provided in this paper provide elegant demonstrations of the power of genetic engineering tools in the creation of novel traits in crops and help us overcome some of the limitations of plant breeding. Each of the two technologies are precise and if carefully used, can avoid off-target or adverse effects. Each technology has its strengths and weaknesses. RNAi can be used to target closely related members of a multi-gene family and more importantly, to obtain silencing in a tissue-specific manner, but it cannot provide 100% silencing of the target gene(s). In contrast, gene editing can completely knock out the function of a target gene, but it cannot easily provide tissue-specificity. However, tremendous progress is being made in further refining and diversifying the uses of these technologies, especially the CRISPR-based systems. It might become possible to use CRISPR to obtain tissue-specificity in the future. It is hoped that the regulatory bodies in every country recognize the potential of such new technologies and permit their deployment to enhance agricultural productivity in an environmentally sustainable manner and to improve the nutritional status of their populace.

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