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VALIDATION OF THE EXPRESSION PATTERNS OF THE (+)-DELTA-CADINENE SYNTHASE GENE FAMILY IN COTTON (*GOSSYPIUM HIRSUTUM* L.) CV. COKER-312 GENOTYPE

B.N. Prashantha[1], I.S. Katageri[1]*, Basavaraj Bagewadi[2] and B.R. Patil[1]

[1]Department of Genetics and Plant Breeding, College of Agriculture, UAS, Dharwad - 580 005, Karnataka, India.

[2]Department of Biotechnology, University of Agricultural Sciences, Dharwad - 580 005, Karnataka, India.

*Corresponding author E-mail : ikatageri@gmail.com

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ABSTRACT

Gossypol, a terpenoid aldehyde commonly found in cotton (*Gossypium hirsutum* L.), is essential for the plant's defense against pests and pathogens. However, its inherent toxicity limits the use of cottonseed in food and feed applications. This study focused on validating the expression patterns of the (+)-delta-cadinene synthase gene family, which plays a crucial role in gossypol biosynthesis. The goal was to use this information to guide genome editing strategies aimed at reducing gossypol levels in cottonseed. Using quantitative real-time PCR (qRT-PCR), we analyzed the expression of 32 (+)-delta-cadinene synthase genes in ovules and leaves across six developmental stages, spanning from 20 to 45 days post-anthesis (DPA) at five-day intervals. Our results revealed that 10 genes were expressed in ovules irrespective of the developmental stages. Among these, six genes: Gohir.A04G023700, Gohir.D05G363800, Gohir.A08G087000, Gohir.D05G363900, Gohir. D05G364000 and Gohir. D05G364300, consistently exhibited significantly higher expression levels across various stages. Notably, Gohir.D05G363900, Gohir.D05G364000 and Gohir.D05G364300 demonstrated slightly higher expression levels across all stages, making them suitable candidates for targeted genome editing. These findings provide valuable insights into the expression dynamics of the (+)-delta-cadinene synthase gene family and identify potential target genes for future genome editing experiments aimed at enhancing the utilization of cottonseed by reducing gossypol content.

Key words : Gossypol, Cotton, (+)-delta-cadinene synthase, Gene expression, Genome editing.

Introduction

Glands are distributed throughout the cotton plant (*Gossypium hirsutum* L.), including in the seeds. These glands produce gossypol and other terpenoid aldehydes that serve as a defense mechanism against pests and pathogens (Bell and Stipanovic, 1977; Scheffler *et al*., 2012). Cottonseed offers significant amounts of oil (21%) and high-quality protein (23%) (Lusas and Jividen, 1987). However, the value of cottonseed as a by-product of fiber production is limited by the presence of gossypol. Gossypol, the primary terpenoid aldehyde in cottonseed glands, is toxic to non-ruminant animals and humans, restricting its use mainly to cattle feed since ruminants can tolerate its effects (Kim *et al*., 1996; Santos *et al*., 2003). For non-ruminants such as chickens, pigs, and fish, gossypol can hinder weight gain and impair reproductive capabilities (Randel *et al*., 1992).

Several strategies have been explored to mitigate the anti nutritional effects of gossypol in cottonseed. Initially, efforts focused on utilizing naturally occurring glandless mutants (McMichael, 1954, 1959, 1960; Lee *et al*., 1968; Hess, 1977; Kohel, 1979; Endrizzi *et al*., 1985). However, these glandless varieties proved commercially unviable due to their increased susceptibility to diseases and insect attacks, as well as the requirement for seed isolation (Hess, 1977; Kohel, 1979; Endrizzi *et al*., 1985; Lusas

and Jividen, 1987). Alternative methods involved mechanical processes (Damaty and Hudson, 1975; Gardner *et al*., 1976; Mayorga *et al*., 1975) and chemical treatments (Pons and Eaves, 1971; Hron and Kuk, 1989; Hron *et al*., 1994; Kuk and Hron, 1998) to extract gossypol from cottonseed products. These methods were not only costly but also reduced the nutritional value of the resulting cottonseed meal and failed to be commercially viable (Frank, 1987; Lusas and Jividen, 1987).

Another notable approach was the introgression of delayed gland morphogenesis traits from Australian wild diploid species into cultivated tetraploid cotton through tri-specific hybridization. However, the inter-specific offspring often displayed reduced pollen fertility, shorter branches, and lower fiber quality compared to commercial upland cotton (Dilday, 1986; Brubaker *et al.*, 1996; Fryxell, 1965; Muramoto, 1969; Bi *et al*., 1998; Altman *et al*., 1987; Rooney *et al*., 1991; Liu *et al*., 2015; Tang *et al*., 2018). The difficulty in maintaining genetic stability across successive generations led most research programs to abandon this approach by the mid-1990s (Dilday, 1986; Zhu and Ji, 2001; Zhu *et al*., 2005).

A significant breakthrough in cotton biotechnology was achieved by Rathore's team at Texas A&M University. Utilizing RNA interference (RNAi) under a seed-specific promoter, they successfully developed ultra-low gossypol cottonseeds (ULGCS) by targeting the (+)-delta-cadinene synthase gene (Sunilkumar *et al*., 2006; Rathore *et al*., 2007; Rathore *et al*., 2012; Palle *et al*., 2013). The release of TAM66274, a cotton variety with significantly reduced gossypol levels approved for human and animal consumption, marks a notable advancement in the field (Rathore *et al*., 2020). However, challenges persist due to stringent global regulations and public acceptance, especially since ULGCS are categorized as transgenic products. In India, only Bt transgenic cotton is approved for commercial cultivation, and no other transgenic crops have been approved for food use. Importing ULGCS from the USA to India involves complex government procedures and regulatory approvals. Given these challenges, alternative strategies are needed.

With advancements in genome sequencing and CRISPR-Cas9 technology, genome editing has proven highly efficient and precise in many crops, including rice (Xu *et al*., 2014; Shan *et al*., 2014), wheat (Shan *et al*., 2014), corn (Liang *et al*., 2014), tomato (Ron *et al*., 2014) and sorghum (Jiang *et al*., 2013; Bortesi and Fischer, 2015; Rinaldo and Ayliffe, 2015). This technology has also been successfully applied in cotton (Chen *et al*., 2017; Gao *et al*., 2017; Janga *et al*., 2017). A significant benefit of the CRISPR-Cas9 system is its ability to develop transgenic products that can eventually be rendered non-transgenic through plant breeding segregation principles. To utilize this genome editing technology to knock out gossypol in seeds while maintaining it in foliar tissues, a better understanding of the expression patterns of different (+)-delta-cadinene synthase gene families during seed development stages is required. There are more than 30 (+)-delta-cadinene synthase gene family members in cotton, and the precise selection of those expressing in seeds is crucial to avoid off-target effects and subsequent consequences.

The aim of this study was to identify and validate the expression patterns of different (+)-delta-cadinene synthase gene families in the Coker-312 genotype. This genotype will be used for genetic transformation, and validating these genes will help pinpoint specific genes with the highest expression levels in seeds, which is crucial for our targeted genome editing experiments. Therefore, the present study conducted to identify the (+)-delta-cadinene synthase genes that need to be knocked down to reduce gossypol synthesis in seeds.

Materials and Methods

Plant material

Cotton (*Gossypium hirsutum* L. cv. Coker-312) plants were grown under controlled greenhouse conditions. The biological samples of ovules and leaves were collected at six developmental stages: 20, 25, 30, 35, 40 and 45 days post-anthesis (DPA).

Total RNA extraction

The process of extracting total RNA from leaf tissues and ovules across six different seed developmental stages (20 DPA to 45 DPA) began with immediate storage at -80°C to preserve RNA integrity. The protocol for total RNA isolation using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich) was followed per the manufacturer's instructions. The purity and integrity of RNA were assessed via denaturing gel electrophoresis (formaldehyde agarose gel) and quantified using a spectrophotometer (Nanodrop, Thermo Scientific). Only high-quality total RNA with an $OD_{260/280}$ ratio between 2.0 and 2.2 was used in subsequent steps.

DNase treatment

Total RNA isolated from different tissues was subjected to RNase-free DNase (Ambion, USA) treatment to ensure the removal of any residual DNA that might interfere with downstream expression analysis. Briefly, RNase-free DNase was added to the total RNA,

and the reaction was incubated at 37°C for 30 minutes, followed by the addition of 1X inactivation buffer. The reaction was then incubated at room temperature (RT) for 5 minutes with intermittent tapping. The samples were centrifuged at 10,000 rpm for 5 minutes at RT and the supernatant was carefully collected without disturbing the pellet. The supernatant contains DNase-free RNA. The RNA quality was checked again using a spectrophotometer and agarose gel electrophoresis and the samples were stored at -80°C until further use.

cDNA synthesis

cDNA was prepared using a Super Script® III first-strand synthesis kit (Invitrogen by life Technologies) according to the manufacturer's protocol. 2 µg of total RNA was used for first-strand cDNA synthesis, along with 1 µL of 50 µM oligo (dT), 1 µL of 10 mM dNTPs, and the total volume was adjusted to 10 µL using DEPC-treated water. The tube was incubated at 65°C for 5 minutes, followed by placing it on ice for at least 1 minute. Then, the following cDNA synthesis mix was prepared by adding each component in the indicated order.

Component	1 Reaction
10X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNaseOUT™ (40 U/µl)	1 µl
SuperScript® III RT (200 U/µl)	1 µl

The 10 µL of the above cDNA synthesis mix were added to each RNA/primer mixture, mixed gently, and incubated for 50 minutes at 50°C. The reactions were terminated by heating to 85°C for 5 minutes and cooling on ice. Reactions were collected via brief centrifugation. Next, 1 µL of RNase H was added to each collected tube, and the tubes were incubated for 20 minutes at 37°C. Finally, the cDNA synthesis reactions were stored at -20°C until further use.

Quantitative real-time PCR analysis

qRT–PCR was performed in optical 72-well plates using a Corbett system (Rotor Gene Q, QIAGEN) and Power SYBR® Green master mix to monitor DNA synthesis. Specific primers for 32 (+)-delta-cadinene synthase genes were designed using NCBI primer blast software. The details of the primers used in the qRT–PCR study are presented in Table 2. All samples were run in four replicates with the following PCR concentrations in a volume of 10 µL: 5 µL SYBR Green master mix (Applied Biosystems, USA), 0.5 µL (5 picomoles) of forward and reverse primers (Euro fins Genomics) and 1 µL of 10X diluted cDNA. The following amplification program was employed: 95 °C for 5 minutes, followed by 40 cycles of 92°C for 15 seconds, annealing at 60°C for 30 seconds and 72°C for 45 seconds. Cotton ubiquitin was used as an internal control and the relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Results

The systematic identification of the (+)-delta-cadinene synthase gene family in cotton (*Gossypium hirsutum* L.) was achieved through extensive genomic analysis using bioinformatics tools. Initially, the Cotton Functional Genome Database (CFGD) provided four whole genome assemblies for *Gossypium hirsutum* (upland cotton): HAU, ZJU, NAU and JGI assemblies. The number of delta-cadinene synthase genes identified varied across these assemblies, with approximately 47 in HAU, 28 in ZJU, 36 in NAU and 33 in JGI. The JGI assembly was selected for detailed analysis, where 33 (+)-delta-cadinene synthase genes were initially identified (Table 1). Sequence analysis through multiple alignments revealed significant genomic differences among 31 of these genes, while the 32nd and 33rd genes were nearly identical, differing by only two nucleotides. Consequently, the 33rd gene was excluded from further research to focus efforts on genes with distinct differences, streamlining the analysis to 32 genes.

Initial examination of RNA-seq data from the Cotton

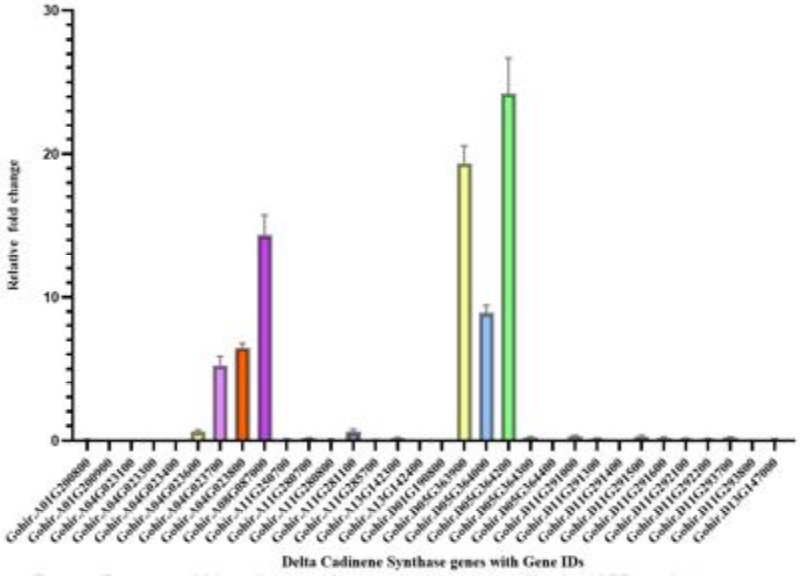

Fig. 1 : Expression of delta cadinine synthase genes at trascriptional level in 20 DPA ovules in comparison to leaf, In X-axis: Delta cadinine synthase genes with their Gene IDs. In Y-axis: level by which more CAD gene was expressed in ovules over leaf in Coker-312 genotypes.

Table 1 : The list of (+)-delta cadinene synthase gene family members in JGI assembly of (*Gossypium hirsutum* L.).

Gene No.	Gene ID	Gene Name	Description	Chromosome	Start	End	Strand
1	Gohir.A01G200800	CAD1-A	(+)-delta-cadinene synthase isozyme A	A01	99,900,667	99,903,174	-
2	Gohir.A01G200900	CAD1-A	(+)-delta-cadinene synthase isozyme A	A01	99,942,998	99,945,419	-
3	Gohir.A04G023100	NA	(+)-delta-cadinene synthase isozyme XC14	A04	3,785,695	3,788,639	+
4	Gohir.A04G023300	NA	(+)-delta-cadinene synthase isozyme XC1	A04	3,876,878	3,879,887	+
5	Gohir.A04G023400	NA	(+)-delta-cadinene synthase isozyme XC14	A04	3,913,890	3,916,683	+
6	Gohir.A04G023600	NA	(+)-delta-cadinene synthase isozyme XC14	A04	4,031,783	4,034,604	-
7	Gohir.A04G023700	NA	(+)-delta-cadinene synthase isozyme XC14	A04	4,131,931	4,134,857	+
8	Gohir.A04G023800	NA	(+)-delta-cadinene synthase isozyme XC1	A04	4,310,541	4,313,526	+
9	Gohir.A08G087000	CAD1-C2	(+)-delta-cadinene synthase isozyme C2	A08	34,440,223	34,443,061	-
10	Gohir.A11G250700	CAD1-A	(+)-delta-cadinene synthase isozyme A	A11	98,152,046	98,154,970	-
11	Gohir.A11G280700	CAD1-A	(+)-delta-cadinene synthase isozyme A	A11	104,849,082	104,853,252	+
12	Gohir.A11G280800	CAD1-A	(+)-delta-cadinene synthase isozyme A	A11	105,095,101	105,104,089	+
13	Gohir.A11G281100	CAD1-A	(+)-delta-cadinene synthase isozyme A	A11	105,214,130	105,217,422	+
14	Gohir.A11G285700	NA	(+)-delta-cadinene synthase isozyme XC14	A11	106,739,259	106,742,977	+
15	Gohir.A13G142300	CAD1-A	(+)-delta-cadinene synthase isozyme A	A13	88,748,467	88,751,160	+
16	Gohir.A13G142400	CAD1-A	(+)-delta-cadinene synthase isozyme A	A13	88,819,082	88,821,671	-
17	Gohir.D01G190800	CAD1-A	(+)-delta-cadinene synthase isozyme A	D01	60,883,452	60,885,957	-
18	Gohir.D05G363900	CAD1-C2	(+)-delta-cadinene synthase isozyme C2	D05	60,156,984	60,160,023	-
19	Gohir.D05G364000	CDN1	(+)-delta-cadinene synthase	D05	60,183,800	60,186,727	-

Table 1 continued....

Table 1 continued....

20	Gohir.D05G364200	NA	(+)-delta-cadinene synthase isozyme XC1	D05	60,221,762	60,224,651	-
21	Gohir.D05G364300	CAD1-C2	(+)-delta-cadinene synthase isozyme C2	D05	60,248,655	60,251,688	-
22	Gohir.D05G364400	NA	(+)-delta-cadinene synthase isozyme XC14	D05	60,325,943	60,328,698	-
23	Gohir.D11G291000	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	63,823,107	63,826,377	+
24	Gohir.D11G291300	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	63,876,646	63,880,403	+
25	Gohir.D11G291400	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	63,882,714	63,885,582	+
26	Gohir.D11G291500	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	63,911,540	63,915,390	+
27	Gohir.D11G291600	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	64,015,315	64,019,003	+
28	Gohir.D11G292100	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	64,156,891	64,159,858	+
29	Gohir.D11G292200	NA	(+)-delta-cadinene synthase isozyme XC14	D11	64,213,905	64,217,223	+
30	Gohir.D11G293700	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	64,586,476	64,590,096	+
31	Gohir.D11G293800	CAD1-A	(+)-delta-cadinene synthase isozyme A	D11	64,594,283	64,597,234	+
32	Gohir.D13G147000	CAD1-A	(+)-delta-cadinene synthase isozyme A	D13	50,869,411	50,872,137	+
33	Gohir.D13G147100	CAD1-A	(+)-delta-cadinene synthase isozyme A	D13	50,898,034	50,900,758	-

Table 2 : The number of (+)-delta-cadinene synthase genes expressed in ovules compared to leaf tissues.

Seed developmental stages	Number of genes expressed in ovules (Fold change value of more than 2)	Range of relative fold change
20 DPA	06	5.25 – 24.21
25 DPA	08	2.05 – 19.47
30 DPA	07	2.75 – 13.50
35 DPA	10	2.00 – 25.16
40 DPA	06	5.90 – 20.60
45 DPA	07	3.05 – 12.20

FGD database indicated widespread expression of these genes throughout nearly all parts of the cotton plant, including various seed developmental stages and different foliar tissues such as leaves, roots, stems and petals. The expression patterns varied significantly across different developmental stages. The expression of these genes was validated in the Coker-312 genotype, intended for subsequent genetic transformation studies. Validation involved RNA extraction from ovules and leaf tissues collected at five-day intervals across developmental stages ranging from 20 to 45 days post-anthesis (DPA). RNA integrity and purity were confirmed via agarose gel electrophoresis and spectrophotometry. Only RNA samples with high integrity and purity were selected for cDNA synthesis and subsequent quantitative real-time PCR analysis.

At 20 DPA, several (+)-delta-cadinene synthase genes exhibited significantly higher expression in ovules compared to leaves (Table 2 and Fig. 1). The fold changes

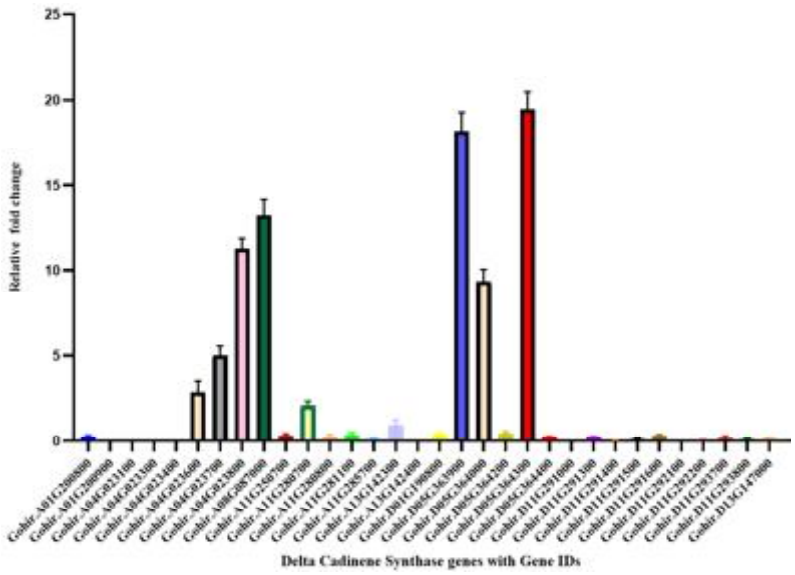

Fig. 2 : Expression of delta cadinine synthase genes at trascriptional level in 25 DPA ovules in comparison to leaf, In X-axis: Delta cadinine synthase genes with their Gene IDs. In Y-axis: level by which more CAD gene was expressed in ovules over leaf in Coker-312 genotypes.

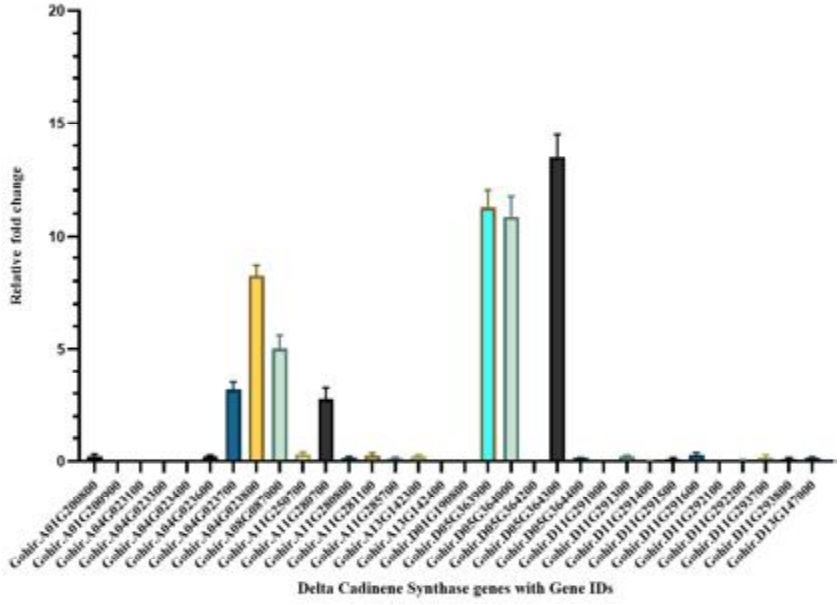

Fig. 3 : Expression of delta cadinine synthase genes at trascriptional level in 30 DPA ovules in comparison to leaf, In X-axis: Delta cadinine synthase genes with their Gene IDs. In Y-axis: level by which more CAD gene was expressed in ovules over leaf in Coker-312 genotypes.

ranged from 5.25 to 24.21. Notably up-regulation was observed in genes Gohir.D05G364200 (24.21-fold), Gohir.D05G363900 (19.36-fold), Gohir.A08G087000 (14.34-fold), Gohir.A04G023800 (6.47-fold), Gohir.D05G364000 (8.92-fold) and Gohir.A04G023700 (5.25-fold). The highest fold change was observed in Gohir.D05G364200 (24.21-fold) and the lowest in Gohir.A04G023700 (5.25-fold).

At 25 DPA, the (+)-delta-cadinene synthase genes continued to show significant up-regulation in cotton ovules relative to leaves, with fold changes ranging from 5.02 to 19.47 (Table 2 and Fig. 2). The expression levels of these genes were substantially higher, with Gohir.D05G364300 (19.47-fold), Gohir.D05G363900 (18.18-fold), Gohir.A08G087000 (13.25-fold), Gohir.A04G023800 (11.26-fold), Gohir.D05G364000 (9.33-fold) and Gohir.A04G023700 (5.02-fold). The highest fold change was observed in Gohir.D05G364300 (19.47-fold) and the lowest in Gohir.A04G023700 (5.02-fold).

Quantitative real-time PCR analysis at 30 DPA revealed sustained up-regulation of (+)-delta-cadinene synthase genes in ovules compared to leaves, with fold changes ranging from 2.00 to 13.50 (Table 2 and Fig. 3). Gohir.D05G364300 (13.50-fold), Gohir.D05G364000 (10.86-fold), Gohir.D05G363900 (11.26-fold), Gohir.A04G023800 (8.24-fold), Gohir.A04G023700 (3.18-fold), Gohir.A11G280700 (2.75-fold) and Gohir.A08G087000 (2.76-fold) exhibited significantly higher expression levels in ovules. The highest fold change was observed in Gohir.D05G364300 (13.50-fold) and the lowest in Gohir.A08G087000 (2.76-fold).

At 35 DPA, the (+)-delta-cadinene synthase genes displayed diverse expression patterns, with fold changes ranging from 2.08 to 25.16 (Table 2 and Fig. 4). Several genes showed significant up-regulation in ovules. Specifically, Gohir.A04G023800 (25.16-fold), Gohir.D05G364300 (17.84-fold), Gohir.D05G363900 (12.08-fold), Gohir.A08G087000 (9.49-fold), Gohir.D11G291300 (3.29-fold), Gohir.A11G250700 (2.49-fold), Gohir.A04G023300 (2.59-fold), and Gohir.D11G291000 (2.08-fold) exhibited significantly higher expression levels in ovules. The highest fold change was observed in Gohir.A04G023800 (25.16-fold) and the lowest in Gohir.D11G291000 (2.08-fold).

At 40 DPA, significant differential expression of (+)-delta-cadinene synthase genes was observed, with fold changes ranging from 5.91 to 20.60 (Table 2 and Fig. 5). Gohir.D05G364300 (20.60-fold), Gohir.D05G363900

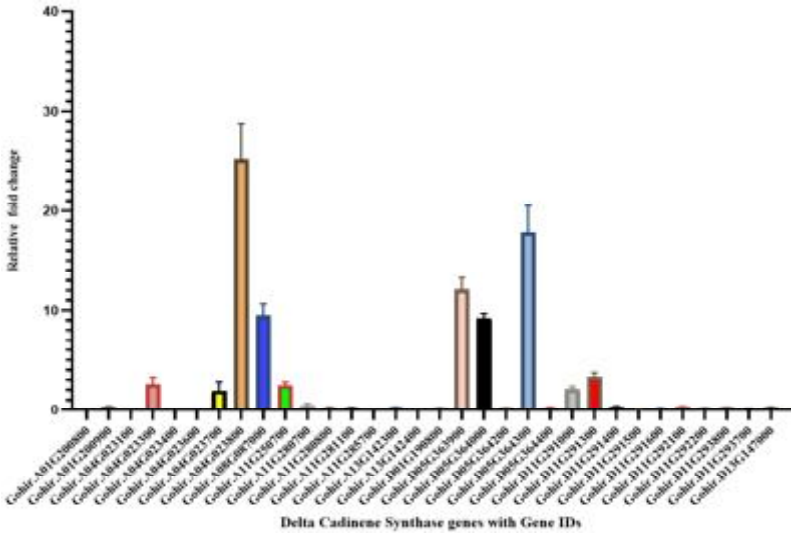

Fig. 4 : Expression of delta cadinine synthase genes at trascriptional level in 35 DPA ovules in comparison to leaf, In X-axis: Delta cadinine synthase genes with their Gene IDs. In Y-axis: level by which more CAD gene was expressed in ovules over leaf in Coker-312 genotypes.

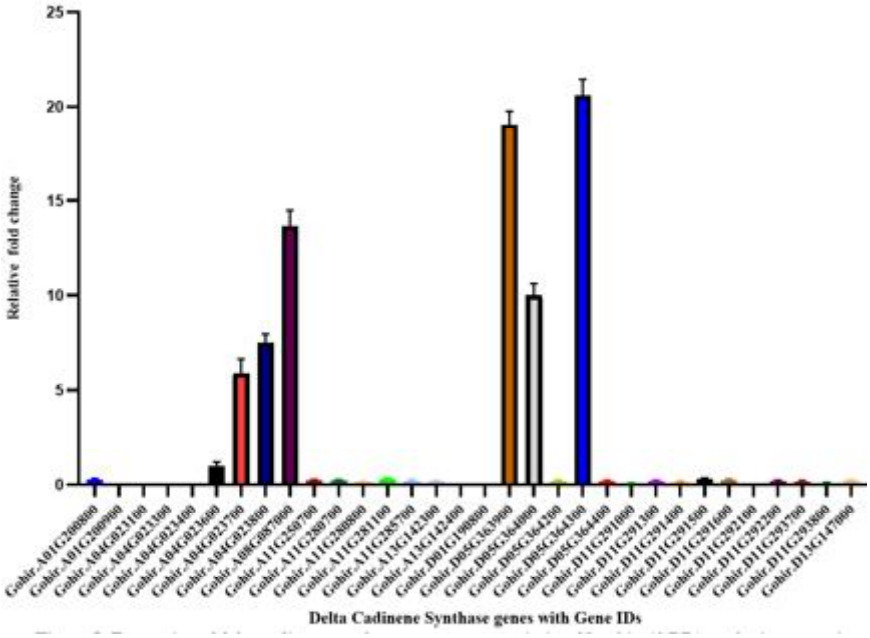

Fig. 5 : Expression of delta cadinine synthase genes at trascriptional level in 40 DPA ovules in comparison to leaf, In X-axis: Delta cadinine synthase genes with their Gene IDs. In Y-axis: level by which more CAD gene was expressed in ovules over leaf in Coker-312 genotypes.

(19.06-fold), Gohir.A08G087000 (13.67-fold), Gohir.A04G023800 (7.47-fold) and Gohir.A04G023700 (5.91-fold) exhibited notable up-regulation in ovules. The highest fold change was observed in Gohir.D05G364300 (20.60-fold) and the lowest in Gohir.A04G023700 (5.91-fold).

At 45 DPA, the (+)-delta-cadinene synthase genes continued to show differential expression between cotton ovules and leaves, with fold changes ranging from 3.05 to 12.20 (Table 2 and Fig. 6). Gohir.A04G023800 (9.94-fold), Gohir.D05G364000 (12.20-fold), Gohir.A04G023700 (8.60-fold), Gohir.D05G363900 (8.19-fold), Gohir.D05G364300 (8.12-fold) and Gohir.A08G087000 (3.05-fold) exhibited varying levels of up-regulation in ovules. The highest fold change was observed in Gohir.D05G364000 (12.20-fold) and the lowest in Gohir.A08G087000 (3.05-fold).

Discussion

Gossypol biosynthesis in cotton (*Gossypium hirsutum*) initiates with the cyclization of farnesyl diphosphate (FPP) into (+)-delta-cadinene catalyzed by delta-cadinene synthase (CDN) enzymes. Subsequent enzymatic transformations convert this intermediate into gossypol (Cai *et al*., 2004). Early strategies to mitigate gossypol's effects included glandless mutants and various extraction methods, but these proved commercially unviable or nutritionally detrimental (McMichael, 1959; Damaty and Hudson, 1975; Frank, 1987). Tri-species hybridization introduced delayed gland morphogenesis traits from wild species but faced challenges with reduced fertility and fiber quality (Dilday, 1986; Brubaker *et al*., 1996). RNA interference (RNAi) targeting the (+)-delta-cadinene synthase gene offered a breakthrough, yielding ultra-low gossypol cottonseeds (ULGCS) like TAM66274, approved for broader consumption (Sunilkumar *et al*., 2006; Rathore *et al*., 2020). However, regulatory hurdles and public acceptance remain barriers, prompting exploration into CRISPR-Cas9 technology for precise genome editing in cotton. A significant advantage of CRISPR/Cas9 over RNAi is that transgenes, such as Cas9 and sgRNA, can be removed from host genomes through simple plant breeding segregation principles (Zhang *et al*., 2014).

Understanding the expression patterns and genomic diversity of delta-cadinene synthase genes is crucial for optimizing strategies to reduce gossypol levels through genetic modification. The JGI assembly was selected for detailed analysis due to the availability of tissue specific

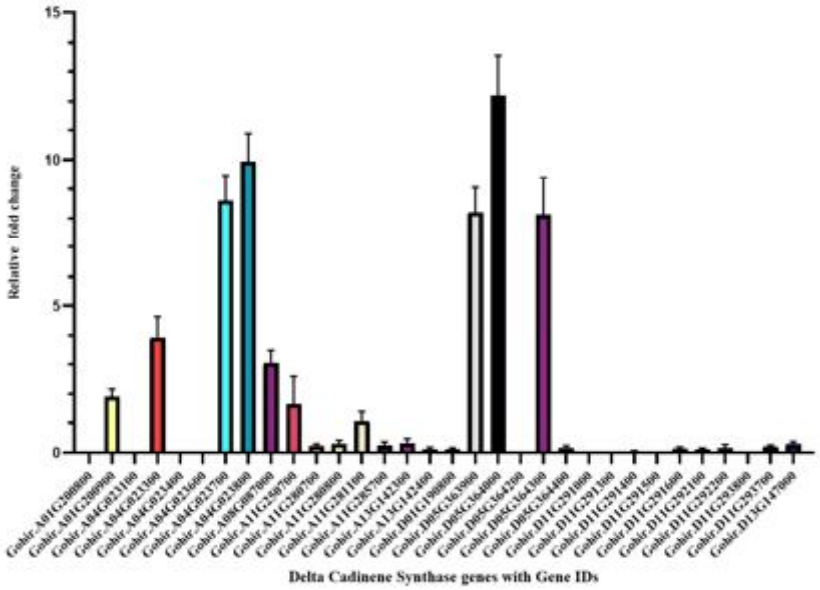

Fig. 6 : Expression of delta cadinine synthase genes at trascriptional level in 45 DPA ovules in comparison to leaf, In X-axis: Delta cadinine synthase genes with their Gene IDs. In Y-axis: level by which more CAD gene was expressed in ovules over leaf in Coker-312 genotypes.

RNA-Seq expression data, essential for validating findings in the targeted genotype. A systematic study identified 32 delta-cadinene synthase genes in cotton using the JGI assembly of the *Gossypium hirsutum* genome, supported by comprehensive genomic analysis. This analysis revealed significant genomic diversity among these genes, suggesting potential functional diversity in gossypol biosynthesis.

RNA-seq data from the database indicated that delta-cadinene synthase genes were not exclusively expressed in ovules, implying broader tissue-specific functions and underscoring the complexity of gossypol regulation. Thorough validation of gene expression patterns across relevant tissues and developmental stages is essential to prevent inefficiencies or failures in genetic modifications and to identify genotype-specific variations in gene regulation. Before implementing genome editing, validating (+)-delta-cadinene synthase (CDN) genes in cotton is crucial to ensure precise targeting and effective reduction of gossypol levels.

The Coker-312 genotype was chosen for validating gene expression due to its demonstrated success in regeneration and its extensive use in transformation studies (Katageri *et al.*, 2007; Jadhav and Katageri, 2017). This cultivar has shown relatively stable and consistent regeneration results despite seed-to-seed variation (Katageri et al., 2007). Its genetic stability is crucial for studying the expression of specific genes like delta-cadinene synthase, ensuring that the results are attributable to genetic modifications rather than inherent genetic variability. Distinct stage-specific patterns of delta-cadinene synthase gene expression were observed between cotton ovules and leaves through quantitative real-time PCR analysis. Our results revealed that 10 genes were expressed in ovules irrespective of the developmental stages. Among these, six genes: Gohir. A04G023700, Gohir.D05G363800, Gohir. A08G087000, Gohir.D05G363900, Gohir. D05G364000 and Gohir. D05G364300, consistently exhibited significantly higher expression levels across various stages. Notably, Gohir.D05G363900, Gohir.D05G364000, and Gohir.D05G364300 demonstrated slightly higher expression levels across all stages, making them suitable candidates for targeted genome editing.

This comprehensive study provides critical insights into the regulation and functional diversity of the delta-cadinene synthase gene family in cotton, guiding the prioritization of potential genetic modification targets aimed at reducing gossypol levels. Strategies such as CRISPR/Cas9-mediated gene editing could selectively target high-expressing genes in ovules, thereby enhancing cottonseed nutritional value without compromising the plant's natural defense mechanisms. By elucidating the expression dynamics of delta-cadinene synthase genes across key developmental stages in cotton, this research lays the groundwork for targeted genetic modifications to improve cottonseed quality and sustainability.

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