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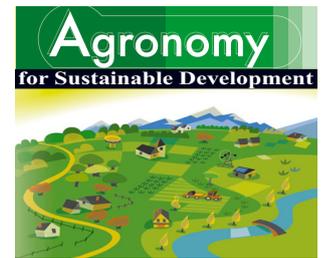
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Review article

Glandless seed and glanded plant research in cotton. A review

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Abstract – Recently the world has been entangled by insufficient food such as the lack of rice which threatens the safety of world food and affect sustainable development of the world economy, resulting in rising of food price. To address this issue, cotton appears as a possible source of both fiber and food. The research in recent years indeed showed bright prospects for this expectation. However, gossypol stored in the glands of cotton is toxic to nonruminant animals and humans, which wastes large amounts of cottonseed protein that could potentially provide the annual protein requirements for half a billion people. *Gossypium* species are characterized by their lysigenous glands containing terpenoid aldehydes, important secondary phytoalexins consisting mainly of gossypol, which constitute one of the important plant's defense system against pests and diseases. The best approach to address this issue is to create glandless seed and glanded plant cotton. A breakthrough in this field would realise the fulfilment of making cotton both a fiber and a food crop, which would be a feat of great magnitude for sustainable development of agriculture. Research on the relationship between glands and their secondary inclusions at the molecular level would be one approach for genetic engineering to control the glands and gossypol content. In this article, we review recent progress on glands and gossypol content for diverse gland types in *Gossypium* species, inheritance of glands and gossypol content, traditional breeding of glandless seeds and glanded plant cotton, the terpenoid aldehyde biosynthesis pathway, molecular cloning of the related genes, the strategy for genetic engineering, and future prospects.

cotton / pigment gland / gossypol / inheritance and breeding / gene cloning and genetic engineering

1. INTRODUCTION

Cotton, genus *Gossypium* L. containing 49 species, is a leading fiber and potential food crop, and cottonseed has compared favorably with other traditional food sources as a source of protein in several human nutritional studies (Lusas and Jividen, 1987). Cottonseed is among the most abundant protein meal and it represents 6.9% of world protein meal production only next to soybeans, rapeseed (Ash and Dohlman, 2006). Global cottonseed production can potentially provide the annual protein requirements for half a billion people, for every kilogram of fiber produced by the plant, 1.65 kg of seed are also produced (Sunilkumar et al., 2006; Bertrand et al., 2005; Gerasimidis et al., 2007). But all cotton species have characteristic lysigenous glands containing terpenoid aldehydes, predominantly the sesquiterpenoid gossypol which is toxic to nonruminant animals and humans (Fryxell, 1968; Bell, 1969; Stipanovic et al., 1977). The pigment glands, as the storage organs of terpenoid aldehydes, including gossypol, are on the surfaces of cotton organs or tissues (Punit et al., 1991).

Gossypol was first characterized in a classic series of studies by Adams et al. in 1938 (Heinstein et al., 1962). Actually

gossypol in cotton is a double-edged sword. It provides constitutive and inducible resistance against a variety of pests and diseases and acts as an important phytoalexin (Wang et al., 2004; Townsend et al., 2005). In vitro, gossypol also acts as an anticancer (Liu et al., 2002; Ye et al., 2007; Oliver et al., 2005), anti-HIV (Lin et al., 1993; Bourinbaiar and Lee-Huang 1994; Keller et al., 2003), antibacterial and nonhormonal male contraceptive (Coutinho, 2002; Tegos et al., 2002; Lopez et al., 2005; Badawy et al., 2007). However, on account of its toxicity, the nutritional value of cottonseed is reduced much, in addition gossypol discolors cottonseed oil (Cherry, 1983) which results in a tremendous waste of seed protein and oil. To reduce gossypol in cottonseed, studies have developed glandless cotton, glandless both in seeds and plants, with no or very low gossypol content (McMichael, 1959; Vaissayre and Hau, 1985). The protein and oil of the glandless seed are free of gossypol and can therefore be used directly as food (Lusas and Jividen, 1987), but plant resistance to pests is reduced and the yield of cotton fibers is reduced due to the absence of gossypol and other terpenoid aldehydes, the glandless cotton has been applied in very limited area (Cherry, 1983; Vaissayre and Hau, 1985).

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Cotton has been anticipated to become a crop that produces both fiber and food for decades, and thus developing a cotton variety that has gossypol within glanded roots, leaves, and stems to maintain the resistance trait but has glandless seeds for safe food utilization is now necessary (Cai et al., 2004). In other words, the ideal cotton cultivar would delay gossypol biosynthesis until germination, a phenotype seen only in some Australian *Gossypium* species (Brubaker et al., 1996). Many studies have examined the relationship between cotton glands and gossypol production, and recent efforts have shown much progress in this field, especially in molecular biology and genetic engineering. Such breakthroughs will revolutionize cotton breeding, cotton planting, industrial processing of cotton seed, and even use as animal feed and human food, and eventually will be beneficial to sustainable development of agriculture and human being and guarantee the safety of the world food.

2. THE RELATIONSHIP BETWEEN GOSSYPOL AND GLANDS: DIVERSITY OF GLANDS OCCURENCE IN *GOSSYPIMUM* SPECIES

Gossypol is synthesized in cotton roots, and transported and stored within pigment glands of cotton above ground (Smith, 1962). The pigment glands are spherical on leaves, bracts, and ovary surfaces; elliptical on stems and stigma surfaces; and spherical to oval on calyx surfaces. Genotypes differ markedly in gland distribution (Punit et al., 1991). The content of gossypol in cotton is dependent on the genetic types of pigment glands. The gland number of cotyledonary leaves is significantly and positively associated with free gossypol and positively associated with the number of gossypol-producing glands and free gossypol content in seeds (Punit et al., 1995).

High performance liquid chromatography, HPLC, analysis of gossypol in different gland genotypes of cotton revealed major differences among glands and species of *Gossypium*, including the glandless and glanded *G. hirsutum* L. and *G. barbadense* L. Especially *G. hirsutum* variety Xiangmian 18 is characterized by glandless seeds with very low gossypol content and low number of glands in foliage (Cai et al., 2004). "Glandless-seeded trait" in Australian *G. sturtianum* Willis reportedly lacks terpenoid aldehydes, and thus may represent an important genetic resource in the development of cottonseed oils and protein free of these toxins (Brubaker et al., 1996). The special trait of glands in this Australian species involves delayed pigment gland morphogenesis (Zhu et al., 1999). The studies on gossypol of the cotyledons during seed germination and the relationship between gossypol and gland formation in five wild Australia *Gossypium* species such as *G. sturtianum*, *G. nandawarensense* Dereda, *G. australe* Mueller, *G. nelsonii* Fryxell, and *G. bickii* showed little gossypol in dormant seeds and cotyledons 24 h before seed germination. However, 48 h after germination, gossypol began to accumulate in cotyledons, although the content was very low. After 1–2 days of growth, the gossypol content in cotyledons increased markedly and was highest 9 days after germination. The dynamics of gossypol accumulation during seed germina-

tion was basically the same in all five wild species, but the time at which gossypol first appeared in the cotyledons differed significantly among them. Presumably some genetic regulation exists between glands and gossypol content (Zhu et al., 1999). Pigment glands in *Gossypium* species have diverse characteristics; glanded cotton normally contains gossypol in both seeds and other plant parts, and the content of gossypol is highly correlated with the number of pigment lands in glanded cotton (Singh and Weaver, 1972). However, in *G. somalense* Hutchinson (Genome E2) and *G. herbaceum* L. (Genome E1), pigment glands are found in the seeds but the gossypol content is unmeasurable.

In general, the following five types of pigment glands are found in *Gossypium* species: glandless cotton, with no or very low gossypol and no glands in seeds, roots, stems, and leaves; glanded cotton, with gossypol within glands in seeds, roots, stems, and leaves; cotton with a delayed gland morphogenesis trait (Zhu et al., 1999), specifically the Australian *Gossypium* species *G. bickii* (Genome G1), *G. australe* (Genome C3), and *G. sturtianum* (Genome C1-n), which contain no or very low gossypol and have no glands in seeds, but have gossypol and glands in roots, stems, and leaves; cotton with glands in seeds, but very low gossypol content, e.g., *G. stocksii*; and cotton that has no or few glands and low gossypol in seeds, but has some glands and gossypol in plant parts. Hence, the diversity of glands in *Gossypium* and the relationship between glands and gossypol content warrant further exploration.

3. INHERITANCE OF GLANDS AND GOSSYPOL

Results on the genetics of glands in cotton have differed because different gland materials have been used. The glandless trait is thought to be controlled by two or three recessive or dominant genes in different gland types from different cotton species. According to McMichael (1960), the glandless trait is controlled by two recessive genes, *gl2* and *gl3*, found in lines of Hopi cotton (McMichael, 1960). In other varieties, however, such as Hai 1 and others, it is controlled by dominant genes such as *GL2e* (Carvalho and de Vieira, 2000). Yet other scientists have reported that the glandless trait is a quantitative trait controlled by minor polygenes (Lee et al., 1968). *GL2e* in *G. barbadense* is dominant and produces glandless plants and seeds. The free and total gossypol contents in F1 seeds from this variety were similar to those of the glandless parent, showing complete dominance of this gene (Carvalho and de Vieira, 2000).

Twenty-one gossypol glanded cultivars from Turkey were crossed with two Turkish gossypol-free cultivars, Suz-86 and 129. Of 5588 plants raised in the F2 generation, 340 were entirely glandless. According to the two independent pairs of genes (glanded: *GL2 GL2 GL3 GL3*; glandless: *gl2 gl2 gl3 gl3*), the expected 1/16 ratio of glandless plants in the F2 generation was in accordance with a chi-square test (Arshad Mahmood et al., 2004).

A mutant of Xiang X9628 (Xiangmian 18) in upland cotton (*G. hirsutum*) has normal glanded leaves and a low gossypol content in seeds (Zhang et al., 2001). Genetic analysis

indicated that the glanded leaves and low gossypol content of the mutant are controlled by two pairs of recessive duplicate genes. Allelic tests showed that one of these is allelic to the *gl2* gene and the other is a multi-allele of the *gl3* loci. This was therefore considered to be a new gland-forming gene in *Gossypium* and was named *gln3*. Data from crosses using line 247-1, which is rich in glands and has a high gossypol content in the flower buds, and plants homozygous for various combinations of gland-determining alleles at the *gl1*, *gl2* and *gl3* loci indicate that the high gossypol content of 247-1 could be explained by the presence of a high-potency allele at the *gl3* locus, plus another factor or factors independent of the other gland-determining alleles (Wilson and Smith, 1976).

Studies on the inheritance of the high-glanding (HG) trait in cotton (*G. hirsutum*) using crosses made among HG, normal glanding (NG) and glandless genotypes, and with isolines of the HG breeding line XG15, which has the genotypes 2 (*G12gl3*) and 2 (*gl2G13*), showed that isoline XG15 *gl2G13* expressed the HG phenotype, suggesting that HG is conferred by a special *G13* allele derived from XG15. This allele, derived originally from Socorro Island cotton, was tentatively designated *G13s* (Calhoun, 1997). Additive effects accounted for more than 90% of the total genetic variance in seed gossypol level in all trials.

Epistatic effects, though small, were frequently significant. In *G. barbadense*, *G12* and *G13* were found to be associated with the production of similar amounts of gossypol, whereas previous trials with cultivated varieties of *G. hirsutum* showed that more than twice in *G12* was expressed compared to *G13*. The higher average productivity of seed gossypol in cultivated *G. barbadense* compared to *G. hirsutum* is attributable to higher activity at the *G13* locus in the former species (Lee, 1973).

Additive and dominance effects and other digenic interactions are associated with gossypol content, with the additive effects and additive × additive epistasis being more prominent. The variety of interactions indicate that the major gland determining the loci *gl2* and *gl3* are not the only determinants of gossypol content, but that the trait shows polygenic inheritance (Singh et al., 1991).

4. TRADITIONAL BREEDING OF GLANDLESS SEED/GLANDED COTTON PLANTS

Since a close relationship exists between gossypol content and pigment glands, tactics to control gossypol through selecting and controlling the gland trait have proven to be appropriate and effective. In cotton breeding, the gland trait has been used for two different purposes: to produce glands rich in gossypol or with high glanding (HG) or to produce glandless plants.

On the one hand, breeding and application of a variety with a high density of glands and high gossypol content can enhance cotton plant resistance to pests and diseases. Such lines and varieties with high gossypol are beneficial as they reduce the cost for pest control and therefore cause less environmental pollution from the use of chemicals (Calhoun and Jones, 1994;

Calhoun, 1997; Lee, 1978; McCarty et al., 1996; Bourland and Benson, 2002; Li et al., 1996).

On the other hand, to make full use of cottonseed protein and oil, scientists bred the glandless cotton 23B, glandless both in seeds and plants, with no or very low gossypol content (McMichael, 1954, 1959, 1960). Subsequently, many more glandless varieties have been bred from the resources created by McMichael (Lusas and Jividen, 1987; Miravalle, 1972; Vaissayre and Hau, 1985). Cottonseed compares favourably to other traditional food sources as a source of protein in several human nutrition studies, and glandless cottonseed is safe and nutritious (Lusas and Jividen, 1987). However, glandless varieties have proven to be commercially unviable as they are more susceptible to insect pests because of the systemic absence of glands that contain gossypol and other protective terpenoids (Sunilkumar et al., 2006). Hence, the best choice currently available is plants with glandless seeds and glanded foliage, and many scientists have attempted to breed such plants.

A new upland cotton (*G. hirsutum*) cultivar has been bred in China, Xiangmian 18, with glandless seed, which has very low seed gossypol and is a glanded plant. This was developed from Xiangmian 10 × Zhong 5655, and was released in China during 2000. The glandless traits of glandless Zhong 5655 originated from Hai 1 (*G. barbadense* L.) This new cultivar can be grown for the production of seed protein and oil as well as lint (Zhang X. et al., 2001).

To transfer the traits for glandless seeds and glanded plants – so-called “delayed pigment gland morphogenesis” or “delayed development of gossypol glands” – Australian species have been bred to cultivated species of *Gossypium* to produce tri-species hybrids. A series of hybrid seeds or materials with the traits for glandless seeds and glanded plants have been obtained, but these have not yet reached the potential for use in commercial cotton production because of many problems such as sterility, with wild traits and other disadvantages (Altman et al., 1987; Kulkarni et al., 2002; Mergeai, 1992; Mergeai et al., 1995, 1997; Dilday, 1986; McCarty et al., 1996; Vroh et al., 1999; Bi et al., 1998, 1999; Zhu et al., 1993, 2004, 2005; Bourland and Jones, 2006; Ahoton et al., 2003).

5. TERPENOID ALDEHYDES IN GLANDS AND THE BIOSYNTHETIC PATHWAY OF GOSSYPOL AND ASSOCIATED TERPENOID

Lysigenous glands in cotton plants contain terpenoid aldehydes (Bell, 1969; Stipanovic et al., 1977). In cultivated cotton, glands in achlorophyllous plant parts of *G. hirsutum* predominantly contain the gossypol and its methyl and dimethyl ethers are found in *G. barbadense*. Glands in young green tissues of *G. hirsutum*, however, contain hemigossypolone as the predominant terpenoid aldehyde, and a new quinone, hemigossypolone-7-methyl ether occurs in *G. barbadense* (Bell et al., 1978; Benedict et al., 2004). A survey of terpenoid quinones and their heliocide derivatives in wild *Gossypium* spp. and related genera in the *Gossypieae* showed considerable diversity. Several cadinane sesquiterpenoids and heliocides

(sesterterpenoids) are deposited in the pigment glands of cotton plants, which function in pathogen and insect resistance (Stipanovic et al., 1999; Martin et al., 2003).

Based on the structure of gossypol, it was suggested that it might be formed metabolically from acetate via the isoprenoid pathway. Heinstein and colleagues examined the location of incorporated ^{14}C and studied the incorporation of mevalonate-2- ^{14}C , a key intermediate in the isoprenoid pathway, into gossypol, as well as the distribution of radioactivity in the gossypol molecule (Heinstein et al., 1962, 1970).

The cotton terpenoid aldehydes and cadalene derivatives are sesquiterpenes (C15) derived from a cytosolic branch of terpenoid metabolism via the mevalonate pathway (Heinstein et al., 1962, 1970). Farnesyl diphosphate (FPP) is generated as the linear carbon skeleton of the sesquiterpenes in cotton (Essenberg et al., 1985; Stipanovic et al., 1986; Liu et al., 1999). The various sesquiterpene synthases cyclize FPP to form the molecular frameworks of different sesquiterpene types (Gershenzon and Crodeau, 1993).

Gossypol is the sesquiterpenoid formed in cottonseed, with only traces of desoxyhemigossypol (dHG) and hemigossypol. In cotton foliage, hemigossypolone is formed from dHG (Stipanovic et al., 1999). The cadinene enzyme was first purified from a glandless cotton mutant by Davis and Essenberg (1995) as a soluble hydrophobic monomer with a molecular mass of 64 to 65 kD.

As reported by Martin et al. (2003), there are several cadinane sesquiterpenoids and heliocides (sesterterpenoids) deposited in pigment glands in cotton plants that function in pathogen and insect resistance (Stipanovic et al., 1999). A proposed pathway for the biosynthesis of these compounds is shown in Figure 1. Infection of cotton stele tissue with conidia of *Verticillium dahliae* that induced the formation of sesquiterpenoid phytoalexins also induced 3-hydroxy-3-methylglutaryl-CoA reducterpenoid aldehydase (HMGR) mRNA and HMGR activity, demonstrating the important role of HMGR in the biosynthesis of the sesquiterpenoids (Bianchini et al., 1994). Studies with specifically labeled mevalonic acid (MVA) or acetate demonstrated the folding pattern of farnesyl diphosphate (FDP) required for gossypol formation. Subsequently, the enzymatic product of the cyclization of E, E-FDP in cotton extracts was identified as (+)- δ -cadinene (CDN) (Benedict et al., 2001; Davis and Essenberg, 1995; Chen et al., 1995). The enzymatic mechanism of CDN synthase for the formation of the cadinane structure of cotton sesquiterpenoids was shown to involve the isomerization of FDP to a nerolidyl intermediate, cyclization to a *cis*-germacradienyl cation, a 1, 3-hydride shift, cyclization to a cadinanyl cation and deprotonation to form (+)- δ -cadinene (Benedict et al., 2001). CDN synthase catalyses the committed step in the formation of the cadinane sesquiterpenoids from FDP at a branch point in the MVA pathway. Gossypol is the sesquiterpenoid formed in cottonseed with only traces of desoxyhemigossypol (dHG) and hemigossypol. In cotton foliage hemigossypolone is formed from dHG (Stipanovic et al., 1999). A Diels–Alder reaction accounts for the cycloaddition of myrcene or β -ocimene to hemigossypolone to form heliocides (Stipanovic, 1992). The monoterpenes or

its precursors are synthesized by the 1-deoxy- -xylulose-5-phosphate (DOXP) pathway in the plastids (Martin et al., 2003; Essenberg et al., 1985; Bell, 1986; Stipanovic et al., 1986; Benedict et al., 1995; Davis and Essenberg, 1995; Alchanati et al., 1998).

In recent years, the biosynthetic pathway of gossypol and its derivatives has been further elucidated. 8-Hydroxy-(+) d-cadinene is a precursor of hemigossypol in *G. hirsutum* (Yan-Hong Wang et al., 2003). A (+) d-cadinene-8-hydroxylase, a cytochrome P450 mono-oxygenase of cotton sesquiterpene biosynthesis (CYP706B1), was expressed in aerial tissues of glanded cotton cultivars, but not or at an extremely low level in the aerial tissues of a glandless cultivar. The expression pattern of CYP706B1 and the position at which it hydroxylates (+) d-cadinene suggest that it catalyzes an early step in gossypol biosynthesis, and thus CYP706B1 holds good potential for manipulating gossypol levels in cottonseed via genetic engineering (Luo et al., 2001). Desoxyhemigossypol is a key intermediate in the biosynthesis of these compounds. A methyltransferase (S-adenosyl-L-Met: desoxyhemigossypol-6-O-methyltransferase) was isolated, purified, and characterized from cotton stele tissue infected with *V. dahliae*. Desoxyhemigossypol-6-methyl ether leads to the biosynthesis of methylated hemigossypol, gossypol, hemigossypolone, or the heliocides (Liu et al., 1999, 2005).

6. MOLECULAR CLONING OF GENES ASSOCIATED WITH GOSSYPOL AND GLANDS

Based on elucidation of the terpenoid biosynthetic pathway, the related genes have also been cloned. The cadinene gene was first cloned and functionally characterized from the A-genome diploid cotton *G. arboreum* and comprise a large multigene family in cotton (Chen et al., 1995; Davis et al., 1996; Meng et al., 1999; Tan et al., 2000) similar to the terpene cyclase genes found in other plants (Facchini and Chappell, 1992; Back and Chappell, 1995). Several allelic and gene family variants of the cotton cadinene genes have since been isolated from both *G. arboreum* (Chen et al., 1996; Liu et al., 1999; Meng et al., 1999; Tan et al., 2000) and the allotetraploid (A + D genomes) species *G. hirsutum* (Davis et al., 1998) (Tab. I).

The cadinene enzyme and transcripts are induced in cotton stems infected with *V. dahliae* (Benedict et al., 1995; Alchanati et al., 1998; Bianchini et al., 1999; Tan et al., 2000), cotton suspension cultures treated with *V. dahliae* elicitors (Chen Z.Y. et al., 1995; Chen X.Y. et al., 1996; Liu et al., 1999), and cotton cotyledons infected with Xcm (Davis and Essenberg, 1995; Davis et al., 1996). cadinene is also developmentally regulated and cadinene transcripts increase during seed development in association with the biosynthesis and deposition of gossypol in the lysigenous storage glands of the embryo (Meng et al., 1999; Martin et al., 2003). Two major subfamilies of the *Gossypium* cadinene multigene family, *cdn1-A* and *cdn1-C*, have been proposed based on sequence relatedness, and appear to be differentially regulated at the transcriptional

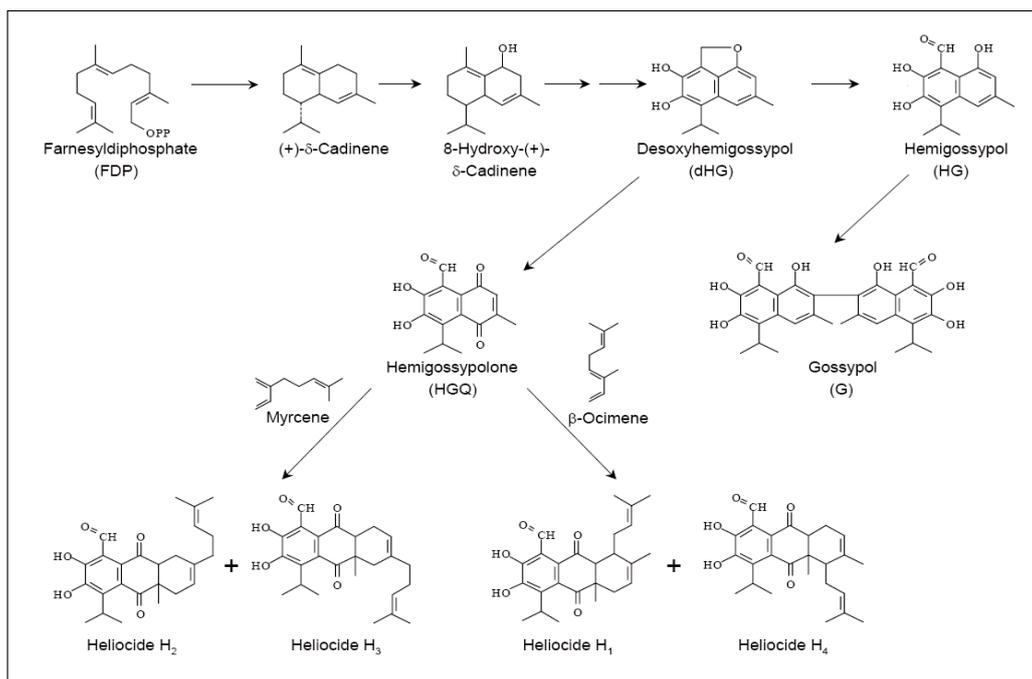


Figure 1. The biosynthesis of gossypol in cottonseed and gossypol, hemigossypolone and helioides₁₋₄ in cotton leaves (Modified from Martin et al., 2003).

Table I. The cadinene genes and complementary DNA cloned in *Gossypium* in GenBank.

Subfamily or gene name	accession number	<i>Gossypium</i> species	author	submitted year
cdn XC14	U23205	<i>G. arboreum</i>	Chen Z.Y. et al.	1995
cdn XC1	U23206	<i>G. arboreum</i>	Chen Z.Y. et al.	1995
cad1-b	X95323	<i>G. arboreum</i>	Chen X.Y. et al.	1996
cdn A	U27535	<i>G. arboreum</i>	Chen Z.Y. et al.	1995
cad1-A	Y18484	<i>G. arboreum</i>	Liang	1998
cad1-A	X96429	<i>G. arboreum</i>	Chen X.Y. et al.	1996
cad1-A	AF456410	<i>G. barbadense</i>	Cai	2002
cad XC14	AF453326	<i>G. hirsutum</i>	Cai	2001
cad1-C2	Y16432	<i>G. arboreum</i>	Meng et al.	1999
cad1-C1	AF174294	<i>G. arboreum</i>	Tan et al.	2000
cdn1	U88318	<i>G. hirsutum</i>	Davis	1997
cdn1-C4	AF270425	<i>G. hirsutum</i>	Townsend	2000
cdn1-C5	AY800106	<i>G. hirsutum</i>	Townsend	2004
cdn1-D1	AY800107	<i>G. hirsutum</i>	Townsend	2004

level (Meng et al., 1999; Tan et al., 2000). A third subfamily, cdn1-B, is represented by a single genomic clone.

A phylogenetic tree of the cotton cadinene genes showed the relatedness of members within the cadinene subfamilies and the distance between the different subfamilies (Fig. 2). cdn1-D1 is sufficiently different from the other cadinene genes of cotton to suggest that it belongs to another subfamily, designated cdn1-D.

The genetic structure of cadinene genes is well conserved, including the number, positions, and sizes of exons and introns, and also corresponds well to the genomic clones of other terpene cyclase genes, such as tobacco (*Nicotiana tabacum* L.) 5-epi-aristolochene synthase. Approximately 2 kb of promoter regions from each cadinene genomic clone were sequenced

and a very low level of sequence conservation was found between all cadinene promoter regions. We found discrete regions of similarity, mostly in the vicinity of the predicted TATA box and transcription start site, but the similarity did not extend appreciably upstream (Back and Chappell, 1995).

Luo et al. (2001) cloned and identified the function of a gene of (+) delta-cadinene-8-hydroxylase, a cytochrome P450 mono-oxygenase of cotton sesquiterpene biosynthesis (Luo et al., 2001). Liu et al. (2005) identified and cloned a 1.9 -kb P450 coding for a 522 amino acid protein that is 48% identical to a soybean cytochrome P450 82 A3 and contains a conserved heme-binding motif and a consensus oxygen-binding pocket sequence (Liu et al., 2005). This P450 is expressed in the leaves of the glanded cotton *G. hirsutum*, but not in the

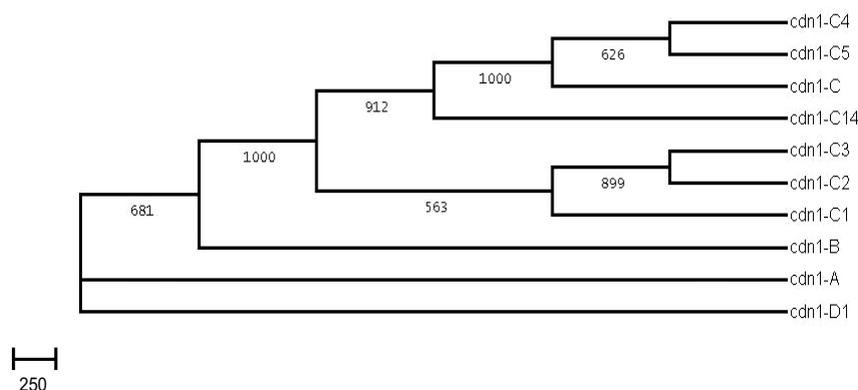


Figure 2. Phylogenetic tree of cadinene protein sequences showing the subfamilies cdn1-A, cdn1-B, cdn1-C, and cdn1-D.

leaves of glandless cotton. The leaves of glandless plants are devoid of glands and the associated terpenoids. This suggests that this P450 enzyme is involved in the terpenoid biosynthetic pathway in cotton.

Studies on gland-morphogenesis-related genes, applying suppressive subtractive hybridization and other methods, have successfully constructed a subtractive library and a normalized cDNA library from a cotton mutant, Xiangmian 18. Some relevant genes, such as the RanBP2 zinc finger protein gene in upland cotton, have been cloned (Cai et al., 2003; Chang et al., 2007; Xie et al., 2007). In addition, the gene of desoxyhemigossypol-6-O-methyltransferase from *G. barbadense* (Liu et al., 1999) and gene of cytochrome P450 related to glands have also been cloned and studied (Liu et al., 2005, 2007).

7. A STRATEGY FOR ELIMINATING GOSSYPOL AND CREATING GLANDLESS SEED-GLANDED COTTON PLANTS

Traditional breeding is still the main approach in the breeding of cotton with glandless seed and glanded plant, but it is limited by, for example, long breeding cycles, frequent attachment to inappropriate genes, and especially, crosses between species. In practice, progress in traditional breeding to create glandless seed and glanded foliage cotton is very slow and large problems still exist. Genetic engineering can hopefully solve that. Advances in understanding the terpenoid biosynthetic pathway and cloning of the relevant genes provide tools to manipulate the biosynthesis of these defense terpenoids through genetic engineering creating glandless seed and glanded foliage cotton. Three aspects are involved in the strategy to eliminate the toxic cottonseed gossypol through genetic engineering: silencing gossypol-related genes in the seeds of glanded cotton using antisense and RNAi method; turning on related foliage genes in glandless cotton; and creating cotton only containing (+) gossypol, the toxicity of which is very low compared to (–) gossypol.

Antisense expression of cadinene genes was envisaged as a way to activate this silencing mechanism in cotton by block-

ing the cadinane-type sesquiterpene pathway and abolishing gossypol production in the transformants. An antisense construct of cdn1-C1 was introduced into cotton plants (Martin et al., 2003), and Southern analyses showed integration of antisense cdn1-C1 cDNA driven by the CaMV 35S promoter into the cotton genome. Northern blots demonstrated the appearance of cdn synthase mRNA preceding CDN synthase activity and the formation of gossypol in developing cottonseed. T2 cottonseed had reduced CDN synthase activity and up to a 70% reduction in gossypol. In T1 leaves, the accumulated gossypol, hemigossypolone, and heliocides were reduced by 92.4, 83.3, and 68.4%, respectively. These results demonstrate that the integration of antisense cdn1-C1 cDNA into the cotton genome leads to a reduction in CDN synthase activity and negatively impacts the biosynthesis of cadinane sesquiterpenoids and heliocides in cotton plants.

Another new cotton variant from the progeny of hemizygous *G. hirsutum* cv. Coker 312 transformed with antisense (+)- delta-cadinene synthase cDNA has reduced the TA levels (Benedict et al., 2004). The gossypol content in seeds of the variant was markedly lower than in seeds of T1 antisense plants. Eighty-nine percent of the variant seed had a 71.1% reduction in gossypol, and foliage of the variant plants had a 70% reduction in gossypol and a 31% reduction in heliocides. Compared to non-transformed plants, no reduction occurred in the number of lysigenous glands in the seed of the variant. The cotton variant showed uncoupling of TA synthesis and gland formation. In this case, the study assumed that the cotton variant may have resulted from somaclonal variation occurring in callus tissue during the transformation-regeneration process.

Constructs for the constitutive or seed-specific antisense suppression of cdn1-C4 were introduced into cotton by *Agrobacterium*-mediated transformation (Townsend et al., 2005). Gossypol levels were not reduced in the seeds of transformants with either construct, and the induction of cadinene expression did not affect stems of the constitutive antisense plants infected with *V. dahliae*. However, the induction of cadinene mRNA and protein in response to bacterial blight infection of cotyledons was completely blocked in the constitutive antisense plants. These results suggest that cdn1-C4 may

be involved specifically in the bacterial blight response. Moreover, the cadinene multigene family may comprise a complex set of genes differing in their temporal and spatial regulation, and they may be responsible for different branches of the cotton sesquiterpene pathway. The antisense approach, however, has been limited or somewhat ambiguous (Martin et al., 2003; Townsend et al., 2005; Sunilkumar et al., 2006).

Recently, a study successfully used RNAi and a seed-specific promoter from the cotton α -globulin B gene to disrupt gossypol biosynthesis in cottonseed by interfering with expression of the cadinene synthase gene during seed development (Sunilkumar et al., 2006). The study demonstrated the possibility of significantly lowering cottonseed- gossypol levels in a stable and heritable manner. Results from enzyme activity and molecular analyses on developing transgenic embryos were consistent with the observed phenotype in mature seeds. Most importantly, the levels of gossypol and related terpenoids in foliage and floral parts were not diminished, and thus their potential function in plant defense against pests and diseases remained untouched. This research provides evidence for spatial and temporal confinement of RNAi-mediated suppression of the s-cadinene synthase gene in cottonseeds that contain the transgene. The results demonstrate the feasibility of a targeted RNAi-based approach to solve an age-old problem of cottonseed toxicity and provide an avenue to exploit the considerable quantities of protein and oil available in the global cottonseed output. The GL genes will be cloned and applied in genetic engineering to obtain glandless seed and glanded foliage cotton.

Since the (+) enantiomer shows little if any toxicity to non-ruminant animals, cottonseed with high -protein content is underutilized due to toxic (-)- gossypol. The data from extracts of the Marie Galante cotton variety and the fact that the intact seed contain 95% (+) gossypol suggest a regio-stereoselective bimolecular coupling of hemigossypol to gossypol (Benedict et al., 2006). This can be used in future genetic engineering to eliminate the toxic (-) gossypol. As the (-) enantiomer has potential medicinal uses, cottonseed with >95% (-) gossypol could have biopharmaceutical applications and can also be created through genetic engineering.

8. CONCLUSION

With the increase in world population, decrease in available arable land, and need for more energy from plants, the world has been threatened by insufficient food. The desire to make cotton both a fiber and a food crop becomes more urgent. People have bended themselves to this research for decades and have attempted many approaches to get rid of toxic gossypol and have made much progress as described above. In recent years, one successful research trend on glands and gossypol in cotton has focused on molecular cloning and genetic engineering to produce glandless seed and glanded foliage to abolish toxic seed gossypol beyond the immediate application of eliminating gossypol from cotton seeds, one can readily anticipate other application for the gene and its promoter. For example, the promoter could be used to introduce pesticides

such as the BT toxin exclusively into the foliage (Liu et al., 2007). One could even use an exotic toxin, for example, from scorpions, since the green tissue special promoter would be directly expressed exclusively in the foliage. Obtaining such a promoter also provides a tool to manipulate the biosynthesis of HQG and heliocides through genetic engineering (Liu et al., 2007). The long-term goal is to elucidate the gene network mechanism controlling glands and gossypol while increasing the resistance of cotton to pests and pathogens, thus expanding utilization and the commercial value of cottonseed. Glandless seed and glanded foliage cotton will bring a revolutionary change in cotton breeding, cotton planting, and industrial processing of cotton seeds, even for use as a food supply. Cotton will become a crop that produces fiber, food, and oil simultaneously, which will help solve the problem of world food supply shortages.

At the same time, the research has also helped us to obtain a better understanding of the relationship between secondary compounds and storage organs (tissues) in plants and the molecular mechanisms controlling them. For example, artemisin and glands in southernwood (an antimalarial) have a similar relationship to gossypol and glands in cotton (Xu et al., 2003). The long-term goal of our research is to clarify the molecular mechanism (s) of the genetic control networks involved in secondary substances and storage organs in plants, and ultimately to control the biosynthesis of useful secondary compounds that will promote the sustainable development of agriculture and benefit humans.

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