

# The genes determining synthesis of pigments in cotton

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## Abstract

Naturally coloured cotton is environmentally friendly, since bleaching and chemical dyeing are not needed during textile production. Studying molecular-genetic mechanisms underpinning pigment production may facilitate breeding cotton with coloured fibre. In the current review we summarize the known data on structural and regulatory genes involved in biosynthesis of flavonoid pigments proanthocyanidins (PAs) in brown and caffeic acid (CA) derivatives in green fibre. The first chapter considers the first studies on fibre cotton inheritance, from the beginning of the last century. Then, we briefly review the biochemical and physico-chemical methods proving the presence of PAs in brown fibre and derivatives of CA in green cotton fibre. The biochemical analysis of coloured cotton fibre was followed by genetic studies of structural genes coding for enzymes participating in PAs and CA biosynthesis, transport and oxidation processes. We summarize the data on the genes coding for transcription factors from the MBW (MYB-bHLH-WD40) regulatory complex, which controls flavonoid biosynthesis in coloured cotton fibre. The regulatory gene most interesting as a target for markers-assisted breeding and genome editing is *GhTT2-3A*.

**Keywords:** brown fibre, caffeic acid, flavonoids, green fibre, *Gossypium*, MBW regulatory complex, proanthocyanidins

## Introduction

Cotton (*Gossypium* L.) is an important crop. The raw materials obtained from cotton are used in the textile and military industries due to the unique properties of cotton fibres, as well as in the pharmaceutical, food, and chemical industries due to the high content of some valuable metabolites. (Gong et al., 2018). Vegetable oils isolated from cotton seeds contain a significant amount of such fatty acids as palmitic, stearic, arachidic, oleic and linoleic acid (Dinesh K. Agarwal et al., 2003). The biological activity of gossypol extracted from cotton root, bolls and floral organs leads to it being used as a valuable pharmaceuticals component (Wang et al., 2009). Cotton fibre serves as an indispensable source of raw materials in the fabric manufacture. However, bleaching and chemical dyeing of fabrics during production contribute to environment pollution. In this regard, naturally coloured cotton fibre which is also called “eco-friendly cotton” can be used for the production of environmentally friendly fabrics (Nimon and Beghin, 1999). Furthermore, the use of naturally coloured cotton reduces the cost of fabric, since expensive bleaching and chemical dyeing are no longer needed.

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**Fig. 1.** The examples of white and brown cotton samples from Herbarium collection of N. I. Vavilov All-Russian Institute of Plant Genetic Resources.

The current review summarizes known data on molecular-genetic mechanisms underpinning pigmentation of fibre during cotton plant development. The metabolic pathways as well as structural and regulatory genes related to pigment synthesis in green and brown cotton fibre are considered.

### 1. Green and brown fibre colour — the first steps in the investigation

Coloured cotton fibres are characterised by different shades from cream to rust, but green and brown fibres are prevalent (Dutt et al., 2004). Derivatives of caffeic acid accumulating in the suberin layer are known to provide green fibre colour (Yatsu, Espelie, and Kolattukudy, 1983; Schmutz et al., 1993; Schmutz, Jenny, and Ryser,

1994; Feng et al., 2017). Brown pigmentation of cotton fibre is caused by the presence of proanthocyanidins (PAs) in cell vacuoles (Xiao et al., 2007, 2014; Feng et al., 2014; Malik et al., 2015) (Fig. 1).

The first investigations related to the genetic aspects of fibre colour date from the beginning of the last century. It was established that the green fibre trait is dominant over the brown and white colour traits (Harland, 1932; Hutchinson and Silow, 1939). Later, it was shown that the green colour fibre trait is regulated by one gene with incomplete dominance (Richmond, 1943).

At the same time, after crossing uncoloured and reddish-brown *Gossypium* plants it was observed that the genotypes with cream and light-brown fibres are heterozygous due to the splitting ratio and incomplete dominance (Balls, 1908; Kottur, 1923; Richmond, 1943;

Zhang et al., 2002). After further research it was shown that the formation of brown cotton fibre is controlled by a single locus containing genes with incomplete dominance (Ware, 1932).

Twelve years later, it was reported that there are six *Lc* genes (*Lc1–Lc6*) that determine fibre colour in the *Gossypium* genome (Silow, 1944). Further monosomic analysis showed that the *Lc1* gene, which is responsible for brown fibre colour, is located in chromosome 7 (En-drizzi and Taylor, 1968).

In 1985 it was first established that condensed tannins or proanthocyanidins (PAs) are responsible for brown pigmentation in cotton fibre (Ryser and Holloway, 1985). In 1994 caffeic acid (CA) derivatives were extracted from green cotton fibre for the first time (Schmutz et al., 1994). After this discovery, the study of the molecular mechanisms controlling the biosynthesis of these pigments began.

## 2. Proanthocyanidins in brown cotton fibre

Flavonoids are plant secondary metabolites derived from the general phenylpropanoids pathway (Schijlen, Ric de Vos, van Tunen, and Bovy, 2004; Koes, Verweij, and Quattrocchio, 2005; Panche, Diwan, and Chandra, 2016). Flavonoids have a broad spectrum of functions in plants including protection against adverse environmental factors such as extremal temperature, ultraviolet radiation damage and pathogens (Alonso-Amelot, Oliveros, and Calcagno-Pisarelli, 2004; Takahashi et al., 2010).

PAs (also condensed tannins) are a class of oligomeric flavonoids with the diphenylpropane common chemical structure (C6-C3-C6), which includes two aromatic rings with a three-carbon bridge that forms a heterocyclic ring (Fig. 2) (Feng et al., 2014; Ma et al., 2016b). PAs have antioxidant and anti-inflammation activities (Salunkhe et al., 1983). In plants, PAs are also responsible for pigmentation of plant tissues and usually accumulate in seed coats, leaves, stems and roots (Prasad, 2000; Peng et al., 2012).

Evidence that PAs are contained in brown cotton fibres was first obtained with the qualitative colour reaction with DMACA (dimethylaminocinnamaldehyde), which is commonly used to detect PAs in plant tissues, leading to blue staining (Harland, 1932; Hutchinson and Silow, 1939; Xiao et al., 2007). The comparative treatment of white and brown cotton fibres with DMACA has demonstrated that brown cotton fibre turned into dark-blue, while the mature white fibre did not show a significant difference in colouration (Li et al., 2013). In addition, the more days after anthesis (DPA) have passed, the darker the brown colour shade of cotton fibre. This observation indicates that the oxidized PAs content increases proportionally with maturation of cotton bolls (Feng et al., 2014).

The structure of PAs units in brown cotton fibre was confirmed using a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS) analysis (Nechepurenko et al., 2009; L. Wang et al., 2016). The results revealed that an initiating unit derived from gallocatechin; all extension units contain 3 hydroxyl groups in B-ring (Bogs, 2005; He, Pan, Shi, and Duan, 2008; Ershik and Buzuk, 2009; Feng et al., 2014; Xiao et al., 2014). The structures of PAs are different in white and brown cotton fibres. The content of prodelphinidin (PD) and proanthocyanidin (PC) are equal in white cotton, while in brown 90% PD and 10% PC was found (He et al., 2008; Feng et al., 2014). Moreover, it was detected that the structure of PAs in white cotton was modified by a galloyl group (Xiao et al., 2014; Feng et al., 2014). Also, the MALDI-TOF MS proteomic analysis of brown cotton fibre identified that 21 proteins were related to secondary metabolism processes and 15 of them were associated with the flavonoid biosynthesis pathway (Li et al., 2013).

### PAs BIOSYNTHESIS DURING THE PHENYLPROPANOID AND FLAVONOID PATHWAY

**Structural genes involved in PAs biosynthesis.** PAs are synthesized during the flavonoid pathway in different plant tissues (Debeaujon, 2003; Bogs, 2005; He et al., 2008). Identified genes involved in this pathway in *G. hirsutum* are listed in Table 1.

Phenylalanine is a precursor for all flavonoids, including PAs (Peng et al., 2012; Li et al., 2013; Thomas and ElSohly, 2016). The phenylpropanoid pathway begins with transformation of phenylalanine by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL) into 4-Coumaroyl-CoA (Fig. 2). 4-Coumaroyl-CoA exposed such enzymes as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3', 5'-hydroxylase (F3'5'H) and dihydroflavonol 4-reductase (DFR) to leucoanthocyanidins (Fig. 2). Leucoanthocyanidins and anthocyanidins are converted into flavan-3-ols by leucoanthocyanidin reductase (LAR) and anthocyanin reductase (ANR), respectively (Fig. 2). Anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR) lead to anthocyanidin formation from leucoanthocyanidin — a PAs precursor (Bogs, 2005; Peng et al., 2012; Jiafu Tan et al., 2013; Malik et al., 2015). PAs represent the polymer based on flavan-3-ol units (Nechepurenko et al., 2009; Feng et al., 2014; Xiao et al., 2014).

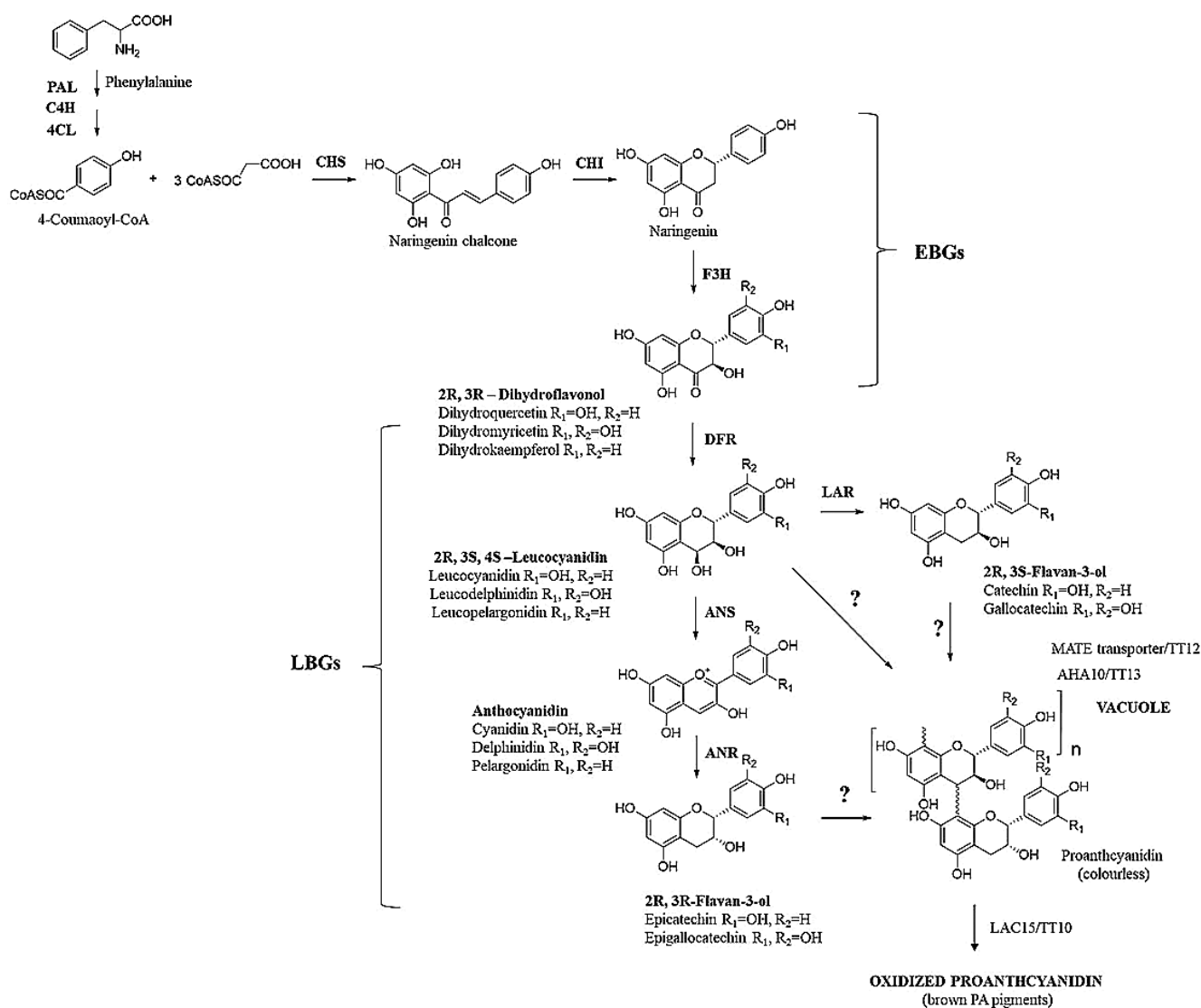
The flavonoid biosynthesis pathway determines pigmentation in cotton fibre (Feng et al., 2013, 2014; Liu et al., 2018). Recently it was demonstrated that overexpres-

**Table 1. The structural genes which are involved in PAs biosynthesis**

<i>G. hirsutum</i> gene	Chromosome; Gene ID (CottonFGD*)	The protein family	Function	References
<i>GhPAL</i>	A01; Gh_A01G1839 D01; Gh_D01G2080	PAL/histidase family	Phenylalanine ammonia-lyase activity	Feng et al., 2017
<i>GhPAL2</i>	A06; Gh_A06G0667 D06; Gh_D06G0758			Qin et al., 2017
<i>GhC4H1</i>	A10; Gh_A10G1590 D10; Gh_D10G1845	Cytochrome P450	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen; iron-ion binding; heme binding	Ling Fan and Wen-Ran Hu, 2012
<i>GhC4H2</i>	A13; Gh_A13G2057 D13; Gh_D13G2458			Ling Fan and Wen-Ran Hu, 2012; Feng et al., 2013
<i>GhCHS</i>	A10; Gh_A10G1079 D10; Gh_D10G1429	Chalcone/stilbene synthases family	Transferase activity (transferring acyl groups other than amino-acyl groups)	Feng et al., 2013
<i>GhCHI</i>	A05; Gh_A05G3491 D04; Gh_D04G1926	Chalcone-flavonone isomerase family protein	Catalyses the intramolecular cyclization of bicyclic chalcones into tricyclic (S)-flavanones	Xiao et al., 2007; Li et al., 2013; Malik et al., 2015
<i>GhF3H</i>	A12; Gh_A12G0549 D12; Gh_D12G0566	2-oxoglutarate-dependent dioxygenases superfamily	Oxidoreductase activity	Xiao et al., 2007; Malik et al., 2015
<i>GhF3'H</i>	A12; Gh_A12G2650 D12; Gh_D12G1798	Cytochrome P450	Oxidoreductase activity	Feng et al., 2013
<i>GhF3'5'H</i>	A07; Gh_A07G1098 D07; Gh_D07G1197			
<i>GhDFR</i>	A05; Gh_A05G1647 D05; Gh_D05G1836	Reductase-epimerase-hydrogenase	Catalytic activity; coenzyme binding	Xiao et al., 2007; Malik et al., 2015
<i>GhANS</i>	A08; Gh_A08G1593 D08; Gh_D08G1902	2-oxoglutarate-dependent dioxygenases superfamily	Oxidoreductase activity	
<i>Gh3GT</i>	A05; Gh_A05G3537 D04; Gh_D04G0070	Glycosyltransferase family	Catalyzes the glycosylation of flavonoids at the 3-O-position	Liu et al., 2018
<i>GhANR</i>	A05; Gh_A05G1424 D05; Gh_D05G1596	Reductase-epimerase-hydrogenase	Catalytic activity; coenzyme binding	Xiao et al., 2007; Malik et al., 2015; Yan et al., 2018
<i>GhLAR</i>	A12; Gh_A12G1558 D12; Gh_D12G1686	NAD(P)-binding domain superfamily	Oxidoreductase activity	Yan et al., 2018
<i>Gh4CL1</i>	A02; Gh_A02G1344 D03; Gh_D03G0479	AMP-binding	Ligase activity	Feng et al., 2017; Ling Fan and Wen-Ran Hu, 2012
<i>Gh4CL2</i>	A02; Gh_A02G1344 D03; Gh_D03G0479			
<i>GhCAD1</i>	A02; Gh_A02G1320 D03; Gh_D03G0457	Zinc-containing alcohol dehydrogenase family	Cinnamyl-alcohol dehydrogenase activity; zinc-ion binding	Ling Fan and Wen-Ran Hu, 2012
<i>GhCAD3</i>	A05; Gh_A05G1579 D05; Gh_D05G1757			
<i>GhCAD7</i>	A11; Gh_A11G2005 D11; Gh_D11G1980	Zinc-containing alcohol dehydrogenase family	Cinnamyl-alcohol dehydrogenase activity; zinc-ion binding	Qin et al., 2017; Ling Fan and Wen-Ran Hu, 2012
<i>GhCAD5</i>	A12; Gh_A12G2203 D12; Gh_D12G2382			
<i>GhCAD6</i>	A12; Gh_A12G0193 D12; Gh_A12G0195			

\* CottonFGD — Cotton Functional Genomics Database





**Fig. 2.** Anthocyanins and PAs biosynthesis during flavonoid pathway (the scheme are modified from Schijlen, et al. 2004). Enzymes are involved in biosynthesis, abbreviated as: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-(hydroxy)cinnamoyl CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. MATE/TT12 multidrug and toxin extrusion family, AHA10/TT13 — H<sup>+</sup>-ATPase isoform 10, LAC15/TT10 — laccase 15. MATE and AHA10 participate in transportation of PAs precursors from cytoplasm to vacuole. Early biosynthetic genes and later biosynthetic genes are designate as EBGs and LBGs, respectively. LBs regulated by MBW complex (TT2-TT8-TT10) in monocots plants, where MBW is Myb-bHLH-WD40 repeat complex.

sion of *Gh3GT* coding the flavonoid 3-glucosyltransferase leads to green cotton fibre formation in brown cotton line (H.-F. Liu et al., 2018). Furthermore, three fibre phenotypes, including brown, white and green colour traits, were observed under downregulation of the *Gh-CHI* gene in brown cotton using the RNAi-mediated inhibition approach (Abdurakhmonov et al., 2016).

The suppression of the *F3H* gene leads to accumulation of naringenin (NAR), which inhibits the rate of fibre development (Tan et al., 2013). Transformation of *F3H*-RNA interference segment into the DNA of brown cotton fibre of the *G. hirsutum* line has demonstrated PA content reduction and a fibre shortening compared

with control samples (Gong et al., 2014). On the other hand, the overexpression of the *F3H* gene has no effect on cotton fibre length. This experiment has shown that flavonoid metabolism could be associated with fibre pigmentation and quality (Dutt et al., 2004; Hua et al., 2009; Tan et al., 2013; Feng et al., 2015; Hande et al., 2017).

Several investigations show that the transcription level of structural flavonoid biosynthesis genes is higher in brown than in white cotton fibre. Transcription analysis reveals that the genes related to the PAs biosynthesis pathway from phenylalanine to flavan-3-ols were up-regulated in brown cotton (Xiao et al., 2014). The expression level of main structural genes such as *GhCHI*,

*GhF3H*, *GhDFR*, *GhANS* and *GhANR* at 8, 16, 20 and 30 DPA was higher in brown compared with white and green cotton fibre (Xiao et al., 2007; Malik et al., 2015). Similar differences in the transcriptional activity of *GhPAL*, *GhCHI*, *GhDFR*, *Gh3GT*, *GhANR* (Li et al., 2013) and *GhC4H*, *GhCHS*, *GhF3'H*, *GhF3'5'H* genes (Feng et al., 2013) in white and brown cotton were noticed.

The high transcription level of *GhANS* and *GhANR* leads to anthocyanidin formation from leucoanthocyanidin (Peng et al., 2012; Malik et al., 2015). Since the expression of the identified genes play a key role in the PA biosynthesis pathway, it might be possible to control PA accumulation and, as a result, to control shades of brown colour during fibre development (Malik et al., 2015).

**The transcription factors in PAs biosynthesis.** Flavonoid biosynthesis is controlled by many transcription factors (TFs) such as R2R3-MYB-type factors, basic helix–loop–helix (bHLH) and WD40 repeat (Koes et al., 2005; Qi et al., 2011; Lloyd et al., 2017). These TFs could form a MBW complex that has a determinant role in the regulation of pigment biosynthesis during the flavonoid pathway (Baudry, Caboche, and Lepiniec, 2006). Using *Arabidopsis* as an experimental model, it was shown that R2R3-MYB proteins activate early anthocyanins and PA biosynthetic genes (EBGs) (Fig. 2). The expression of late biosynthetic genes (LBGs) is controlled by MYB-bHLH-WD40 repeat (MBW) complex (Fig. 2) (Li, 2014).

It was reported that in *Arabidopsis* the R2R3-MYB-coding gene *AtTT2* (TRANSPARENT TESTA 2) initiates the PAs biosynthesis (Nesi et al., 2001). Two homologous candidate genes — *GhMYB10* and *GhMYB36* (later *GhTT2-3A*) — were isolated from tetraploid cotton using BLAST analysis and related to R2R3-type MYB transcription factors (Table 2) (Lu, Roldan, and Dixon, 2017). Transactivation assays shown that these genes are the part of the regulative MBW complex that could activate *LAR* and *ANR* promoters (Albert et al., 2014; Jun et al., 2015; Li, Chen et al., 2016; Li, Dong et al., 2016; Liu, Jun, and Dixon, 2014). The simultaneous presence of both proved that MYBs from the *G. hirsutum* genome lead to more effective PAs biosynthesis (Lu et al., 2017).

The *GhTT2-A07* gene (later *GhTT2-3A*), which is related to brown colour cotton fibre as *Lc1* locus, was identified (Table 2) (Hinchliffe et al., 2016). Using transgenic analysis and expression levels, the *GhTT2-3A* gene has been shown to regulate the formation of brown fibres (Yan et al., 2018).

The *Arabidopsis* bHLH-type gene *AtTT8* (TRANSPARENT TESTA 8) is transcriptionally activated by *AtTT2*; it has a further synergic cooperative influence on the PAs structural genes and enhances their expression level (Baudry et al., 2004; Koes et al., 2005; Baudry, Caboche, and Lepiniec, 2006; Shangguan, Yang, Zhang, and Wang, 2016). Similar observations were reported for

**Table 2. Regulatory genes which are involved in PAs biosynthesis pathway in cotton**

<i>G. hirsutum</i> gene	Chromosome; Gene ID (CottonFGD*)	The protein family	References
<i>GhMYB10</i>	A06; Gohir.A06G075700 D06; Gohir.D06G074700	MYB domain	Lu, Roldan, and Dixon, 2017
<i>GhTT2-3A</i> ( <i>GhMYB36</i> )	A07; Gh_A07G2341 D07; Gh_D07G0169		Hinchliffe et al., 2016
<i>GhbHLH130D</i>	A11; GH_A11G1273 D11; GH_D11G1302	bHLH domain	Yan et al., 2018
<i>GhTTG1</i>	A05; Gohir.A05G415900	WD-repeat	Humphries et al., 2005
<i>GhTTG3</i>	D04; Gohir.D04G000300		

\* CottonFGD — Cotton Functional Genomics Database

*GhTT2-3A* and *GhbHLH130D* (*AtTT8* ortholog) cotton genes — it was established that *GhTT2-3A* activates the *GhbHLH130D* gene (Table 2) (Gong et al., 2018). Dual-luciferase assays demonstrated that the *GhTT2-3A* and *GhbHLH130D* genes affect the PAs biosynthesis structural genes, *GhANR* and *GhLAR*, and consequently induce PAs accumulation in cotton fibre (Yan et al., 2018).

It was established that WD40-coding gene *AtTTG1* (TRANSPARENT TESTA GLABRA 1) in *Arabidopsis* is responsible for seed coat production, root hair development and control of anthocyanins biosynthesis (Oppenheimer et al., 1991; Galway et al., 1994; Liu, Osbourn, and Ma, 2015). Among four *GhTTG1-GhTTG4* genes known to be involved in the cotton fibre growing initiation, *GhTTG1* and *GhTTG3* demonstrate close sequence similarity to the known *AtTTG1* anthocyanin regulatory gene (Table 2) (Walker et al., 1999; Mehboob-ur-Rahman et al., 2012; B. Liu, Zhu, and Zhang, 2015). Furthermore, a transient analysis determined that the *GhTTG1* and *GhTTG3* genes were able to complement the purple pigment in anthocyanin-deficit *ttg1 Arabidopsis* mutants, in contradistinction to *GhTTG2* and *GhTTG4* (Humphries et al., 2005; Marinova et al., 2007). Thus, WD-repeat *GhTTG1* and *GhTTG3* genes play a significant role during cotton fibre formation and in PAs biosynthesis regulation (Humphries et al., 2005).

**Other genes.** Structural genes involved in PAs modification, transport and oxidation processes have also been identified and investigated in the *G. hirsutum* genome. In particular, the *TT12* gene encodes proteins related to MATE (MULTIDRUG AND TOXIN EXTRUSION) family proteins which are responsible for the transfer of epicatechin-3'-O-glucoside, a precursor of PAs, into a central vacuole (Marinova and Pourcel, 2007; Zhao and Dixon, 2009). The *GhTT12* gene was studied using genetic engineering manipulations (Gao et al., 2016). A full-length cDNA of *TT12* from brown

cotton fibre was cloned in *N. tabacum* to study the expression level. The results have confirmed the potential participation of the *GhTT12* gene in PAs transportation (Gao et al., 2016).

Phylogenetic analysis supposes that cotton's MATE proteins are merged in the group with the TT12-like MATE transporters in *Arabidopsis* participating in PA transportation from the cytoplasmic matrix into the vacuole (Xu et al., 2018). The *GhMATE12*, *GhMATE16* and *GhMATE38* genes identified using phylogenetic analysis share a similar transcription activity with the *GhTT12* gene at specific development stages. As a result, detected genes could participate in transportation of PAs into the vacuole along with *GhTT12* (Xu et al., 2018). Besides, it was shown that the transcript level of the *GhMATE1a* and *GhMATE1b* genes was higher in brown than in white cotton (Feng et al., 2014). The highest value was enriched to 21 DPA for the *GhMATE1a* gene and to 27 DPA in the case of *GhMATE1b* (Feng et al., 2014).

It is known that Aha10 (H<sup>+</sup>-ATPase isoform 10) belongs to p-type ATPase family plasma membrane H<sup>+</sup> pump and is involved in the PAs accumulation process in the vacuole (Baxter et al., 2005; Appelhagen et al., 2015). *Aha10* mutants are characterized by the formation of a large number of small vacuoles instead of one big central vacuole. In addition, production of final metabolites in the flavonoid biosynthesis pathway such as PAs, anthocyanidins and epicatechins breaks down while the primary products are present (Baxter et al., 2005).

The *AtTT10* gene of *Arabidopsis* is responsible for a laccase-like 15 enzyme (LAC15) synthesis, involved in conversion of colourless epicatechin monomer and oligomers to brown oxidized tannins in the seed coat (Pourcel et al., 2005; Lloyd et al., 2017). The gene orthologue to *AtTT10* was identified in *G. hirsutum* (Hinchliffe et al., 2016).

The *AtTT13* gene from *Arabidopsis* is responsible for PA production in the seed coat endothelium (Gonzalez et al., 2016; Lloyd et al., 2017). The *GhTT13* gene, an orthologue to *AtTT13*, was detected in upland cotton *G. hirsutum* (Hinchliffe et al., 2016).

Thus, the control of PAs biosynthesis is controlled by factors which are also associated with anthocyanin biosynthesis. The main structural genes, as well as the components of the MBW complex controlling the synthesis of PAs, are currently discovered.

### 3. Caffeic acid derivatives in green cotton fibre

Caffeic acid (CA) represents a cross-linked phenolic polymer that derived from three types of phenylpropane units: coniferyl, sinapyl and *p*-coumaryl alcohol (Fig. 3) (Schmutz et al., 1994; Ma et al., 2016a). CA is involved in lignin biosynthesis, which is a main structural com-

ponent of the secondary cell wall in higher plants (Riley and Kolattukudy, 1975; Liu, Luo, and Zheng, 2018). It was reported that the secondary cell wall of cotton fibre contains mainly cellulose (about 94%) and 0.4–1% lignin (Fan et al., 2009).

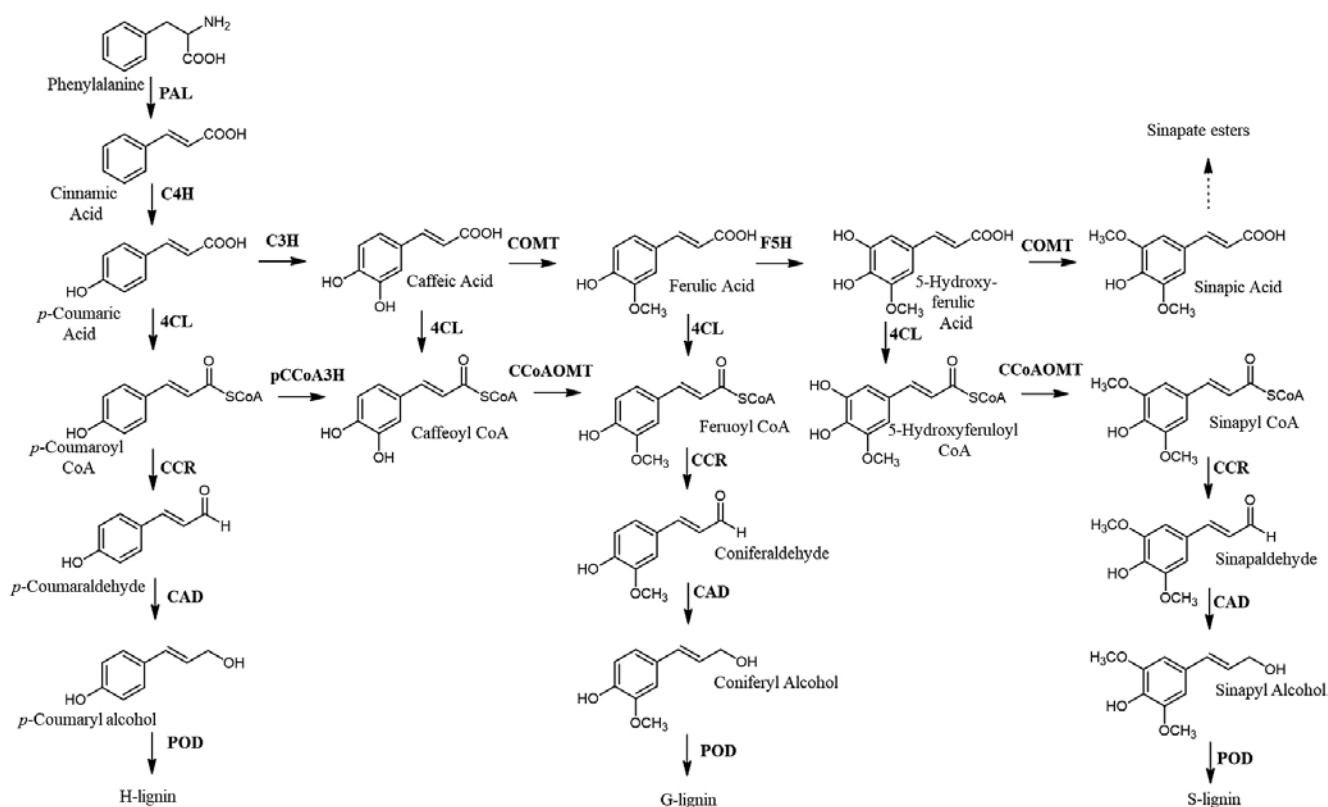
It was shown that CA derivatives are responsible for the formation of green coloured cotton fibre: about 70% of  $\omega$ -hydroxydocosanoic acid and 25% of docosanedoic acid were isolated from green cotton fibre, compared with 0.5% in white fibre (Schmutz et al., 1993, 1994). Using an ultra-violet spectroscopy and a nuclear-magnetic-resonance (<sup>1</sup>H-NMR) spectroscopy it has been shown that glycerol, CA and esterified CA constitute the main part of the wax fraction in green cotton fibre (Schmutz et al., 1993).

Green cotton fibre demonstrates the significant increase in UV-absorption properties — the radical-scavenging activity rate was approximately 20 times more than white cotton fibre due to the accumulation of CA derivatives (Re et al., 1999; Ma et al., 2016a; Masek, 2016). A decrease in the ability of radical scavenging capacities is observed under alkaline solution treatment of green and brown fibre cotton due to enclashing of fibre hydrophilic properties leading to barrier-free penetration of free radicals to the active component inside the fibre. The reason is alkaline hydrolysis of the ester bond and the release of water-soluble CA (Ma et al., 2016b). Moreover, the significant changes in fibre colour have been observed after alkaline treatment (Hinchliffe et al., 2015). This emphasizes the essential role of CA and its derivatives in fibre pigmentation and indicates the necessity of the phenylpropanoid pathway in colouration of green cotton fibre (Fan et al., 2009).

### SUBERIZATION OF GREEN COTTON

Suberin and cutin biopolymers usually occur on the surface of higher plants — suberin accumulates into inner cell-wall and cutin is found at the surface of epidermal cells (Moire et al., 1999; Nawrath, 2002; Graça, 2015). These compounds protect plant leaves from adverse environmental factors (Kolattukudy, 1980; Yatsu et al., 1983). Suberin is formed from poly-functional fatty acids that are bound by ester bonds with glycerol (Kolattukudy, 1980; Matzke and Riederer, 1991; Graça, 2015). Epidermal cells of cotton seeds with green fibres produce both cutin and suberin (Ryser et al., 1983; Yatsu et al., 1983; Schmutz et al., 1993; Stankovič Elesini et al., 2002).

Suberization of the cotton seed coat is characteristic feature of *Gossypium* species (Ryser et al., 1985). However, a suberin layer has not been found in the seed coat of brown and white cotton fibres (Ryser et al., 1983, 1985; Schmutz et al., 1993). Multiple concentric rings of laminar ultrastructure similar to the suberin layer in the cell wall of green cotton fibre were demonstrated using electron microscopy



**Fig. 3.** Phenylpropanoid pathway of caffeic, ferulic and sinapic acids biosynthesis (modified from Humphreys, et al. 1999). The involved enzymes: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, *p*-Coumarate 3-hydroxylase; 4CL, 4-(hydroxycinnamoyl CoA ligase; COMT, caffeic acid 5-hydroxyferulic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; pCCoA3H, *p*-Coumaroyl CoA 3-hydroxylase; CCoAOMT, caffeoyl CoA O-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamoyl alcohol dehydrogenase; POD, peroxidase.

(Yatsu et al., 1983). Simultaneously, the white fibre cotton cell wall had a thin outer cuticle (Yatsu et al., 1983).

Phenylalanine ammonium-lyase is recognized to be an important agent in converting phenylalanine into trans-cinnamic acid. Results of an experiment with the addition of 2-aminoindan-2-phosphonic acid, known as an inhibitor of phenylalanine ammonium-lyase, to cultivating ovules have shown that an esterified CA is covalently linked with suberin and plays a significant role in the attachment of suberin to a cellulosic secondary wall (Schmutz et al., 1993). Moreover, green fibre colour could not be observed if the suberin formation is inhibited during tissue development.

There are some studies describing an accurate chemical structure of aliphatic components of the suberin layer (Schmutz et al., 1993; Ma et al., 2016a; Feng et al., 2017). It was demonstrated by reverse-phase analytical high-pressure liquid chromatography (HPLC) that extracted yellow pigment from the wax of green fibre cotton contains two major components and several minor ones. Namely, 22-O-caffeoyl-22-hydroxydocosanoic acid, glycerol ester, and 22-O-caffeoyl-22-hydroxydocosanoic acid were identified as main components of yellow pigment extract. According to the ultra-violet visible spectroscopy data, the minor components could be referred

to CA derivatives (Ma et al., 2016a). Also, several fatty acids including dimethyl-2-hydroxysuccinate monoglyceride and heptacosanoic acid monoglyceride were isolated from green cotton fibre (Schmutz et al., 1993; Ma et al., 2016a; Feng et al., 2017). The structures were established by NMR spectroscopy on nuclei  $^1\text{H}$  and  $^{13}\text{C}$ , H-H Correlation Spectroscopy (H-COSY), Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) and were coincided with earlier data (Schmutz et al., 1994; Torresdepinedo et al., 2007; Feng et al., 2017). It was found that from the isolated fatty acids, 22-O-caffeoyl-22-hydroxydocosanoic acid and 22-O-caffeoyl-22-hydroxydocosanoic acid (yellowish and a green-yellow powder, respectively) were demonstrated to be responsible for pigmentation (Feng et al., 2017). It was observed that the higher the concentration of 22-O-caffeoyl-22-hydroxydocosanoic acid, the deeper green colour of the cotton fibre (Feng et al., 2017).

#### STRUCTURAL GENES RELATED TO CA AND ITS DERIVATIVES BIOSYNTHESIS

CA biosynthesis is based on the phenylpropanoid pathway (Fig. 3) (Humphreys, Hemm, and Chapple, 1999; Boerjan, Ralph, and Baucher, 2003; Heldt and Heldt,



2005; Fan et al., 2009; Li et al., 2015). Identified genes involved in the CA biosynthesis pathway for *G. hirsutum* are listed in Table 1.

Trans-hydroxycinnamic acid is a product of the first stage catalysed by PAL, which transformed into *p*-coumaric acid due to para hydroxylation with C4H (Fig. 3). It should be noted that *p*-coumaric acid is a main precursor of other natural phenols (Boerjan et al., 2003; Ramawat and Mérillon, 2013; Shahidi and Yeo, 2018). Addition of OH-group is catalysed by *p*-coumarate 3-hydroxylase (C3H) at the ortho-position with CA formation, which serves as an initial compound in ferulic and sinapic acids biosynthesis (Fig. 3) (Heldt and Heldt, 2005).

Gyaiacyl lignin (G-lignin) consists of such monolignols as coniferyl alcohol, syringine lignin (S-lignin) and *p*-Hydroxyphenyl lignin (H-lignin), formed from sinapyl alcohol and *p*-Coumaryl alcohol through the phenylpropanoid pathway, respectively (Fig. 3) (Humphreys et al., 1999; Boerjan et al., 2003; Vanholme et al., 2010; Li, Pu, and Ragauskas, 2016). The ratio of monolignols affects the physical properties of lignin polymer (Macmillan et al., 2013).

From two phenylalanine ammonium-lyase genes, *GhPAL* and *GhPAL2*, the expression of *GhPAL* has a higher level during initiation of fibre secondary cell wall thickening (Ling Fan, Wen-Ran Hu, 2012; Feng et al., 2014; Qin et al., 2017). It has been shown that the *GhC4H1* and *GhC4H2* genes involved in lignin and flavonoid biosynthesis processes are expressed in developing cotton fibres (Ling Fan and Wen-Ran Hu, 2012).

*Gh4CL1-Gh4CL4* cotton genes encode 4-Coumarate CoA ligases (4CL), which are responsible for converting *p*-coumaric acid to *p*-Coumaroyl CoA and its derivatives such as ferulic, caffeic and sinapic acids into CoA esters (Figs. 2, 3). These compounds take part in the phenylpropanoid pathway, providing the plant secondary metabolites formation (Hamberger and Hahlbrock, 2004; Ling Fan, Wen-Ran Hu, 2012; Li et al., 2015). Four isoforms of the *Gh4CL1-Gh4CL4* genes were identified in the genome of white and green *G. hirsutum* L. The expression profiles studding of 4CL structural genes in white and green cotton fibre were assayed by RT-PCR (Feng et al., 2017). *Gh4CL3* and *Gh4CL4* demonstrate a higher transcript level in roots and hypocotyls than in flowers and leaves and have a similar expression profile in white and green fibre gradually increased to 24 days of growing. *Gh4CL1* expression decreased with fibre development in white and green cotton. The highest expression level among all considered genes was observed in *Gh4CL2* in green fibre (Feng et al., 2017).

The catalytic properties of proteins related to 4CL cotton structural genes displayed that *Gh4CL2* has the highest turnover rate for caffeic, cinnamate and ferulate in comparison to *Gh4CL3* and *Gh4CL4* (Feng et al., 2017). *Gh4CL1* showed a low turnover rate also, but

demonstrated a preference for 4-coumarat synthesis (Feng et al., 2017). Considering the results of expression level studding and enzyme activity of corresponding genes, it can be proposed that the *Gh4CL2* gene is involved in the metabolism of CA derivatives and is responsible for pigmentation of green cotton fibres (Ling Fan and Wen-Ran Hu, 2012).

It was found that among seven *GhCAD1-GhCAD7* genes similar to *AtCAD5* in *A. thaliana*, only the *GhCAD6* gene showed up-regulation during cotton fibre development. This result displays the involvement of this gene in the phenylpropanoid pathway (Fan et al., 2009; Ling Fan and Wen-Ran Hu, 2012).

Thus, the nucleotide sequences of most genes involved in CA derivatives biosynthesis and modification have not yet been identified.

## Conclusion

The article provides comprehensive knowledge of historical aspects and describes the various approaches associated with the research of phenolic pigments isolated from coloured cotton. Structural and regulatory genes involved in PAs and CA biosynthesis have been characterized in the current review. Biosynthesis of these plant phenolic compounds occurs via the phenylpropanoid pathway. The metabolism of PAs is most fully studied at the genetic level. However, there are no studies concerning transcriptional regulation of CA biosynthesis. In addition, we have a limited understanding of the pleiotropic influence of the genes responsible for colour on the fibre quality attributes. Further study of the genes related to PAs and CA biosynthesis in combination with available data will make it possible to reconstruct the complete genetic regulatory network of the biosynthesis of these compounds.

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