INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–335/2015/17–1–205–210
http://www.fspublishers.org

Full Length Article

Transcriptome Analysis of Pigment Related Genes in Colored Cotton

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Abstract

The biochemical basis of pigmentation has been well studied in naturally colored cotton but little is known about the molecular basis of color development in cotton fibers. The differences in pigmentation between brown, green and white cotton fiber can be mined by the molecular study of structural genes of flavonoid biosynthesis pathway. The transcriptome analysis of five flavonoid biosynthesis related structural genes, encoding chalcone isomerase (GhCHI), flavanone 3-hydroxylase (GhF3H), dihydroflavonol 4-reductase (GhDFR), anthocyanidin synthase (GhANS), and anthocyanidin reductase (GhANR) was performed at two fiber developmental stages i.e. 10 DPA and 20 DPA in brown, green and white cotton. The transcript level of all structural genes was higher in brown cotton fiber as compared to green and white at both 10 DPA (days post anthesis) and 20 DPA. The decline in the transcript level of all structural genes except *GhANS* was observed at 20 DPA as compared to 10 DPA. The transcript accumulation of *GhANS* was negligible in green and white cotton fibre at both stages. Correlating these findings with the flavonoid biosynthetic pathway revealed that *GhANR* and its substrate *GhANS* are crucial for the synthesis of proanthocyanadines, which is a brown pigment and transcripts of structural genes are directly proportional to the brown pigment. In conclusion, *GhANS* gene is strongly associated to the brown color development and modulation in the transcript level of this gene could improve the pigmentation of brown cotton fibers.

Keywords: qRT-PCR; Structural genes; Falovonides; Anthocyanidin synthase; Proanthocyanidins

Introduction

The physiological and molecular mechanisms of pigmentation in cotton fibers are not well researched. Traditional genetic analysis revealed that brown fiber color was controlled by six loci and only one locus is involved in the inheritance of green fiber color (Kohel, 1985). Extraction of pigments from naturally colored fiber has revealed the fact that flavonoids are involved in the development of fiber color (Hua *et al*., 2007).

The flavonoids are secondary metabolites that are found throughout the plant kingdom (Koes *et al*., 1994). These are involved in pigmentation of flowers, fruits and seeds (Koes *et al*., 1994), and development of fiber color (Hua *et al*., 2007). The flavonoids provide protection from ultraviolet light, microorganisms, senescence of tissues and also act as signal molecules in plant microbe interaction (Dooner *et al*., 1991; Dixon and Paiva, 1995). Flavonoid biosynthesis pathway has been explored and many regulatory and structural genes involved in flavonoid biosynthesis have been cloned from various plants i.e. maize, Antirrhinum, tobacco, Petunia and Arabidopsis (Holton and Cornisch, 1995). In the last decade many ornamental plants with new types of pigmented flowers have been developed by modification in the expression of structural genes involved in the flavonoid biosynthesis pathway (Aida *et al*., 2000a, b; Zuker *et al*., 2002; Fukui *et al*., 2003).

The genes involved in the biosynthesis of flavonoids are divided into two classes, the structural genes, which are directly involved in the synthesis of flavonoids and the regulatory genes that control the expression of structural genes during the flavonoids biosynthesis pathway (Forkmann and Heller, 1999). Flavonoid biosynthesis pathway can be illustrated on the basis of structural genes involved in the synthesis of precursors for different classes of flavonoids (Takos *et al*., 2006). The basic precursors involved the biosynthesis of flavonoids are malonyl-CoA and p-coumaroyl-CoA, which are derivatives of carbohydrates and phenylepropanoid pathways respectively (Forkmann and Heller, 1999).

Flavonoid biosynthesis is initiated by an enzymatic

To cite this paper: Malik, W., A.A. Khan, H.M.N. Cheema, U. Aslam, M.Z. Iqbal, A. Qayyum, A. Yasmeen and N. Bibi, 2015. Transcriptome analysis of pigment related genes in colored cotton. *Int. J. Agric. Biol.,* 17: 205–210

step catalyzed by chalcone synthase (CHS) that results in production of yellow color chalcone (naringenin chalcone). This yellow color product is not the end product of flavonoid biosynthesis pathway, but keeps on producing different classes of flavonoids in the presence of several other enzymes (Schijlen *et al*., 2004). The CHS structural genes have been attended by plant scientists (van der Meer *et al*., 1992; Jorgensen *et al*., 1996; Deroles *et al*., 1998) and the down regulation of these genes turned the colored flower to white. After the formation of naringenin chalcone, flavanones are produced by another enzyme known as chalcone isomerase CHI (Fig. 1). A mutation in CHI genes modify the flower colour in *Diathus cayophyllus*, while it led to change in seed coat color in Arabidopsis (Forkmann and Heller, 1999). Transformation of CHI genes also enhanced the flavonoids contents in tomato (Muir *et al*., 2001). Flavonone-3-hydroxylase is also one of the important enzymes of flavonoid biosynthesis pathway found to be highly conserved among different plant species (Britsch *et al*., 1993). A mutation in F3H gene could prevent the progression along flavonoid biosynthesis pathway and consequently produce no colour (Martin *et al*., 1991; Britsch *et al*., 1992). The next enzymes DFR (dihydroflavonols 4-reductase) in flavonoid biosynthesis pathway catalyze the reduction of dihydroflavonols to leucoanthocynidines (Kristiansen and Rohde, 1991). The anthocyanidin synthase (ANS) catalyzes the conversion of leucoanthocynadine (colorless) into anthocyanidin and if anthocyanidin reductase (ANR) catalyzed the reaction, proanthocyanidins (pigmentation) is produced (Nakatsuka *et al*., 2005; Takos *et al*., 2006).

Naturally colored cotton is getting popularity for its advantage of circumventing the dying process in the textile industry. Exploring the mechanism of pigmentation can pave the way of pigment engineering for a variety of color in cotton fiber. The purpose of this research was to investigate the putative involvement of flavonoid biosynthesis pathway in the pigmentation of colored cotton fiber. The transcript level of five flavonoids biosynthesis pathway related structural genes i.e. *GhCHI*, *GhF3H*, *GhDFR*, *GhANS* and *GhANR* (Xiao *et al*., 2007) was studied at two fiber development stages in brown, green and white cotton fiber.

Materials and Methods

Plant Growth

The seeds of brown, green and white cotton genotypes were delinted in 10% H_2SO_4, washed with tap water and dried for 48 h at room temperature. The seeds were grown in a field during cotton season (from May to November, 2011). The flowers were tagged at 0 DPA (days post anthesis) for sample boll collection and continued till the third week of flowering.

Sample Collection and Total RNA Isolation

The samples of developing fibers of brown, green and white cotton were collected at 10 DPA and 20 DPA, wrapped in aluminum foil and preserved in liquid N_2 until RNA extraction. The bench clean up protocol was followed to avoid possible contamination and RNA degradation as all the materials were sterilized with bleach, ethanol and RNAse away solution (Invitrogen, USA). The Eppendorf tubes (1.5 mL) and microtips were treated with 0.01% DEPC (diethyl pyrocarbonate). The fruiting body wall (outer covering of cotton boll) of the samples was removed by gentle strokes of pestle in a mortar containing liquid N_2 and the fibers form 10 DPA and 20 DPA bolls were separated from the ovules using pre-chilled fine forceps. The isolated fibers were ground to fine powder and total RNA was extracted using Plant RNA Purification reagent (Invitrogen, USA). Quality of total RNA was determined by electrophoresis on 1% agarose gel containing 0.05% EthBr and observed under UV light. Contamination of genomic DNA was removed by DNAse (Promega, USA) treatment and RNA was quantified spectrophotometrically (Nano Drop-2000, Thermo Scientific, USA).

cDNA Synthesis

The first strand of cDNA (complementary DNA) was synthesized from 2 µg RNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA) with Oligo $(dT)_{18}$ primers according to manufacturer's instructions. The reaction was prepared in sterile water provided with kit. The reaction mixture was briefly centrifuged incubated at 65°C for 5 min followed by cDNA synthesis step at 42°C for 60 min and reaction was stopped by heating at 70°C for 5 min. The cDNA was diluted in final volume of 20 µL.

Primer Designing and Validation

The cDNA sequences of three internal control genes, *UBQ7*, *Gbpolyubiquitin* and *Histone-3* (Table 1) and flavonoid biosynthesis related structural genes; *GhCHI, GhF3H, GhDFR, GhANS* and *GhANR* (Table 2) were retrieved from NCBI database. The primers were designed using PRIMER 3.0 software by targeting Exon regions to avoid contamination by amplification from genomic DNA.

Prior to quantitative reverse transcriptase PCR (qRT-PCR) analysis, assay validation was carried out to obtain 100% amplification efficiency by serial dilution and optimum annealing temperature by gradient PCR. It eliminates the primer dimer formation and signal to noise ratio. This validation was performed using cDNA of brown, green and white cotton fibers at10 and 20 DPA.

Real Time RT-PCR Analysis

Normalized expression of five target genes *GhCHI*, *GhF3H*, *GhDFR*, *GhANS* and *GhANR* was measured by real time RT-PCR analysis using Syber Green chemistry.

Table 1: Housekeeping genes used as internal control for real time PCR analysis

Gene name	Gene bank Accession No	Primer sequences		Amplicon length (bp)
		Forward	Reverse	
UBQ7	DQ 116441	5'-AAGCCCAAGAAGATCAAGCA-3'	5'-CGCATTAGGGCACTCTTTTC-3'	114
Gbpolyubiquitin-1	AY375335	5'-CAAAGAAGGAATTCCCCCAG-3'	5'-CACCACGAAGACGAAGAACA-3'	131
Histon3	AF024716	5'-GTGAAATTGCCCAGGACTTC-3'	5'-GATCCTACGAGCCAACTGGA-3'	176

Table 2: Flavonoid structural genes related to pigmentation in coloured cotton for real time PCR analysis

Gene name	Gene bank Accession No	Primer sequences		Amplicon length (bp)
		Forward	Reverse	
GhCHI	EF187439	5'-GCCATTAACGGGTCAACAAT-3'	5'-AAGGAACCTTGGCCTGAAAT-3'	183
GhF3H	EF187440	5'-TAAGCCAGAGGGTTGGATTG-3'	5'-TCAGGTTGAGGGCATTTAGG-3'	183
GhDFR	EF187441	5'-TGGTGGTCGGTCCATTTATT-3'	5'-ACCTTCCGCTTTTGGATTCT-3'	173
GhANS	EF187442	5'-ACAATGCTAGTGGGCAGCTT-3'	5'-GCAGTTGCCTTGCATACTCA-3'	138
GhANR	EF187443	5'-TGGACCGATATCGAGTTC-3'	5'-TATGCTGCTGGGGACAAT-3'	180

Chalcone isomerase (*GhCHI*), Flavanone 3-hydroxylase (*GhF3H*), Dihydroflavonol 4-reductase (*GhDFR*), Anthocyanidin synthase (*GhANS*), Anthocyanidin reductase (*GhANR*)

The assay was normalized by using three housekeeping reference genes; *UBQ7*, *Histone-3* and *Gbpolyubiquitin*. Reaction was performed in 96 well PCR plate. Three biological and four technical replicates of brown, green and white fibers cDNA were used for each of primers to conduct the assay. The PCR were performed in 25 µL reaction volume containing 40 ng/µL cDNA template, 30 ng of each primer, 1X SYBER Green master mix (Thermo-Scientific, USA), and double distilled deionized water (d_3H_2O) to make up the reaction volume.

Real Time PCR was performed in Real Time PCR Detection System CFX96 (Bio Rad, USA) in a 96 well PCR plate. Cycling conditions for the PCR were: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, with an additional melt curve step at the end of each cycle, followed by a final extension for 8 min at 72°C. Specificity of the amplification was verified by Melt Curve analysis using CFX Manager Software.

Statistical Analysis

The C_t values (cycle threshold) obtained from Real Time PCR results were used to measure the normalized relative gene expression ($\Delta\Delta C_t$) of all target gene at 10 DAP and 20 DPA using CFX Manager software (BioRad, USA). Three housekeeping genes (*UBQ7*, *Gbpolubiquitin-1* and *Histone3*) were marked as reference genes, while rests of the genes were marked as target in the plate set up data entry. The $\Delta\Delta C_t$ values, calculated by inbuilt software tool, were exported. The relative quantification was carried out using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) along with standard deviation and graphs were generated using Microsoft Excel software.

Results

The qRT-PCR analysis provided normalized relative gene expression of five flavonoid related structural genes *GhCHI*, *GhF3H*, *GhDFR*, *GhANS* and *GhANR*. The data regarding internal control genes showed similar expression in all types i.e. brown, green and white cotton fibers at two different fiber development stages (Fig. 2 and 3).

The qRT-PCR showed that expression level of the five genes was 2.73 fold higher in brown cotton fiber as compared to green and white at 10 DPA (Fig. 4). Similar pattern was observed for all the genes at 20 DPA in brown cotton fibers as compared to green cotton fibers (Fig. 5). A comparison of flavonoid structural gene's transcript level at 10 DPA and 20 DPA in brown, green and white cotton fibers showed decline in transcript accumulation of *GhCHI*, *GhF3H*, *GhDFR* and *GhANR* in brown fibers at 20 DPA. The structural gene (*GhANS*) involved in the synthesis of Anthocyanidin showed higher transcript level at 20 DPA as compared to 10 DPA in brown cotton fibers. It was also observed that expression level of flavonoids structural genes was higher at 20DPA than 10DPA in white cotton fiber. The transcript accumulation of GhANS was almost negligible in green and white cotton fiber at both 10 DPA and 20 DPA. In green cotton fiber the transcripts of *GhF3H* and *GhDFR* were higher at 20 DPA but is was far less than brown cotton fiber at both fibre development stages i.e. 10 DPA and 20 DPA. In white cotton fiber all genes showed negligible expression at 10 DPA while expression of two genes *GhDFR* and *GhANR* was comparatively high at 20 DPA.

Discussion

Various genes are found to be involved in pigmentation during fiber development (Li *et al*., 2005; Lee *et al*., 2006; Shi *et al*., 2006; Xiao *et al*., 2007). Comparison of various genes transcript level by qRT-PCR is a dependable method to elucidate their role in pigmentation. However, main problem of this technique is the wrong interpretation of results due to variable concentrations and quality of RNA and efficiencies of retrotranscription during the synthesis of cDNA from RNA (Vandesompele *et al*., 2002).

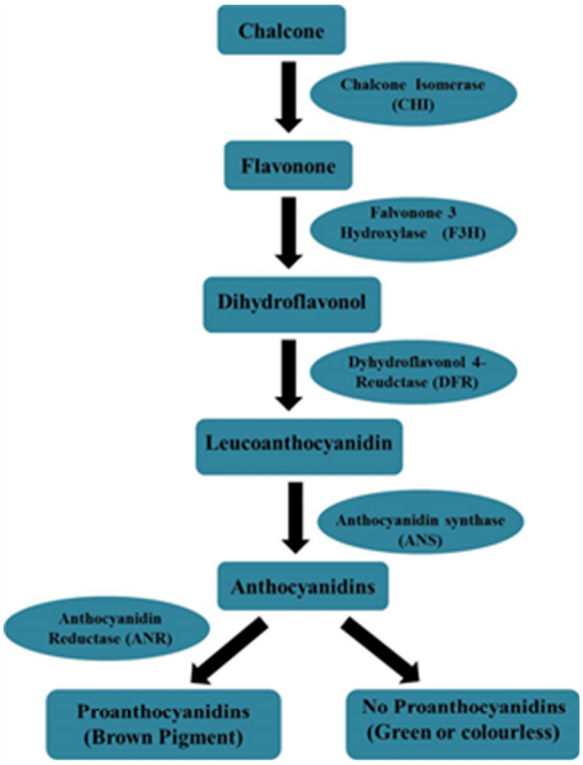

Fig. 1: Flavonoids biosynthesis pathway in cotton fiber

Therefore, expression is normalized against a housekeeping gene but there is a possibility of variation in expression of housekeeping genes due to dynamic changes in a biological system in response to environmental effects (Thellin *et al*., 1999; Czechowski *et al*., 2005). Transcriptome profiling during fiber morphogenesis showed variable expression of genes at various developmental stages (Indrais *et al*., 2011). Keeping in view all these factors, it is necessary to normalize real time RT-PCR data of cotton fiber against several internal control genes, especially when comparing two different developmental stages (Tu *et al*., 2007). Therefore, a combination of three internal control genes *UBQ7*, *Histone-3* and *Gbpolyubiquitin-1* were used to get clear picture of normalized relative expression of flavonoid biosynthesis related structural genes at various fiber morphogenesis stages.

The spatial and temporal gene expression is reported in single celled cotton fiber during its development (Applequist *et al*., 2001; Kim and Triplett, 2001; Iqbal *et al*., 2008). In naturally colored cotton the maximum pigment biosynthesis occurred during 5 DPA to 20 DPA. After 20 DPA a decline in the biosynthesis of pigmentation in cotton fiber is reported (Dutt *et al*., 2004; Hua *et al*., 2007). Different studies showed that pigmentation in cotton fiber is due to flavonoid biosynthesis during the course of fibre development (Dutt *et al*., 2004; Zhao and Wang, 2005; Hua *et al*., 2007). Therefore, normalized transcriptome analysis of flavonoid biosynthesis related five structural genes i.e.,

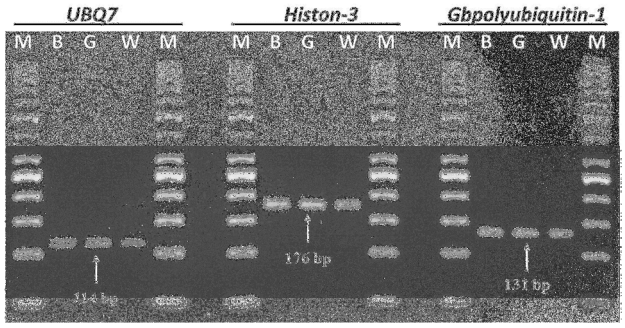

Fig. 2: Validation of reference genes, *UBQ7*, *Histone-3* and *Gbpolyubiquitin*, showed similar transcript level at 10 DPA in brown, green and white cotton fibers, M = 50 bp DNA ladder, B = brown cotton fiber, G = green cotton fiber, W = white cotton fibers

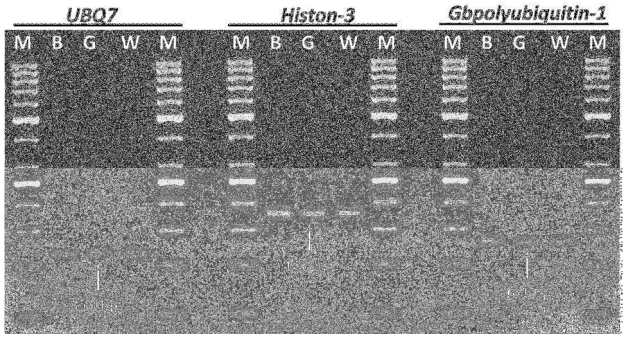

Fig. 3: Validation of reference genes, *UBQ7*, *Histone-3* and *Gbpolyubiquitin*, showed similar transcript level at 20 DPA in brown, green and white cotton fibers, M = 50 bp DNA ladder, B = brown cotton fiber, G = green cotton fiber, W = white cotton fibers

GhCHI, *GhF3H*, *GhDFR*, *GhANS* and *GhANR* was carried out at 10 and 20 DPA to find their role in cotton fiber pigmentation. The highest transcript level at 10 and 20 DPA of brown fiber as compared to green and white cotton fiber suggested the role of flavonoids in pigmentation of brown cotton and these flavonoids were not involved in pigmentation of green cotton fiber. These results also showed the relationship of phenotype with changed transcript level (Takos *et al*., 2006; Xiao *et al*., 2007). In white cotton fiber higher expression of flavonoid structural genes at 20 DPA than 10 DPA (Fig. 5) might be due to the involvement of flavonoids and their derivatives in the formation of lignin during cotton fiber cell wall synthesis at 20 DPA (Kim and Triplett, 2001; Hua *et al*., 2007). At this stage an ample amount of carbohydrates is required for cellulose synthesis, which is also involved in the signal transduction pathway. It leads to the activation of genes involved in flavonoid biosynthesis and resultantly higher transcript level of flavonoids structural genes was observed at 20 DPA (Solfanelli *et al*., 2006). The decline in transcript level of all genes except *GhANS* from 10 and 20 DPA in

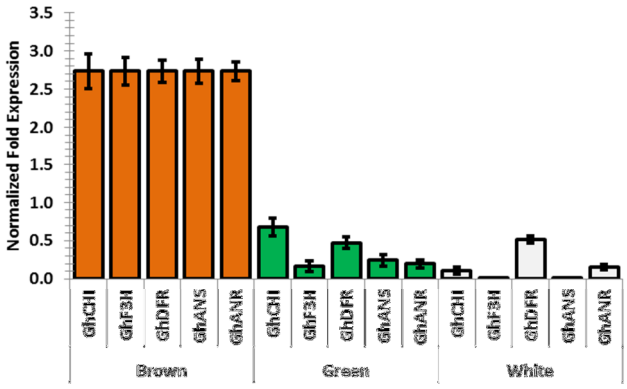

Fig. 4: Transcript level of five flavonoid related structural genes at 10 DPA

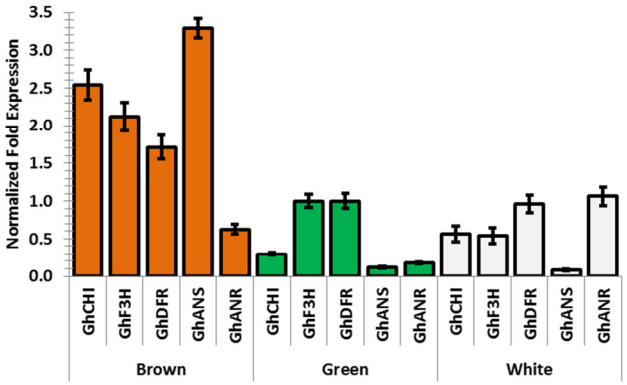

Fig. 5: Transcript level of five flavonoid related structural genes at 20 DPA

brown cotton indicated the temporal regulation of these genes at different stages of fiber development (Xiao *et al*., 2007). A high expression of *GhANS* at 20 DPA probably involved regulatory genes, which are responsible for the expression of any structural gene (Holton and Cornisch, 1995). The temporal expression of flavonoid structural genes in brown cotton fiber was also consistent with the time course of pigment accumulation in naturally pigmented cotton fibers (Wang and Li, 2002; Dutt *et al*., 2004; Hua *et al*., 2007).

Flavonoids are secondary metabolites and complex path way is involved in the synthesis of flavonoids (Fig. 1). In brown cotton fibers, proanthocyanidins (brown pigments) is the resultant product of flavonoid biosynthesis pathway (Xiao *et al*., 2007). Among many enzymes, chalcone isomerase (CHI) is located at the upstream of pigment biosynthesis pathway which catalyzes the cyclization of Chalcone into flavanone (Nishihara *et al*., 2005). Therefore the expression of chalcone isomerase related structural gene *GhCHI* in brown fiber might be the main cause of biosynthesis of proanthocyanidins (brown pigments) or these genes could serve as precursor for flavanone as well as pigment biosynthesis. Metabolic engineering of CHI (chalcone isomerase) showed that enhanced expression of CHI related gene influenced the flavonoid metabolism and resultantly more amount of flavonoid contents (Muir *et al*., 2001; Li *et al*., 2006). Although all structural genes involved in the biosynthesis of fiber pigmentation has their own importance, expression of *GhANS* and *GhANR* could play a pivotal role in the production of proanthocyanidins (brown pigments) in cotton fibers. This study showed a higher transcript level of *GhANS* in brown than green cotton fiber. Similarly the expression level of this gene was almost negligible in white cotton fiber. It suggested that more transcript level of *GhANS* will lead to enhanced biosynthesis of anthocyanidin from leucoanthocyanidin and this in turn will be converted into proanthocyanidins (brown pigments).

It is concluded that *GhANS* (anthocyanidin synthase) and *GhANR* (anthocyanidin reductase) genes might be the cause of change in fiber color from white to brown. The gene manipulation strategy to modulate the transcription of *GhANS* and *GhANR* could improve the pigmentation of brown cotton fibres by proanthocyanidins accumulation. This study also confirmed that brown and green pigmentation of fiber is not the result of same flavonoids biosynthesis pathway in cotton fiber.

Acknowledgements

The authors acknowledge the Higher Education Commission of Pakistan (HEC) for providing financial support to conduct these studies under "Indigenous Fellowship Program Batch IV".

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(Received 25 April 2014; Accepted 08 July 2014)