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PHYSICAL, CHEMICAL SCIENCES AND ENGINEERING

Manuscript info:
Received June 12, 2018., Accepted July 17, 2018., Published August 20, 2019.

ANALYSIS OF GENES EXPRESSION LEVEL IN DIFFERENT COTTON VARIETIES

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http://dx.doi.org/10.26739/2573-5616-2019-8-20

Abstract: It has been studied expression level of various genes in different cotton varieties: "Cocker 312" (control) and gene-knockout varieties Porloq-1 and Porloq-2, obtained by RNA interference (RNAi) of phytochrome A1 (PHYA1) gene. The obtained results has shown that the genes expression level in modified varieties was significantly differed compared to initial variety Coker-312.

Key words: cotton, PHYA1, RNAi, genes expression, differentially expressed genes.

Recommended citation: Khurshida Ubaydullaeva, Venera Kamburova, Zabardast Buriev. ANALYSIS OF GENES EXPRESSION LEVEL IN DIFFERENT COTTON VARIETIES. 7-8. American Journal of Research P. 215-221 (2019).

Light is one of the most important factors affecting plant growth and development (Wells and Stewart, 2010). The direction, duration and wavelength of light are registered by plants using three classes of photoreceptors: phytochromes (phys) (600-750 nm), cryptochromes / phototropins (320-500 nm) and UV-B (282-320 nm) (Rao et al., 2015; Sheerin and Hiltbrunner, 2017). Plant phytochromes are a family of red / far red light photoreceptors that

carry a linear tetrapyrrole chromophore attached through their cysteine residue to their N-terminal domain (Casal et al., 2014).

In cultivated cotton, the phytochrome gene family is of particular importance. Thus in a number of studies it has been shown that in cotton phys mediate fiber elongation, cellulose accumulation, expression of fiber development genes, fiber quality and fiber color (Qian et al., 2016). At the same time, it was previously shown that silencing ("switching off") of the phytochrome A1 genes (PHYA1) using RNAi technology has led to the creation of the first cotton biotechnological lines with high fiber quality, high yields, early maturation and resistant to abiotic and biotic stresses (Abdurakhmonov et al., 2014).

Taking into account all the above, the main purpose of the study was investigation of comparative gene expression of phytochromes A1 (PHYA1) and B (PHYB), as well as differentially expressing genes in commercial Porloq cotton varieties, obtained using the RNAi technology.

Material and methods.

The study object and experiment design. Plants were grown under standard conditions in a phytotron. Pre-treated and sterilized cotton seeds, Gossypium hirsutum L., were planted in a sterile nutrient medium. Seeds were incubated in dark conditions and at a temperature of 28° C for three days. Three days later, Petri dishes with germinated seeds were converted into photoperiodic light/dark conditions with a duration of 16/8 hours, respectively. The power of light was equal to 5000 lux. After the appearance of 3-4 true leaves of the plant was transferred to the soil. All plants were subjected to genetic verification to confirm the presence of the RNAi construct.

Isolation of total RNA. Total RNA was isolated using the Wu method (Wu et al., 2002). RNA purity was verified in a 1% agarose gel containing 2.2 M formaldehyde in the presence of ethidium bromide. RNA samples were digested with RNAase-free DNase I (Ambion, USA) according to the manufacturer's instructions, and were purified by an additional purification step using phenol-chloroform (5 : 1; Ambion) and precipitation with ethanol. The concentration of total RNA was measured on a spectrophotometer (GENESYS 10UV, Thermo Scientific, USA).

Quantitative RT-PCR. The qRT-PCR reaction was carried out on lightcycler 480 (Roche, Basel, Switzerland) with the following cycling profile: 95°C for 5 min, following by 45 cycles of 10 sec at 95°C, 15 sec at 60°C and 15 sec at 72°C. Each qRT-PCR reaction mixture contained 10 μl of SYBR Green I Master (Roche, Basel, Switzerland), 1 μl of forward primer (10 χM), 1 μl of reverse primer (10 χM), 2 μl of cDNA (4 x fold dilution), and 6 χl of ddH2O. The transcript of cotton Ubiquitin 7 gene

(GhUBQ7) was used to normalize the expression levels of RT-PCR products (Fu, 2015). All reactions were performed with three replicates. The $2^{-\Delta\Delta Ct}$ method (ΔCt = Ct (differentially expressed gene) - Ct (UBQ7), $\Delta\Delta Ct = \Delta Ct$ (RNAi) - ΔCt (Coker 312), $2^{-\Delta\Delta Ct}$ = Relative Expression) was used to calculate relative expression of differentially expressed genes (Livak and Schmittgen, 2001). Primers for RT-PCR, presented in Table 1, were used in the work.

Results and methods.

As can be seen from Fig. 1, the expression level of the PHYA1 gene in parental varieties AN-Bayavut-2 (for variety Porloq-1) and C-6524 (for variety Porloq-2) in comparison with the reference variety Coker-312 was increased by 2.8 and 1.3 times, respectively. While the expression level of the PHYB gene in variety C-6524 was reduced by 65% compared to Cocker-312, while in variety AN-Bayavut-2 it was slightly decreased (about 5%).

Herewith analysis of the expression level of the PHYA1 gene in Porloq gene-knockout varieties has shown that, in the Porloq-1 variety the PHYA1 transcripts were suppressed by 50% compared to the original Cocker-312 variety, and by 80% compared to the parent form AN-Bayavut-2. At the same time, the level of expression of PHYA1 in Porloq-2 variety was reduced by ~ 33% compared to the variety Coker-312 and by 40% in comparison with the parent form C-6524.

However, taking into account the fact that an increase in PHYB gene expression was previously shown in cotton RNAi lines with PHYA1 gene knockout (Abdurakhmonov et al., 2014), and also that the effects of these phys in the cell are antagonistic (Hennig et al., 2001), comparative expression of the PHYB gene in unmodified and gene knockout cotton varieties has been studied.

The results showed that the expression of the PHYB gene in gene-knockout cotton variety Porloq-1 was increased by 1.5 and 1.6 times in comparison with the original variety Coker-312 and the parent variety AN-Bayavut-2, respectively (Fig. 1, A). At the same time, in the RNAi variety Porloq-2 the level of PHYB transcripts was increased by 1.3 and 1.4 times compared to the original variety Coker-312 and the parent variety C-6524, respectively (Fig. 1B).

It is interesting to note that under the normal conditions phys regulate the elongation of hypocotyl in Arabidopsis thaliana due to the regulation of gene expression of phytohormones, including brassinosteroids (BR), auxin (IAA), gibberellins (HA), cytokinins (CK), ethylene and abscisic acid (ABA) (Sheerin and Hiltbrunner, 2017; Casal et al., 2014; Lymperopoulos et al., 2018). Herewith, phyA regulates transcription at the molecular level, forming a complex with a family of phytochrome-interacting factors (Lymperopoulos et al., 2018; Kong and Okajima,

2016). PhyA interacts with PIF, causing their degradation, which leads to inhibition of elongation of the hypocotyl. At the same time, in response to the phytohormone signal, the DELLA proteins are ubiquitinated and decompose, releasing PIF to bind to target promoters and modulate gene expression related to the elongation of the hypocotyl (Ahmed et al., 2018).

Thus, the effects of phyA and phytohormones in signal transduction are antagonistic and, therefore, inhibition of PHYA expression should naturally lead to an acceleration of cell growth. In addition, it is known that most phytohormones regulate the development of cotton fibers (Liao et al., 2010; Shangguan et al., 2010; Ahmed et al., 2018). At the same time, IAA, HA, ethylene and BR stimulate the elongation of cotton fibers, which is also confirmed by the accumulation of transcripts of genes regulating the synthesis of these hormones at different stages of fiber development (Liao et al., 2010; Shangguan et al., 2010).

Summarizing these data and the results of the expression of the PHYA1 and PHYB genes, it can be assumed that the effects of PHYA1 gene silencing can be mediated through the activation of PIFs by phytohormones associated with fiber elongation or by over-expression of the PHYB gene.

Based on this assumption, a comparative study was conducted on the change in the expression of differentially expressed genes (DEG) in gene knockout and unmodified cotton varieties.

The obtained results have shown that the transcription levels of many genes in gene knockout varieties were significantly changed. For example, expression of the gene encoding ABA - 8?-hydroxylase (Gh_D08G1639), a type of cytochrome P450 monooxygenase, which catalyzes the first stage of the oxidative degradation of ABA, has been increased (Fig. 2 A, B).

In addition, the effect of PHYA1 gene silencing on some proteins and enzymes involved in the process of biogenesis and organization of the cell wall was revealed. For example, expression of the gene encoding the xyloglucan endotransglucosylase hydrolase family of proteins (Gh_D02G0992) was increased in Porloc series varieties both in comparison with the original Coker-312 variety and the parental varieties AN-Bayavut-2 and C-6524 (Fig. 2 A, B).

At the same time, a comparative study of the level of DEG transcripts in gene knockout varieties has shown that expression of genes encoding ole e1 allergen and extension family protein (Gh_D05G0805), cytosolic sulfotransferase 12-like protein (Gh_D13G0889), probable ccr4-associated factor 1 homolog 11 (Gh_D09G0953), ein3-binding f-box protein 1-like (Gh_D05G0148), alcohol dehydrogenase (Gh_D02G0733), lipoxygenase-3 (Gh_D10G2595), pectinesterase

inhibitor-like protein (Gh_D11G 2932), и hypothetical protein M569_00222 (Gh_Sca016160G01), was increased in both knockout varieties compared to the control variety Cocker-312. Expression of other genes, including Gh_D05 G3509 (scarecrow-like protein 21) и Gh_A05G0931 (probable protein phosphatase 2c 25), was reduced in RNAi varieties in comparison with Coker-312 (Fig. 2 A, B).

Thus, summing up the data on the comparative gene expression of phytochromes A and B, as well as DEG in the leaves of gene knockout varieties, it can be assumed that the RNAi effects of the PHYA1 gene are realized through a system of phytohormonal regulation, which contributes not only to the development of cotton fiber, but also to increased resistance of studied cotton genotypes to water and salt stress.

Acknowledgment. The authors thank Academy of Sciences of Uzbekistan and Ministry of Innovative Development of the Republic of Uzbekistan for Research Grants nos. FA-F5-025.

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Table 1. Primers used for qRT-PCR.

№	Primer name	Primer sequences (5'→3)	Encoded protein
1.	Gh_D10G2595F	CCATAACCGCGACCTTAACT	Lipoxygenase-3
2.	Gh_D10G2595R	CGGCAACTTCTTCACTGTTTC	
3.	Gh_D02G0992F	TCGAGTTCCTCGGGAATTTAAG	Xyloglucan endotransglucosylase hydrolase family protein
4.	Gh_D02G0992R	CAGTTGGGTCAAACCAGAGATA	
5.	Gh_D02G0733F	GCAGAAGGATGAAGTCCGTATTA	Alcohol dehydrogenase
6.	Gh_D02G0733R	GACCTAGTATGCGAGGAAACAG	
7.	Gh_D08G1639F	GGTGTCCCTGTGTGATGATT	Abscisic acid 8'-hydroxylase 1-like
8.	Gh_D08G1639R	TGCCCAACATCCTCTCTTTAC	
9.	Gh_D05G3509F	GAGGTCCTTAT GTGCGGATTAC	scarecrow-like protein 21
10.	Gh_D05G3509R	CTGCTCGGATAGTTCCATCAAA	
11.	Gh_A05G0931F	TCCGTCCGGGTCTCTATTATC	Probable protein phosphatase 2c 25
12.	Gh_A05G0931R	TTGGCCTCTTCCTCTTCAATATC	
13.	Gh_D09G0953F	GAGGAGGCAAGGGATTGATTT	Probable ccr4-associated factor 1 homolog 11
14.	Gh_D09G0953R	CCCAACTCACCGACTCATTAC	
15.	Gh_D05G0148F	CTCCTGTGCTTGTGTCTCTAAG	ein3-binding f-box protein 1-like
16.	Gh_D05G0148R	CGTTCTCCTTGACCACCTTT	
17.	Gh_D11G2932F	CTTTGGCACTTCACGAACAAG	Pectinesterase inhibitor-like protein
18.	Gh_D11G2932R	AACTCACGCCTCTGGTAAAC	
19.	Gh_D13G0889F	CTTTGGCACTTCACGAACAAG	Cytosolic sulfotransferase 12-like protein
20.	Gh_D13G0889R	AACTCACGCCTCTGGTAAAC	
21.	Gh_Sca016160G01F	AGTGCTTTACAACCCGAAGG	Hypothetical protein M569_00222
22.	Gh_Sca016160G01R	ACACGGTCCAGACTCCTAC	
23.	Gh_D05G0805F	GTGGACCAAACCAGCTATACA	ole e1 allergen and extension family protein
24.	Gh_D05G0805R	AGGGCATGAAGTGGAGAAAG	
25.	Gh_UBQ7F	GAAGGCATTCCACCTGACCAAC	Ubiquitin
26.	Gh_UBQ7R	CTTGACCTTCTTCTTCTTGTGCTTG	

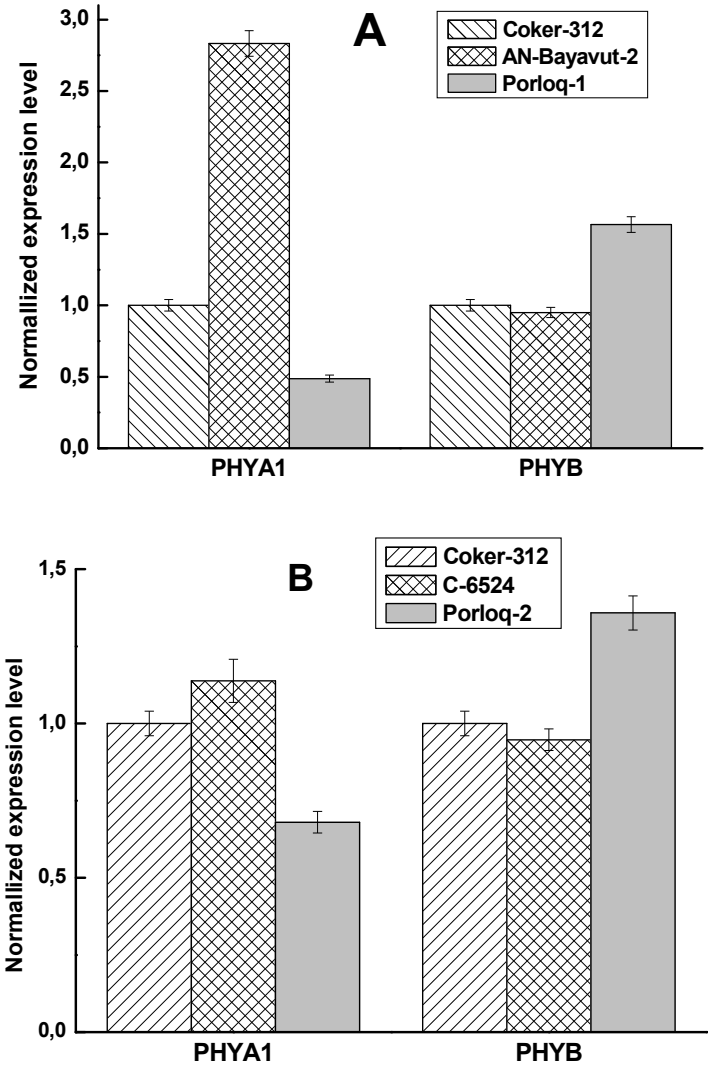

Fig. 1. Comparative expression of phytochrome genes in unmodified and gene-knockout cotton varieties. Gene expression levels were normalized using the ubiquitin GhUBQ7 gene as a reference. $P \leq 0.05$.