GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Temporal and tissue expression of genes involved in buds of earliness cotton cultivar

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Genet. Mol. Res. 14 (3): 7386-7394 (2015)
Received August 19, 2014
Accepted April 8, 2015
Published July 3, 2015
DOI http://dx.doi.org/10.4238/2015.July.3.14

ABSTRACT. Gene sequences previously identified in *Arabidopsis* buds were used as references in order to estimate temporal and tissue expression in buds, leaves, stem, and root tissues in cotton plants. Buds were evaluated during 3 phases: 2-8, 10-12, and 14-20 mm. Primers were designed for the *ARF6*, *ATFY*, and *SEUSS* genes for use in semi-quantitative reverse transcription-polymerase chain reaction and quantitative reverse transcription-polymerase chain reaction. Different levels of expression of the 3 genes were confirmed in cotton buds as well as in other tissues. The peak of gene expression was observed in buds sized 10-12 mm, after which expression decreased in larger buds. The gene *GhFYPP3* was the most promising for further prospection of promoter regions, with regular expression

patterns observed in bud sizes 10-12 and 14-20 mm. This trait was not observed in others genes.

Key words: Bud development; *Gossypium hirsutum*; Reverse-transcription-polymerase chain reaction; Semi-quantitative expression

INTRODUCTION

The agricultural sector is a major driver of the world economy in both developed and developing countries. Important agricultural commodities such as corn, soybeans, and cotton have generated billions of dollars in the seed and derivatives market, benefited by competitive cultivars developed through conventional breeding or by transgeny. Most of these cultivars cluster pyramidal traits, making them less vulnerable to biotic and abiotic disorders (Lopes et al., 2012).

Despite the severe damage that abiotic stresses causes to crops, such as the biotic stresses caused by pests, diseases, weeds, and insects, are more harmful because of the high costs to control these problems or cause further environmental damage (Oerke, 2006; Edwards and Gatehouse, 2007; DeVilliers and Hoisington, 2011). During insect infestation, losses are greater when pests feed on reproductive structures as this directly affects crop yield (Santos et al., 2002; Boxall et al., 2002; Sori and Ayana, 2012). Conventional breeding techniques are limited for acquiring insect-resistant cultivars in some species because of the complexity of resistance factors and the lack of resistant genetic resources, particularly for biting insects.

Currently cultivated genetically modified crops have expressly contributed to the minimization of this lack of resources, although most crops are resistant only to Lepidoptera insects. These plants contain insecticidal genes regulated by constitutive promoters and showing limited expression in floral structures (Benfey et al., 1989; Wilkinson et al., 1997; James, 2013).

Because most harmful insect pests feed on buds, identifying and using promoters that regulate their expression in these structures is an essential step in the development of efficient genetic engineering methods, such as using promoters that control the level, location, and timing of gene expression.

The latest advances in genomic technology have generated large amounts of information, and thus, several banks of nucleotide sequences were created and have become available for use by researchers (Alvarenga et al., 2011; Lovén et al., 2012).

The cotton genome contains several important genes associated with physiological processes, particularly those involved in the defense of reproductive organs, as these organs are directly responsible for fiber and seed yields. Knowledge of the function and expression of these genes would contribute to the understanding of their biosynthetic pathways. These promoters could be used to develop constructs for bioengineering of plant defense.

Thousands of cotton genes have been described and deposited in CottonDB (cottondb.org), most of which have been functionally characterized. Among these, some were found to be involved in floral structures, such as in the anther, ovule, and petal (Wang and Li, 2009; Shi et al., 2009; Pang et al., 2010), and in fiber development steps, from initial trichome formation to their elongation (Walford et al., 2011; Zhu et al., 2011; Li et al., 2011). Pinheiro et al. (2013) identified sequences expressed in cotton associated with microsporogenesis until boll formation.

To increase the understanding of genes expressed in cotton buds, we studied the temporal and spatial expression of 3 bud-sequence genes deposited in GenBank using an earliness cotton cultivar developed by Embrapa and adapted to a semiarid environment.

MATERIAL AND METHODS

In silico analysis of genes expressed in cotton buds

In silico searching was conducted in NCBI-GenBank (http://www.ncbi.nlm.nih.gov) in order to select genes involved in bud development in herbaceous species. Among the selected sequences, 3 candidates from *Arabidopsis thaliana* were compared with those contained in the Cotton Genome Database (http://cottondb.org/) for further alignment performed in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/) with the following criteria: up to 20 base pairs, 45-50% GC content, 60°C annealing temperature, and amplicons close to 200 base pairs. Primer sequences were as shown in Table 1.

Table 1. Primer synthesized from gene sequences involved in bud development, deposited in NCBI-GenBank.

Gene	Accession No. (GenBank)	Sequence
GhARF6	NM 001036038	F: 5'-GAGTTGGCACCTGATAGTGA-3' R: 5'-CCGAAGGACCCTGATTTATA-3'
GhATFY	NM 112888	F: 5'-GGTTAAAGAAGGCCAGCATC-3' R: 5'-CTCGATCAACAAAATCTCCC-3'
GhSEUSS	NM 001123962	F: 5'-CAAATGCGAAGAAGAAGTGG-3' R: 5'-CGGGGCATATCAACATAAAG-3'

F = foward; R = reverse.

Experimental bioassay

Seeds of earliness cv. CNPA 8H (*Gossypium hirsutum* var. *latifolium*) were sown in greenhouse, in 20-L pots containing soil that had been NPK-fertilized. At the blooming phase (35 days after emergence), young buds of different sizes were collected (Table 2) as well as the young leaves, main stem, and roots for RNA extraction.

Table 2. Phenological events associated to bud development in *Gossypium hirsutum*.

Bud diameter (mm)	Shape[1] and phenological event[2] associated
2-8	Pinhead and matched-head squares. Pollen begin to differentiate; grains are larger and numerous; developing of ovules in progress
10-12	Square growth midpoint. Tapetum degenerated; starch grains gradually accumulated in pollen grains; accumulation of proteins, lipids and carbohydrates in cytoplasm of vegetative cell
14-20	Candle. Anther is fully mature, with peak of starch concentration in the grains, indicating imminent anther dehiscence

[1]Ritchie et al. (2004), [2]Santos et al. (2005).

RNA extraction and cDNA synthesis

Total RNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek, Hayward, CA, USA) following manufacturer recommendations. RNA purity and concentration were estimated by 0.8% agarose gel electrophoresis and by spectrophotometry. cDNA synthesis was performed using an ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI, USA), using 1 µg total RNA and following manufacturer recommendations. The reverse transcription reaction was performed in a volume of 44 µL as follows: 11 µL reaction (1 µg total RNA + 0.5 µg Oligo dT_{15}), 1X ImProm-II™ Reaction Buffer, 6 mM $MgCl_2$, 0.5 mM dNTPs, 1 U/µL Recombinant RNasin Ribonuclease Inhibitor, and 1.25 U/µL ImProm-II™ RT. Samples were incubated in a thermal cycler, first at 25°C for 5 min and then at 42°C for 1 h and 70°C for 15 min.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Reactions were performed in a volume of 25 µL containing 1 µg cDNA, 0.2 mM of each forward and reverse primers (Table 1), 0.04 U *Taq* polymerase, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 1X kit buffer. RT-PCR thermal conditions were as follows: pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 2 min. A final extension was added at 94°C for 5 min. The β-actin gene was used as a constitutive control (F: 5'-GATCTGGCATCACACCTTC-3' and R: 5'-AGGAAGCTCGTAGCAGCTCTT-3'). The amplicons were analyzed by 0.8% agarose gel electrophoresis and using a 100-base pair marker (Invitrogen, Carlsbad, CA, USA). The gel images were photodocumented.

RT-quantitative PCR (qPCR) assays

RT-qPCR was performed only in flower buds of pre-set sizes as shown in Table 2. RNA extraction was performed as described above, and cDNA synthesis was performed using the SuperScript™ III Reverse Transcriptase (Invitrogen) according to manufacturer guidelines. RT-qPCR was performed in a volume of 10 µL containing 5 µL Evagreen (Biotium, Inc., Hayward, CA, USA), 0.2 µM of each forward and reverse primers (Table 1), 2 µL 1:20 (v/v) cDNA, and ultra-pure water. The amplification steps were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and finally, 60°C for 1 min. Primers for the β-actin (F: 5'-GATCTGGCATCACACCTTC-3' and R: 5'-AGGAAGCTCGTAGC AGCTCTT-3') and ubiquitin (F: 5'-CAACGCTCCATCTTGTCCTT-3' and R: 5'-TGATC GTCTTTCCCGTAAGC-3') genes were used as constitutive controls.

The graphics, Cqs, and melt curve were automatically generated by the thermocycler software of the Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) based on the normalization method with a reference gene, ΔΔCq (Livak and Schmittgen, 2001). The expression pattern was estimated by relative quantification.

RESULTS

Selection of candidate genes in cotton buds

The bud genes *GhARF6*, *GhFYPP3*, and *GhSEUSS* were selected from sequences

deposited in the NCBI GenBank and later compared with sequences deposited in CottonDB. Similarity analysis, conducted using the BLASTn tool, revealed 17 sequences homologous to *GhARF6* and expressed in the chloroplasts (5), nuclei (17), and fruit (1), 9 sequences homologous to *GhFYPP3* and expressed in the pollen tube (3) and nuclei (6), and 10 sequences homologous to *GhSEUSS* and expressed in the nuclei (2) and flower (6).

Semi-quantitative RT-PCR

Although the selected genes were isolated from flower buds, the results obtained by semi-quantitative RT-PCR revealed that all genes were also expressed in other tissues of cotton, but showed different levels of expression (Figure 1).

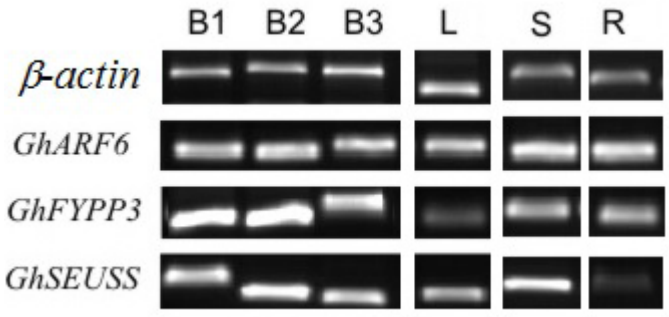

Figure 1. Products of semi-quantitative RT-PCR on 0.8% agarose gel from different tissues of cotton BRS 8H. B, bud (sizes: B1 = 2-8 mm; B2 = 10-12 mm; B3 = 14-20 mm); *lane L* = leaf; *lane S* = stem; *lane R* = root.

GhARF6 showed similar expression levels in all tissues studied; *GhFYPP3* was more highly expressed in the buds, stem, and roots, but showed low expression in the leaves. *GhSEUSS* showed similar expression levels in the buds, leaves, and stems, and was very low in the roots.

Analysis of the expression pattern of the 3 genes only in flower buds revealed that all genes showed similar expression patterns. Semi-quantitative PCR could not be used to assess at which stage expression differed during development.

RT-qPCR

The pattern of expression of the 3 genes based on RT-qPCR results confirmed the results of the semi-quantitative assays (Figure 1), showing better definition of the temporal activity of each gene. Peak of expression occurred at stage 10-12 mm (Figure 2) in all genes, and then decreased during the phase preceding flower opening.

One of the goals of this study was to isolate the promoter regions controlling genes showing substantial expression in buds. The *GhFYPP3* (Figure 2A) appeared to be the most suitable for this purpose, as it showed consistently high expression pattern in all 3 bud phases, with a slight decrease of 9% in 14-20 mm; in *GhARF6* (Figure 2B) and *GhSEUSS* (Figure 2C), the reductions were 64 and 70%, respectively, in the same phases.

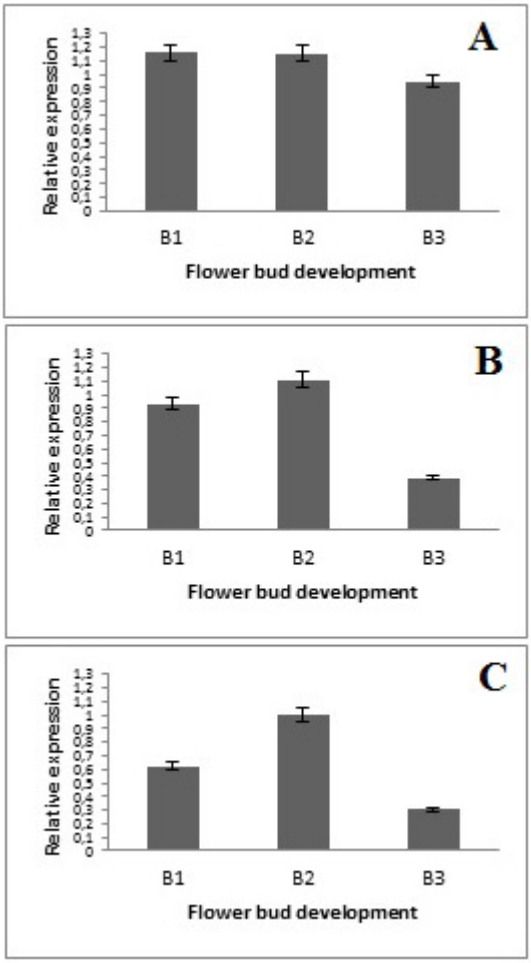

Figure 2. Gene expression in cotton buds via RT-qPCR. **A.** *GhFYPP3*, **B.** *GhARF6*, and **C.** *GhSEUSS*. B1 = 2-8 mm; B2 = 10-12 mm; B3 = 14-20 mm. Statistical analysis were performed using the program of Eco™ Real-Time PCR System ($P \leq 0.05$).

DISCUSSION

The genes used in this study have been reported to be factors that are directly involved in the ontogeny of *A. thaliana* buds; however, based on the results obtained in this study in *G. hirsutum*, their action is not only restricted to reproductive structures, but also in other tissues, although information regarding their functions is limited.

In particular, although all 3 genes have established functions in *A. thaliana*, the greatest expression was verified in 10-12 mm plants at the points of buds. This may be related to the high cellular activity of gametes during the several mitotic divisions to form the pollen and embryo sac. At this stage, the deposition of starch is high, as it is a source of energy for

the gradual development of pollen (Santos et al., 2005). Soon after, with the phase preceding flower opening (14-20 mm), all physiological and biochemical structuring of gametes is already established, and metabolic activity is less accentuated, corresponding to the reduced levels of expression as shown in Figure 2 for all 3 genes.

The pattern of stability observed in the expression of *GhFYPP*, with a slight decrease in the 14-20 mm stage, suggests that this gene is present in the steps that occur after anthesis, involving the stages of fruit initiation and ripening. The *A. thaliana FYPP3* gene is also involved in all steps related to flowering, suppressing, or accelerating the steps according to the level of expression (Kim et al., 2002). Physiologically, *FYPP3* encodes for enzymes involved in the translocation of phytochrome A and B, which are involved with flowering (Kircher et al., 1999; Nagy et al., 2000).

According to Dai et al. (2012), the *FYPP* gene is also expressed in the root, which was observed in this study in *G. hirsutum* in the semi-quantitative assay (Figure 1). Because the physiological processes involving the root development depend on the absence of light, this gene may also be involved in events associated with photosensitivity. Kim et al. (2002) reported that the cascades of cell signaling by light energy have been widely studied, and that the FYPP protein family participates actively in cellular signaling related to blooming.

The other genes detected in cotton buds, *GhARF6* and *GhSEUSS*, showed very similar expression patterns, demonstrating a more temporal bias and with peaking at 10-12 mm, which was followed by a marked reduction soon after this stage (Figure 2B and C). In *A. thaliana*, *ARF6* is involved in the response to stimuli by auxin, a hormone that is essential to tissue differentiation and the regulation of adventitious rooting (Gutierrez et al., 2009; Tabata et al., 2009). During the growth of floral structures in *A. thaliana*, apical meristems are activated to produce floral meristems by high concentrations of auxin. Some genes, such as *PIN-1*, *DAD1*, *KNOX*, and *ARF*, are mediators of these processes and are important during the initial differentiation of cells that will form the tissues (Benková et al., 2003; Tabata et al., 2009). *ARF6* is also involved in regulating the biosynthesis of jasmonic acid, an essential hormone for plant growth and the defense against stress. As these functions constitutively involve the whole plant, the expression pattern of all genes as shown in Figure 1 is consistent.

SEUSS is more directly involved with the female gametes. Its main function is in transcriptional co-regulation, together with *LEUNIG*, in a series of events related to the floral organs and with pronounced expression at the beginning of flowering (Franks et al., 2002; Vaniyambadi et al., 2006; Hashimoto et al., 2008; Bao et al., 2010; Grigorova et al., 2011). This agrees with the results shown in Figure 2, as the expression pattern of this gene was reduced by 70% when buds were next to the anthesis and therefore when all gametic structures had been generated. In *A. thaliana*, functional analysis indicated that *SEUSS* plays other roles in flower development, specifically in the carpel margin meristems, a vital meristematic structure located at the margins of the fused carpels and that gives rise to several structures in the female reproductive system, including the eggs (Franks et al., 2002; Azhakanandam et al., 2008; Grigorova et al., 2011; Kamiuchi et al., 2014).

In this study, we conducted semi-quantitative analysis and RT-qPCR and found that the *GhFYPP3* gene is the best candidate for further studies involving the isolation of promoter regions, which will be a valuable resource in developing gene constructs to examine the defense of floral structures.

ACKNOWLEDGMENTS

Research supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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