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Expression of NCED gene in colored cotton genotypes subjected to water stress

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gas exchange
semi-quantitative RT-PCR
qRT-PCR

ABSTRACT
Considering that the NCED gene acts on the biosynthetic cascade of ABA, a hormone involved in the functioning of stomata and consequently in the regulation of transpiration, the aim of this research was to analyze the expression of this gene in colored cotton genotypes subjected to water stress at the beginning of plant growth. Four colored cotton genotypes were used, subjected to two managements, with and without water stress, beginning the treatments when the blade of the first true leaves reached an area that allowed the evaluation of gas exchange. For the studies of the expression of the NCED gene, via RT-qPCR, leaves were collected on three distinct dates: at 4 and 6 days of water stress, and after the plants regained their turgor. The differential expression of NCED was found in all genotypes, with higher levels of expression related to six days of water stress. When the stomatal conductance was around 25%, there was overexpression in the genotype CNPA 2009.13, followed by CNPA 2009.6, BRS SAFIRA and CNPA 2009.11, confirming the data obtained in the semi-quantitative RT-PCR. The NCED gene is involved in the response to water stress in the vegetative phase of colored cotton.

Palavras-chave:
Gossypium hirsutum L.
trocas gasosas
RT-PCR semiquantitativa
qRT-PCR

Expressão do gene NCED em genótipos de algodoeiros coloridos submetidos a estresse hídrico

RESUMO
Considerando que o gene NCED atua na cascata biossintética do ABA, hormônio envolvido no funcionamento dos estômatos e, consequentemente, na regulação da transpiração objetivou-se, com esta pesquisa, analisar a expressão desse gene em genótipos de algodão colorido submetidos a déficit hídrico no início do crescimento das plantas. Foram utilizados quatro genótipos de algodão colorido (CNPA 2009-6, CNPA 2009-11, BRS SAFIRA e CNPA 2009-13) submetidos a dois manejos sem e com estresse hídrico iniciando-se quando o limbo das primeiras folhas verdadeiras atingiu uma área que permitiu a avaliação de trocas gasosas. Para os estudos de expressão do gene NCED via RT-qPCR foram coletadas folhas em três datas distintas: aos quatro e seis dias de estresse hídrico e após as plantas recuperarem a turgescência. Em todos os genótipos foi constatada expressão diferencial de NCED com maior nível de expressão relativa aos seis dias de estresse; quando a condutância estomática estava em torno de 25% ocorreu superexpressão no genótipo CNPA 2009.13 seguido de CNPA 2009.6, BRS SAFIRA e em CNPA 2009.11 confirmando os dados obtidos na PCR semiquantitativa. O gene NCED está envolvido na resposta ao estresse hídrico na fase vegetativa de algodoeiro colorido.

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Introduction

Cotton (*Gossypium hirsutum* L. var. *latifolium* Hutch.) is one of the main agricultural crops in many parts of the globe, and more than 35 million hectares are annually planted in over 60 countries, moving about $ 12 billion (ABRAPA, 2014). In the Northeast, colored cotton cultivation is expanding, supported by genetic enhancement and improvement of crop management techniques. However, in order to increase the yield of colored cotton, it will be necessary to obtain more genotypes adapted to drought, a prevalent condition in the Northeast region (Meneses et al., 2006).

Several studies have been conducted on water stress in cotton plant, researching about the zoning for its cultivation (Mann et al., 1996), growth and development (Menezes et al., 2006), production and quality of fibers (Faulkner et al., 2012) and gas exchange (Clement et al., 2011), besides studies involving gene expression, such as those conducted by Ackerson (1981), Payton et al. (2011) and Park et al. (2012), who related some genes to the defense mechanism of cotton to water stress, including the NCED gene (cis-epoxycarotenoid dioxygenase-9).

The abscisic acid (ABA) plays an important role in the tolerance to stresses in some plant species (Zhang et al., 2008; Khodambashi et al., 2013). In part, this characteristic is due to the key function of the NCED gene in the biosynthesis of ABA for cleaving cis-epoxycarotenoids, 9'-cis-neoxanthin and 9-cis-violaxanthin in the intermediates C15 (xanthoxin) and C25 (Zhang et al., 2008; Hwang et al., 2010). Xanthoxin, in turn, is transported to the cytosol and used in the biosynthesis of ABA (Chernys & Zeevaart, 2000; Thompson et al., 2000).

Considering the above, this study investigated the expression of the NCED gene in genotypes of cotton in the vegetative stage, under water stress conditions.

Material and Methods

The experiment was carried out in a greenhouse (07º 13' 50'' S; 35º 52' 52'' W; 551 m) from April to May 2013, at the Center of Technology and Natural Resources (CTRN) of the Federal University of Campina Grande (UFCG), in Campina Grande, PB, Brazil.

Four cotton genotypes from the Active Germplasm Bank of the Embrapa Cotton (CNPA 2009-6, CNPA 2009-11, CNPA 2009-13 and BRS Safira) were investigated under two managements, with and without water stress. Three seeds were planted in each tube (288 mL), filled with commercial substrate (humus, ground green coconut, wood chips, cattle manure and chemical fertilizer). After ten days of seedlings emergence, thinning was performed, leaving only one plant per tube.

Irrigation was daily performed, in the morning, through the application of an amount of water sufficient for the beginning of the drainage. In one group of plants, irrigation was applied on a daily basis during the entire experiment, corresponding to the treatment without water stress, and in the other treatment with water stress, irrigation was suspended from the 13th day after emergence (DAE), when the blade of the first true leaf reached minimum dimension of 6 cm^2.

From the 13th DAE on, readings of gas exchanges were performed on the 1st, 3rd, 4th and 6th day of water stress, using an Infra-Red Gas Analyzer (IRGA – LCpro+, ADC Bioscientific®), in order to identify changes in the values of stomatal conductance (gs). Plants remained under stress until the cotyledonary leaves wilted and hung down, with abscission of 50%. This fact varied among the genotypes, being of 10 days for CNPA 2009-6, 13 days for CNPA 2009-11 and BRS SAFIRA and 15 days for CNPA 2009-13. Then, plants were rehydrated and reevaluated with respect to gas exchanges, when the leaves recovered turgor, which occurred between 3 and 4 days after the restart of irrigation. Stomatal conductance readings were performed at 8 a.m., by regulating the flux density of photosynthetic photons at 1,200 μmol m^{-1} s^{-1}, air temperature corrected to 25 ºC and internal CO_2 concentration at the level of the environment (Magalhães Filho et al., 2008).

For the analyses of gene expression, the leaves collected in plants with 4 and 6 days of stress and after turgor recovery were immediately frozen in liquid nitrogen and maintained at -80 °C.

For the extraction of total RNA, the leaves were macerated in liquid N_2 using mortar and pestle; then, total RNA was extracted using the Invisorb Kit (Invitek). The total RNAs were analyzed in agarose gel at 0.8% and quantified in spectrophotometer (Eppendorf®, model BioPhotometer plus). For the removal of any genomic DNA, 1 μg of total RNA of each sample was treated with 1 U μL^{-1} of DNase I (BioLab). The synthesis of cDNA was performed using 1 μg of total RNA treated with DNase I using the ImProm-II™ Reverse Transcription System (Promega). All procedures followed the recommendations of the manufacturers.

In the semiquantitative RT-PCR, specific oligonucleotides were used for the NCED gene (GenBank: HM145908.1) (F- 5'ATGATCCACGATTTCGCCAT3' and R- 3'TCCCAAGCATTCCAAAGATG5') and for the constitutive gene Ubiquitin (F- 5'CAACGCTCCATCTTGTCCTT3' and R- 3'TGATCGTCTTTCCCGTAAGC5'). The reactions were conducted according to the following parameters: 1 μL of cDNA; 0.2 μM of each oligonucleotide; 2 U of Taq; 0.2 mM of dNTP; 2 mM of $MgCl_2$ and 1X of buffer. The conditions of the PCRs were: pre-denaturation at 96 ºC for 1 min, followed by 35 cycles of denaturation at 96 ºC for 1 min, annealing at 58 and 55 ºC for 1 min, for NCED and Ubiquitin, respectively, extension at 72 ºC for 1 min and final extension at 72 ºC for 10 min. The reactions were analyzed in agarose gel 1% and photo-documented.

Each reaction of RT-qPCR used 5 μL of Evagreen (Biotium), 0.2 μM of each oligonucleotide, 1 μL of cDNA of leaves of plants with four and six days of stress and water for the final volume of 10 μL. The amplification conditions were: 50 ºC for 2 min, 95 ºC for 10 min and 40 cycles of 95 ºC for 15 s; then, 60 ºC for 1 min. The graphs, Melt curves and Cqs were automatically generated by the thermocycler Eco™ Real-Time PCR System (Illumina), based on the method of normalization with one gene of reference, ΔΔCq (Livak & Shimittgen, 2001); the generated pattern was analyzed using the relative quantification. The constitutive genes Actin (F- 5'TTGCAGACCGTATGAGCAAG3' and R- 5'ATCCTCCGATCCAGACACTG3') and Ubiquitin were used as endogenous references.

Results and Discussion

With one day of water stress, there was no significant difference in the stomatal conductance between the genotypes, or even between both water treatments. However, at 3, 4 and 6 days of water stress, despite the lack of significant difference between the genotypes, there was variation in stomatal conductance ($p < 0.01$) between the water treatments (Table 1).

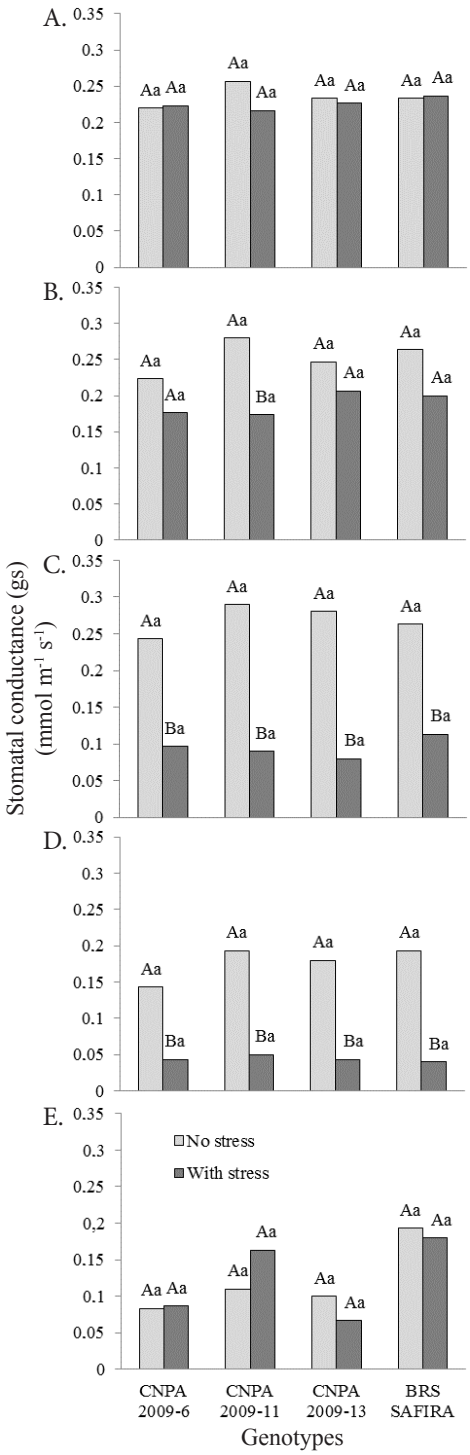
In the recovery stage, there was no significant difference between the treatments; however, there was difference between the genotypes ($p < 0.01$). Regarding the interaction between the factors, significant effect ($p < 0.05$) was only observed in the recovery (Table 1).

One day after water stress started, stomatal conductance was not affected, possibly due to the high moisture content of the substrate inside the tube (Figure 1A). With three days of water stress, the genotype CNPA 2009-11 significantly ($p < 0.05$) decreased stomatal conductance. For the other genotypes, there was also reduction in the conductance, but in lower intensity (Figure 1B). On the fourth day of water stress, all genotypes significantly decreased stomatal conductance, with a mean reduction from 73.41 to 33.99%. This is a noticeable sign of water restriction resulting in stomatal closure (Figure 1C).

Six days after water stress started, the genotypes were only with a mean of 24.88% of stomatal conductance, with clear similarity of response between the four genotypes (Figure 1D).

After rehydration, the plants under stress recovered and, in some cases, managed to surpass the stomatal conductance of those that were not exposed to water stress (control), although they did not differ significantly (Figure 1E). However, the time of recovery varied among the genotypes and was equal to three days for CNPA 2009-6 and four days for the others (CNPA 2009-11, BRS Safira and CNPA 2009-13). After the plants were exposed to water stress for a certain period and rehydrated, cell turgor increased and the leaves recovered the normal form and reopened their stomata, which was confirmed by the stomatal conductance data. The reduction in stomatal conductance is one of the adaptive mechanisms of the plants to water stress, in the attempt to decrease the loss of water, although it also results in reduction of CO_2 assimilation (Taiz & Zeiger, 2013).

The recovery of the genotypes after rehydration, notably CNPA 2009-6, is an evidence of the tolerance of cotton to water stress, corroborating with results obtained by Ennahli & Earl (2005), cultivating plants of this species in soils with relative moisture contents varying from 75 and 5%. These authors also observed recovery of gas exchanges, 48 h after the plants were irrigated.



Uppercase letters for genotypes between water treatments (F test) and lowercase letters for genotypes in the same water treatment (Scott-Knott)

Figure 1. Stomatal conductance in cotton genotypes subjected to water stress. (A) One day of stress (DWS); (B) Three DWS; (C) Four DWS; (D) Six DWS; (E) After rehydration

Table 1. Summary of the analysis of variance for stomatal conductance (gs) evaluated on the 1st, 3rd, 4th and 6th days of water stress (WS) and in the recovery after water stress

Source of variation	DF	Mean square				
		WS 1st day	WS 3rd day	WS 4th day	WS 6th day	Recovery
Genotype (G)	3	0.000272 ns	0.001238 ns	0.001513 ns	0.000917 ns	0.014515**
Treatment (T)	1	0.000600 ns	0.024704**	0.182004**	0.106667**	0.000038 ns
Interaction GxT	3	0.000633 ns	0.001349 ns	0.001338 ns	0.000811 ns	0.002060*
Block	2	0.005579**	0.000788 ns	0.004154 ns	0.000029 ns	0.000717 ns
Residual	14	0.000751	0.002273	0.0027226	0.000005	0.000521
Total	23	0.024983	0.065862	0.038158	0.125983	0.058496
CV (%)		11.87	21.55	28.67	28.61	18.58

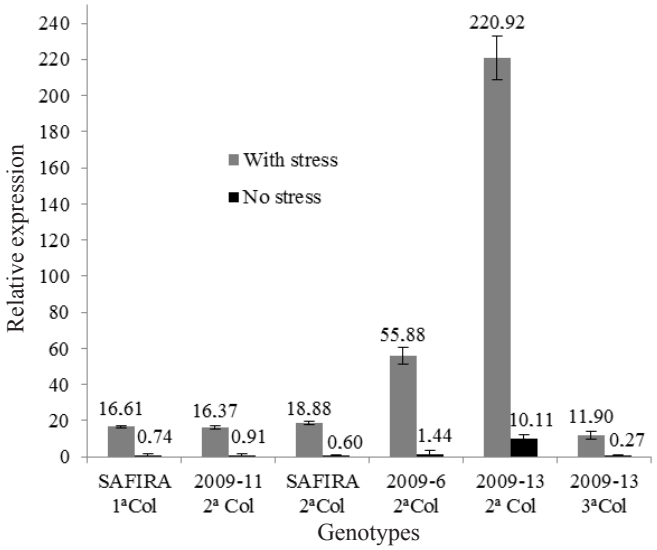
Ko & Pccinni (2009) also mention the relationship between soil moisture and stomatal conductance, in the characterization of gas exchanges in cotton plants under limited irrigation conditions. These authors cite that the physiological stage of water-stressed plants can be observed not only through measurements of soil moisture, but also through gas exchanges.

In the analyses of the expression of the NCED gene through semiquantitative RT-PCR, on the 4th day, there was an increase in the differential expression only in BRS Safira, while a reduction of expression was observed in the other genotypes (Figure 2).

The expression of the NCED in BRS Safira on the fourth day of water stress occurred when there was a reduction in stomatal conductance from 73.41 to 33.99%, which suggests the induction of NCED expression and the participation and influence of the reduction in stomatal conductance as a consequence of the water stress.

Other researchers attributed the stomatal closure to various factors, such as Plumbe & Willmer (1986), who reported the existence of phenolic compounds capable of causing stomatal closure almost as efficiently as ABA. The greatest example of these phenolic compounds is the acetylsalicylic acid present in the plant, not only under water stress, but also under saline stress, at high or low temperatures or in the defense of the plant against the attack of pests or pathogens (Carvalho et al., 2007; Freitas et al., 2009).

At six days of water stress, all genotypes showed increase in the relative expression of the NCED gene (Figures 2 and 3), with a relative overexpression in the genotype CNPA 2009-13, followed by moderate to low expression, in the genotypes CNPA 2009-6, BRS Safira and CNPA 2009-11 (Figure 3). The increase in the days and intensity of water stress led to an increment in NCED expression, suggesting the involvement of this gene in the highest tolerance to water deficiency, since the increase in its expression follows, in this stage, the decrease of stomatal conductance in all genotypes (Figure 1D).

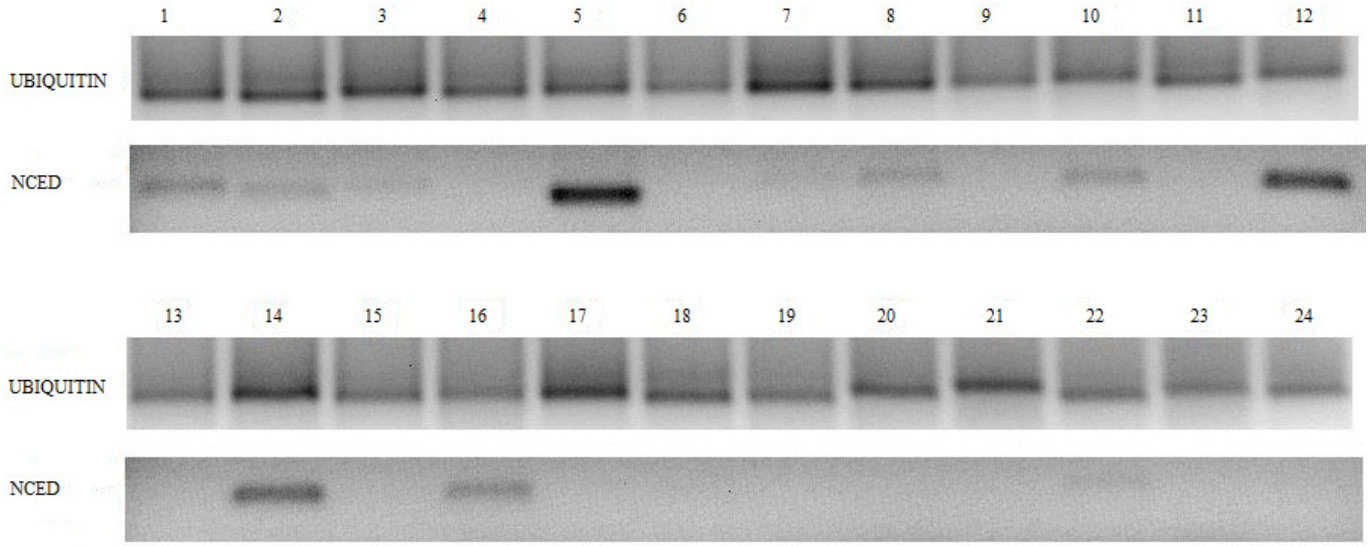


Treatments: WWS- with water stress; NWS- no water stress

Figure 3. Relative expression via RT-qPCR of the NCED gene in leaves of the four cotton (*G. hirsutum* L.) genotypes with four (1 st collect) and six days (2 nd collect) of water stress

Hwang et al. (2010) also supports the idea that the overexpression of NCED in some genotypes may promote greater tolerance to drought. For Shamim et al. (2013) and Clément et al. (2011), this can be explained, because the increase in NCED expression influences the synthesis of ABA, which in turn is related to stomatal closure. Other researchers have also reported the action of ABA on the stomatal closure of stressed plants, promoting greater tolerance to the limiting conditions of the water stress (Setter et al., 2011; Shatil-Cohen et al., 2011).

After the stress stopped, stomatal conductance returned to the level of the control plants and NCED expression was no longer observed (Figure 2). These data are the confirmation that the reduction in stomatal conductance and overexpression of NCED along the water stress causes the plant to tolerate water scarcity, not affecting its capacity of recovery.



Plants with four DWS (days of water stress): 1 and 2 – Genotype CNPA 2009-6 NWS (no WS) and WWS (with WS); 3 and 4 - Genotype CNPA 2009-11 NWS and WWS; 5 and 6 - Genotype CNPA 2009-13 NWS and WWS; 7 and 8 - Genotype BRS SAFIRA NWS and WWS; Plants with six DEH: 9 and 10 – Genotype CNPA 2009-6 NWS and WWS; 11 and 12 - Genotype CNPA 2009-11 NWS and WWS; 13 and 14 - Genotype CNPA 2009-13 NWS and WWS; 15 and 16 - Genotype BRS SAFIRA NWS and WWS; Plants after recovery from WS: 17 and 18 – Genotype CNPA 2009-6 NWS and WWS; 19 and 20 - Genotype CNPA 2009-11 NWS and WWS; 21 and 22 - Genotype CNPA 2009-13 NWS and WWS; 23 and 24 - Genotype BRS SAFIRA NWS and WWS

Figure 2. Electrophoresis in agarose gel at 0.8% of the semiquantitative RT-PCR with the oligonucleotides for the genes Ubiquitin (constitutive) and NCED (specific)

Conclusions

1. The genotype CNPA 2009-6 recovers faster with the rehydration after water stress.

2. Stomatal conductance is a good indication of the perception of soil moisture deficiency by cotton.

3. The NCED gene was more expressive in the genotypes CNPA 2009-6 and BRS SAFIRA in the induction of stomatal conductance after the fourth day of water stress and on the sixth day, in the genotype CNPA-13.

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