



Short Communication

Expression studies of transcription factors under moisture stress in Cotton (*Gossypium hirsutum* L.)

S. A. Yadav, M. P. Jadhav, Sateesh Adiger, Sonam Singh¹, Amolkumar U. Solanke¹, I. S. Katageri* and H. M. Vamadevaiah

Department of Biotechnology, University of Agricultural Sciences, Dharwad 580 005; ¹ICAR-National Research Centre on Plant Biotechnology, Pusa Campus, New Delhi 110 012

(Received: January 2018; Revised: July 2018; Accepted: August 2018)

Abstract

The process of plant adaptation to the environmental stresses is controlled by orchestration of complex molecular networks regulated by transcription factor (TF) genes. In the present study, JK-4(G₁₁), CPD-433(G₁₅) and 5433A2A03 N83(G₁₆) recorded higher relative water content under moisture stress condition as compared to other genotypes with higher level of proline content indicating their role in drought tolerance. Further, expression analysis of TF genes from cotton (*GhWRKY19*, *GhLIM*, *GhNAC*, *GhGeBP*, *GhWRKY70* and *GhC2H2*) through qRT-PCR in 27 different cotton genotypes showed differential expression under stress condition indicating their role in stress tolerance.

Keywords: Abiotic stresses, cotton, moisture stress, qRT-PCR, TF genes.

Drought is one of the most widespread environmental stresses, which affect the cotton production worldwide. It affects morphological, physiological, biochemical and molecular processes in plants resulting in growth inhibition, stomata closure with consecutive reduction of transpiration, decrease in chlorophyll content and inhibition of photosynthesis and protein changes to cope up with osmotic changes in the tissues (Zhu and Zhang, 2003). Drought tolerance is a genetically controlled complex physiological process of plant adaptation that involves multiple genes and pathways (Shinozaki and Yamaguchi-Shinozaki, 2007), and literature shows that the expression levels of hundreds of genes are altered in response to drought (Zhou et

al. 2007). Despite the lack of proper understanding of various drought tolerance mechanisms, physiological and molecular studies have been documented in several plants (Vasquez-Robin et al. 2008). In the last decade several stress responsive genes and TFs responsible for induction of stress tolerance in crop plants were identified through molecular and biochemical studies (Abdeen et al. 2010). These TF genes are the major components of gene regulatory network involved in drought tolerance.

WRKY transcription factors have diverse biological functions in plant disease resistance, abiotic stress responses, nutrient deprivation, senescence etc. WRKYs can act as transcriptional activators or repressors, in various homo- and hetero dimer combinations (Bakshi and Oelmüller, 2014). *LIM* TF plays an important role during fibre initiation stage and is involved in the actin cytoskeleton organization for cell growth and development under drought. Proteins of NAM, ATAF, and CUC (*NAC*) TF family contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain (Olsen et al. 2005). *NAC* proteins are also found to participate in plant responses to pathogens, viral infections and environmental stimuli (Kim et al. 2007). *GeBP*/*GPL*s play role in pathogen resistance and can induce HR-like responses when over expressed as constitutively active form. *GeBP*/*GPL* genes are involved in indirect regulation of cytokinin response genes, like *ARR6* (Chevalier et al. 2008) and

*Corresponding author's e-mail: katageris@uasd.in

have a specific role between stress and the cytokinin pathway (Perazza et al. 2011). In plants, the *C2H2* proteins are involved in various processes including development and organogenesis along with the response to stress and defense pathways and other abiotic stresses. Understanding the presence of genetic variability for differential expression these genes among cotton genotypes would be useful in breeding for moisture stress resistance in cotton. Therefore, the present study was aimed at studying the expression pattern of these genes among 27 cotton genotypes showing diverse response to moisture stress. The expression levels of six selective TF genes was also measured through qPCR in well watered and water stressed leaf tissues. In present investigation we have studied 27 cotton genotypes, out of which CCH-1831, 5433 A2 A03 N83, PH-1009 and JK-4 identified as drought tolerant and G.cot-16, Suraj, RHC-0811, HLS-321729, CPD-2007-4 and CPD 464 susceptible.

The cotton seeds of all the 27 genotypes treated with Imidacloprid (10g/kg seed) were sown in the pots. Watering was withheld after 45DAS (Days after sowing) in half of the pots of each genotype. The soil samples from each pot were taken with the help of screw agar. Relative water content was estimated as per the method of Barrs and Weatherly (1962). Proline content (mg/g fresh wt.) in the leaf tissues of cotton genotypes were estimated by Bates et al. (1973).

Leaves from plants under moisture stress (20 days after withholding water) and control were collected separately. Total RNA was isolated and about 2 μ g of total RNA in a single 20 μ l reaction was quantitatively converted to single-stranded cDNA. The reaction mixture was divided into triplicates of 8 μ l as technical replications. In the present study ubiquitin (UBQ) was used as reference gene. A Stratagene Mx3000P qPCR instrument was used for real-time PCR amplifications. The mathematical model delta-delta Ct method (Livak and Schmittgen, 2001) was used to determine relative expression ratio (fold change) (Caldana et al. 2007).

In the present study, moisture stress was induced by withholding water 45DAS. Plants experiencing moisture stress were indirectly ensured by presence 50% soil moisture in the pots not watered 45 DAS as compared to normally watered. At that stage (20 days after withholding water), significant variation for relative water content (RWC) in leaves among genotypes was recorded. Irrespective of genotypes the RWC was higher in normal condition than stress. JK-4, CPD-433 and 5433A2A03 N83 recorded higher RWC in the moisture stress condition

and showed less per cent change in RWC between normal and stress, indicating their tolerance to moisture stress (Table 1). In contrast, PS 20-2-1, 543403A03 N106 and G.cot-16 Surat recorded significantly lower RWC under similar condition but showed high per cent change in RWC between stress and normal condition than the other genotypes reflects their susceptibility to moisture stress (Table 1). Several studies reported that the relative water content in leaves decreased significantly in drought susceptible genotypes than resistant genotypes (Chen et al. 1990).

Slow utilization of proline for protein synthesis and stimulation of glutamate conversion to proline during stress may be the possible reason for its accumulation (Stewart, 1974). In the present study, irrespective of genotypes there was higher proline content in drought condition than normal condition (Table 1, Fig. 1). Genotypes, 5433A2A03 N83, EC 560392 and 211412 W247-85-D14-26 showed a highest per cent increase in proline content in drought condition indicating as drought tolerant genotypes. The expression levels of selected six TF genes were analyzed in water stressed condition in comparison to normal condition considering the Ct value of reference gene (Ubiquitin). A differential up- and down-regulation of selected genes was recorded (Supplementary Table S1, www.isgpg.org). The alterations in the expression of TF genes was observed in both drought tolerant and susceptible genotypes.

Out of six TF genes, *GhNAC* and *GhWRKY70* showed up-regulation at 1% level of significance commonly in 5433 A2 A03 N83 and PH-1009 and down-regulation in CCH-1831 which are the previously reported drought tolerant genotypes. Similar results were obtained by Zhu et al. (2013), which showed that the up-regulation of WRKY TF is related to the abiotic stress tolerance in cotton. *GhC2H2* and *GhGeBP* gene showed up-regulation at 1% level of significance in 5433 A2 A03 N83 and PH-1009. The role of *GeBP* under stress and helps in cell wall metabolism and showed that overexpression of *C2H2* type zinc finger proteins induce the expression of several stress-related genes, conferring enhanced tolerance to salt, dehydration and cold stresses Perazza et al. 2011; Xu et al. 2008). Tran et al. (2004) reported the up-regulation of three *NAC* genes, *ANAC019*, *ANAC055*, and *ANAC072*, by drought, salinity, and low temperature and the transgenic *Arabidopsis* plants and over expressing these genes showed improved stress tolerance compared to the wild-type.

Table 1. Effect of moisture stress on RWC in leaf sample (%) and proline content in leaf samples (µg/g fresh wt.)

Genotype	RWC (%)			Proline (µg/g fresh wt.)		
	Control	Moisture Stress	% Change	Control	Treated	% Change
G.cot-16 surath (G ₁)	72.12	45.49	-36.92	36.61	69.36	+47.21
HBS-128 (G ₂)	80.33	65.55	-18.40	40.14	79.93	+49.79
Abhadita (G ₃)	80.54	54.38	-32.47	37.75	71.29	+47.05
CPD 464 (G ₄)	80.19	60.57	-24.46	32.29	60.83	+46.92
CPD 2007-4 (G ₅)	81.15	64.20	-20.89	33.66	63.67	+47.14
AK 23 B (G ₆)	84.67	59.39	-29.85	40.48	74.70	+45.81
RAJ-2 (G ₇)	81.48	62.46	-23.35	41.61	76.63	+45.70
NH 615 (G ₈)	85.58	69.85	-18.38	33.31	66.63	+50.00
543374 A 02 N68 (G ₉)	77.43	57.21	-26.11	43.21	79.93	+45.95
RDT-17 (G ₁₀)	75.65	61.42	-18.81	42.52	75.95	+44.01
JK-4 (G ₁₁)	86.73	76.82	-11.43	44.80	84.48	+46.97
PH 1009 (G ₁₂)	73.41	58.29	-20.60	41.84	82.32	+49.17
CPD 446 (G ₁₃)	92.34	78.54	-14.94	44.57	81.86	+45.56
IC35701Coker 417-68 (G ₁₄)	77.27	54.14	-29.93	38.43	76.63	+49.85
CPD 433 (G ₁₅)	80.33	70.46	-12.29	37.29	71.86	+48.10
5433 A2 A03 N83 (G ₁₆)	81.75	71.68	-12.31	39.11	81.07	+51.75
HBS 123 (G ₁₇)	93.55	65.44	-30.04	40.02	80.73	+50.42
HLS 321729 (G ₁₈)	85.30	58.86	-30.99	34.57	64.47	+46.38
CCH 1831 (G ₁₉)	82.40	64.63	-21.57	38.32	70.38	+45.56
AKA 081 (G ₂₀)	80.28	68.04	-15.24	42.07	79.25	+46.92
IC 359963 (G ₂₁)	76.30	51.83	-32.07	35.59	66.97	+46.86
PS-20-2-1 (G ₂₂)	77.37	44.73	-42.19	39.45	78.68	+49.86
543403 A03 N106 (G ₂₃)	84.16	52.45	-37.68	34.91	70.38	+50.40
RHC 0811 (G ₂₄)	83.44	57.22	-31.43	37.41	70.84	+47.19
211412W247-85-D14-26 (G ₂₅)	89.53	57.17	-36.15	35.36	71.52	+50.56
JBWR 23 (NSP-18) (G ₂₆)	79.90	56.77	-28.94	39.34	77.54	+49.27
EC 560392 (G ₂₇)	79.35	67.94	-14.38	36.61	74.70	+50.99
Mean	81.58	61.32		38.57	74.17	
CV	0.95			0.99		
	CD	SEm±		CD	SEm±	
	1.02	0.37		0.68	0.24	
	0.28	0.10		0.19	0.07	
	1.45	0.52		0.96	0.34	

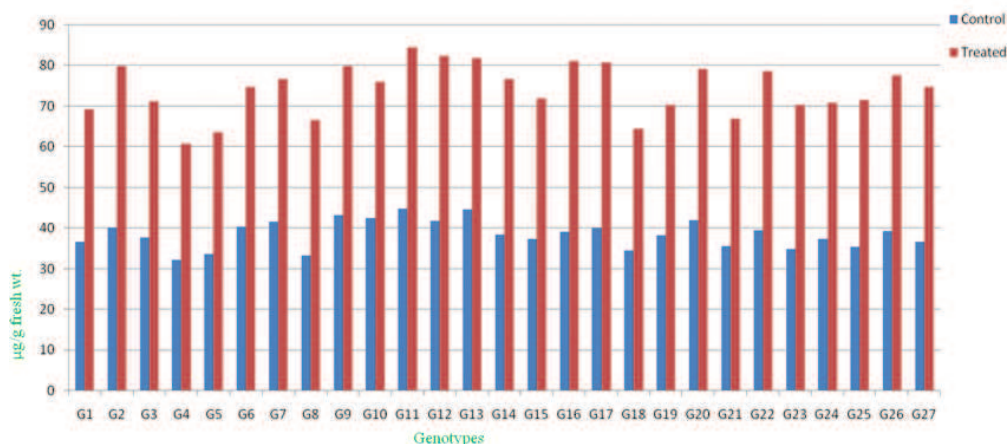


Fig. 1. Effect of moisture stress on proline content in leaf samples

Table 2. Relative expression profiling of *WRKY 19*, *LIM*, *NAC*, *GeBP*, *WRKY 70* and *C2H2* genes

Genotypes	Fold expression					
	<i>WRKY 19</i>	<i>LIM</i>	<i>NAC</i>	<i>GeBP</i>	<i>WRKY 70</i>	<i>C2H2</i>
G.cot-16 surath (G ₁)	185.6790	3068.5329	2.5609	0.7220	0.6926	0.4313
HBS-128 (G ₂)	2.9485	0.1553	166.1873	12.3234	0.2582	50.0975
Abhadita (G ₃)	0.1300	0.0589	0.0070	0.1801	0.3860	0.1564
CPD 464 (G ₄)	0.0634	0.0976	1.6320	2.2868	0.2376	0.0679
CPD 2007-4 (G ₅)	0.1397	0.1426	29.1078	1.9453	0.0943	1.2283
AK 23 B (G ₆)	0.0747	0.8847	0.3728	2.6027	0.1723	0.5471
RAJ-2 (G ₇)	421.6786	0.0542	66.1040	0.1634	0.2112	126.5298
NH 615 (G ₈)	0.2059	1.8834	1.8834	0.5797	0.0333	5.7491
543374 A 02 N68 (G ₉)	0.6213	0.6974	7.1602	0.1780	0.2212	13.8007
RDT-17 (G ₁₀)	0.3164	0.4920	21.5060	13.3306	9.1684	7.9631
JK-4 (G ₁₁)	1.0210	0.1081	145.6808	7.3785	1.3318	3.3096
PH 1009 (G ₁₂)	916.5057	13.2385	97.4552	183.546	657.1141	53.9419
CPD 446 (G ₁₃)	0.0143	2.1238	0.0303	4.8121	1.1355	0.0153
IC35701Coker 417-68 (G ₁₄)	0.1768	0.4886	120.537	34.3761	10.6050	12.3805
CPD 433 (G ₁₅)	2.1634	2.9966	4.3873	2.9554	2.0279	0.8566
5433 A2 A03 N83 (G ₁₆)	72.8404	5819.448	190.458	3.5801	84.6438	99.0442
HBS 123 (G ₁₇)	4.0935	0.7561	110.660	0.9075	11.5782	8.2630
HLS 321729 (G ₁₈)	0.1250	0.8685	7.2267	0.9862	0.0203	0.3686
CCH 1831 (G ₁₉)	0.3660	0.0529	0.2553	0.1041	0.3660	0.0956
AKA 081 (G ₂₀)	0.7337	3.0314	0.6990	1.8067	0.4414	0.6628
IC 359963 (G ₂₁)	9.2749	0.6242	2.4396	0.1630	0.2132	0.0123
PS-20-2-1 (G ₂₂)	1.2541	0.1882	1.4175	0.2655	0.0313	239.962
543403 A03 N106 (G ₂₃)	2.6329	1.0257	30.9815	1.8446	0.3376	0.9330
RHC 0811 (G ₂₄)	9.6911	0.1975	146.017	0.1463	0.1657	3.5064
211412W247-85-D14-26 (G ₂₅)	157.951	3532.972	23.3713	12.6115	34.6953	15.2070
JBWR 23 (NSP-18) (G ₂₆)	0.3360	0.9954	8.6738	3.7668	6.1050	1.3410
EC 560392 (G ₂₇)	5.9518	0.1514	8.5347	0.0325	0.1381	5.3765

ÄCt: (Ct of target gene-Ct of reference gene); Table t value (1 %, df: 2) = 4.30265

These TF genes were dramatically showed the down-regulation in some of the previously reported susceptible genotypes G.cot-16 Surath and RHC 0811 and CPD 2007-4 and in some newly studied genotypes during this investigation also showed the up and down-regulation of these TF genes whose transcription profile has been shown in Table 2. Although the findings are in close agreement with the previous report, those genotypes showed deviation from the previous reports showed further need to be validated. Specially there is need to reconfirm the tolerant nature of CCH-1831 genotype. The role of other transcription factors which are not considered in this study may also be responsible for its tolerance.

Authors' contribution

Conceptualization of research (ISK, AVS, H MV); Designing of the experiments (ISK, H MV, SAY); Contribution of experimental materials (ISK, H MV); Execution of field/lab experiments and data collection (SAY, MPJ, SA); Analysis of data and interpretation (SAY, MPJ, SA); Preparation of manuscript (ISK, SKY, MPJ, SA, H MV).

Declaration

The authors declare no conflict of interest.

References

- Abdeen A., Schnell J. and Miki B. 2010. Transcriptome analysis reveals absence of unintended effects in drought-tolerant transgenic plants over-expressing the transcription factor ABF3. *BMC Genomics*, **11**: 69-76.
- Bakshi M. and Ralf Oelmüller R. 2014. WRKY transcription factors: Jack of many trades in plants. *Plant Signaling & Behavior*, **9**: 1-18.
- Barrs M. D. and Weathery P. E. 1962. A re-examination of relative turgidity for estimating water deficit in leaves. *Australian J. Biolog. Sci.*, **15**: 413-428.
- Bates L. S., Waldeen R. P. and Teare I. D. 1973. Rapid determination of free proline for water stress studies. *Plant Soil*, **39**: 205-207.
- Caldana C., Scheible W. R., Mueller-Roeber B. and Ruzicic S. A. 2007. Quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. *PL. Methods*, **3**: 1746-1748.
- Chen J., Gu W. L. and Dai J. Y. 1990. Effects of drought on permeability and fatty acid composition of membrane lipids in leaves of *Zea mays*. *Plant Physiol Communic.*, **6**: 39-41.
- Chevalier F., Perazza D., Laporte F., Le He´nanff G., Hornitschek P., Bonneville J. M., Herzoq M. and Vachon G. 2008. *GeBP* and *GeBP*-like proteins are noncanonicalleucine-zipper transcription factors that regulate cytokinin response in *Arabidopsis*. *Plant Physiol.*, **146**: 1142-1154.
- Kim S. G., Kim S. Y. and Park C. M. 2007. A membrane-associated *NAC* transcription factor regulates salt-responsive flowering via Flowering Locus T in *Arabidopsis*. *Planta.*, **226**: 647-654.
- Livak K. J. and Schmittgen T. D. 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2-DDCT method. *Methods*, **25**: 402-408.
- Olsen A. N., Ernst H. A., Leggio L. L. and Skriver K. 2005. *NAC* transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci.*, **10**: 79-87.
- Perazza D., Laporte F., Balague C., Chevalier F, Remo S, Bourge M., Herzoq M. and Vachon G. 2011. *GeBP/ GPL* transcription factors regulate a subset of cpr5-dependent processes. *Plant Physiol.*, **157**: 1232-1242.
- Shinozaki K and Yamaguchi-Shinozak K. 2007. Gene networks involved in drought stress response and tolerance., *J. Exp. Botany.*, **58**: 221-227.
- Tran L S., Nakashima K., Sakuma Y., Simpson S D., Fujita Y., Maruyama K., Fujita M., Seki M., Shinozaki K. and Yamaguchi-Shinozaki K. 2004. Isolation and functional analysis of *Arabidopsis* stress inducible *NAC* transcription factors that bind to a drought responsive cis-element in the early responsive to dehydration stress1 promoter, *Plant Cell*, **16**: 2481-2498.
- Vasquez-Robinet C., Mane S. P., Ulanov A. V. et al. 2008. Physiological and molecular adaptations to drought in andean potato genotypes. *J. Exp. Bot.*, **59**: 2109-2123.
- Xu D. O., Huang J., Guo S. Q., Yang X. and Bao Y. M. 2008. Overexpression of TFIIIA-type zinc finger protein gene ZFP252 enhances drought and salt tolerance in rice (*Oryza sativa* L.). *FEBS Lett.*, **582**: 1037-1043.
- Zhou S., Bechner M C., Place M., Churas C P., Pape L., Leong S. A., Runnheim R., Forrest D. K., Goldstein S., Livny M. and Schwartz D. C. 2007. Validation of rice genome sequence by optical mapping. *BMC Genom.*, **8**: 270-278.
- Zhu Y. N., Shi D. Q., Ruan M. B., Zhan L. L., Meng Z. H, Liu J., Yang W. C. 2013. Transcriptome analysis reveals crosstalk of responsive genes to multiple abiotic stresses in cotton (*Gossypium hirsutum* L.), *PLOS ONE*, **8**: e0080218-1-13.
- Zhu X. Y. and Zhang C. L. 2003. Main functional proteins in plant responses to water stress. *Acta Botanica Boreali-Occidentalia Sinica*, **23**: 503-508.

Supplementary Table S1. Up and down-regulated TF genes in cotton genotypes

Genotype	TF genes up-regulated	TF genes down-regulated
G.cot-16 surath (G ₁)	<i>WRKY 19, LIM, NAC</i>	<i>GeBP, WRKY 70, C2H2</i>
HBS-128 (G ₂)	<i>NAC, GeBP, C2H2</i>	<i>LIM, WRKY 70</i>
Abhadita (G ₃)	-	<i>WRKY 19, LIM, NAC, GeBP, WRKY 70, C2H2</i>
CPD 464 (G ₄)	-	<i>WRKY 19, LIM, WRKY 70, C2H2</i>
CPD 2007-4 (G ₅)	<i>NAC, GeBP</i>	<i>WRKY 19, LIM, WRKY 70</i>
AK 23 B (G ₆)	-	<i>WRKY 19, NAC, WRKY 70</i>
RAJ-2 (G ₇)	<i>WRKY 19, NAC, C2H2</i>	<i>LIM, GeBP, WRKY 70</i>
NH 615 (G ₈)	<i>LIM</i>	<i>WRKY 19, GeBP, WRKY 70</i>
543374 A 02 N68 (G ₉)	<i>NAC</i>	<i>GeBP, WRKY 70</i>
RDT-17 (G ₁₀)	<i>NAC, WRKY 70, C2H2</i>	<i>WRKY 19, LIM</i>
JK-4 (G ₁₁)	<i>NAC, WRKY 70</i>	<i>LIM</i>
PH 1009 (G ₁₂)	<i>WRKY 19, NAC, GeBP, WRKY 70, C2H2</i>	-
CPD 446 (G ₁₃)	<i>LIM, GeBP</i>	<i>WRKY 19, C2H2</i>
IC35701Coker 417-68 (G ₁₄)	<i>NAC, GeBP, WRKY 70, C2H2</i>	<i>WRKY 19</i>
CPD 433 (G ₁₅)	<i>LIM, NAC</i>	-
5433 A2 A03 N83 (G ₁₆)	<i>WRKY 19, LIM, NAC, GeBP, WRKY 70, C2H2</i>	-
HBS 123 (G ₁₇)	<i>WRKY 19, NAC</i>	-
HLS 321729 (G ₁₈)	<i>NAC</i>	<i>WRKY 19, WRKY 70, C2H2</i>
CCH 1831 (G ₁₉)	-	<i>WRKY 19, LIM, NAC, GeBP, WRKY 70, C2H2</i>
AKA 081 (G ₂₀)	<i>LIM</i>	<i>WRKY 70, C2H2</i>
IC 359963 (G ₂₁)	-	<i>GeBP, WRKY 70, C2H2</i>
PS-20-2-1 (G ₂₂)	<i>C2H2</i>	<i>LIM, GeBP, WRKY 70</i>
543403 A03 N106 (G ₂₃)	<i>NAC, GeBP</i>	<i>WRKY 70</i>
RHC 0811 (G ₂₄)	<i>NAC</i>	<i>LIM, GeBP, WRKY 70</i>
211412W247-85-D14-26 (G ₂₅)	<i>WRKY 19, LIM, NAC, WRKY 70, C2H2</i>	-
JBWR 23 (NSP-18) (G ₂₆)	<i>NAC, WRKY 70</i>	<i>WRKY 19</i>
EC 560392 (G ₂₇)	<i>WRKY 19, NAC</i>	<i>LIM, WRKY 70</i>