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Research Article

Expression of NAC transcription factor is altered under intermittent drought stress and re-watered conditions in *Hevea brasiliensis*

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Abstract Drought stress is one of the important factors that restrict the expansion of *Hevea brasiliensis* cultivation to non-traditional regions experiencing extreme weather conditions. Plants respond to drought stress by triggering expression of several drought responsive genes including transcription factors which in turn trigger expression of various downstream signalling pathways and adaptive networks. Expression of such drought responsive genes may revert back to their original level upon re-watering. However, no reports are available on such phenomenon in *Hevea* and hence, this study was initiated. For this purpose, NAC transcription factor (*NAC tf*) was chosen as candidate gene. Its expression levels were monitored under intermittent drought as well as irrigated conditions in two clones (RRII 105 and RRIM 600) of *H. brasiliensis* with contrasting tolerance level. Copy number of *NAC tf* was found similar in both the clones. Expression of *NAC tf* was found highly up-regulated in RRIM 600 (a relatively drought tolerant clone) than in RRII 105 (a relatively drought susceptible clone) throughout the drought incidences which upon re-watering, reached back to its original levels in both the clones. The study indicated the existence of an association between expression of *NAC tf* and drought tolerance trait exhibited by the tolerant clone RRIM 600. The study also proves the influence of drought and re-watering on the leaf photosynthesis and expression of *NAC tf* in *H. brasiliensis.*

Keywords *Hevea brasiliensis*, intermittent drought stress and watering, NAC transcription factor, quantitative expression analysis

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Abbreviations

tf:	Transcription factor
RRII:	Rubber Research Institute of India
A:	CO_2 assimilation rate
gs:	Stomatal conductance
qPCR:	quantitative PCR
RQ:	Relative quantification
Ct:	threshold cycle

Introduction

Drought is one of the most devastating abiotic stresses, which negatively influences plant growth and development in general (Deikman et al. 2012) and it is the most important factor that restricts the expansion of *Hevea brasiliensis* cultivation to newer areas in several rubber growing countries (Sethuraj, 1986). Soil and atmospheric drought, high atmospheric temperature, high light and low relative humidity occurring at the same time severely affect the growth and yield of natural rubber (Chandrasekhar et al. 1990; Jacob et al. 1999; Devakumar et al. 1998). Different genotypes adopt different survival mechanisms to acclimatize to extreme climatic conditions.

Plants have evolved various survival strategies to overcome water deficit conditions and at the molecular level, several transcription factors are triggered which function as central regulators and molecular switches for gene expression in stress signaling and adaptation networks (Zhang et al. 2011). Transcription factors (Tfs) play important roles in plant stress responses by regulating various signalling pathways through their binding to the *cis*-acting elements located in promoter region of downstream target genes, thereby activating or repressing them. Many Tfs *viz.* WRKY (Rushton et al. 2012), zinc finger (Huang et al. 2009), AP2/ERF2 (Sakuma et al. 2002), MYB (Abe et al. 1997), ZmDREB2A

(Qin et al. 2007) and NAC (Tran et al. 2004) have been characterized as drought-responsive.

NAC transcription factors comprise one of the largest gene families, which are only found in plants. NAM (no apical meristem), ATAF (Arabidopsis transcription activation factor), CUC (cup-shaped cotyledon) or NAC domain proteins possess a highly conserved N-terminal DNA binding domain (NAC) and a variable C-terminal transcription regulation region (TRR) which are known to activate or suppress transcription of many target genes (Ernst et al. 2004). The C-terminal regions of some *NAC tf*s also contain transmembrane motifs (TMs) which anchor to the plasma membrane (Tran et al. 2004; Nakashima et al. 2007). NAC genes have been reported to be involved in organ development and boundary maintenance, cell division, secondary wall synthesis, senescence, defence against pathogens and also act as master regulators in abiotic stress responses (Takada et al. 2001; Guo and Gan 2006; Kim et al. 2006; Uauy et al. 2006; Zhong et al. 2006; Puranik et al. 2012). In *Hevea brasiliensis*, along with expression of several drought responsive transcripts (Thomas et al. 2005; 2011; 2012; Sathik et al. 2011; Luke et al. 2015), differential expression of *NAC tf* also was observed under drought stress. Further under drought stress, it was found significantly up-regulated in relatively drought tolerant *Hevea* clones while its expression was at minimal in the relatively susceptible clone (Thomas et al. 2011). Even though molecular effects of drought stress on plants are well documented, responses in plants to intermittent drought and re-watering are relatively unknown. The information generated on gene expression pattern under drought stress as well as associated with recovery from stress during re-hydration would provide a degree of cross verification of genes regulated by drought stress (Huang et al. 2008). To evaluate the level of expression of drought responsive transcripts in *Hevea* under drought and re-watered conditions, *NAC tf* expression was taken as a candidate gene. Identification of drought responsive genes and validation of their association with drought tolerance are pre-requisites to establish if they can be employed in crop improvement programmes. Even though reports on gene response to drought stress and subsequent re-watering are available in other plants (Filippou et al. 2011), no such studies were reported in *Hevea*. Hence this study was conducted in *Hevea* with an aim to assess the molecular response to drought stress and re-watering with reference to a specific drought responsive gene *viz NAC tf*. Prior to validation of expression in different clones of *Hevea* with varying levels of drought tolerance, their copy number in clone RRII 105 and RRIM 600 was assessed. Expression of *NAC tf* under drought and during re-watering was measured and the results are discussed.

Materials and Methods

Plant material and stress induction

Two *Hevea* clones, RRII 105 (relatively drought susceptible) and RRIM 600 (relatively drought tolerant) were chosen for the present study. The plants were produced by bud-grafting of seedlings with clonal buds collected from *Hevea* budwood nursery maintained at Rubber Research Institute of India (RRII) farm at Kottayam. The budded stumps were later transferred to polythene bags (size, 65 × 35 cm) and were grown in open field conditions at RRII as per the recommended package of practices (Mercykutty, 2008). After growing for six months (two to three whorl stage) in open field conditions, the plants were transferred to glass house for treatment. One group of plants was subjected to drought stress for five days and another group for ten days and both the groups were subsequently re-watered for another five days. This was followed by another similar cycle of drought stress and re-watering. The control plants were irrigated on alternate days to saturation level throughout the study period. Photosynthetic gas exchange parameters were recorded after each treatment (5 and 10 days after drought and five days after re-watering) during the three cycles. After each treatment, leaf samples were collected in liquid N_2 for gene expression analysis.

Physiological parameters

The degree of impact of drought stress on young plants was assessed by measuring the net CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI-6400 XT), LI-COR, U.S.A. All the gas exchange measurements were made at a constant CO_2 concentration of 400 ppm using a CO_2 injector (LI-6400-01, LI-COR, USA) and at 500 μmol m^{-2} s^{-1} of light intensity using red LED source (with 10 % blue light) attached with the leaf chamber.

Gene expression analysis

Total RNA from the leaf samples was extracted using Spectrum Plant Total RNA Kit (Sigma-Aldrich) followed by cDNA synthesis (4 μg of total RNA as starting material) using Superscript III reverse transcriptase (Invitrogen) following

Table 1 Genes and the corresponding primers used for qPCR analysis. *GAPDH* gene was used as internal control and *HbCOI1* gene was used for comparison of expression

Sl. No.	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
1	HbDRT5b (NAC tf)	TCAAACACTGTCATGTCCAAGAAA	GAATCAGGGCAACCTTTTAAACC
2	HbCOI1	AGGTATTTGTGGGTGCAAGGTT	GGCGAGCCATTGCTAGAAGA
3	GAPDH	GCCTGTGATAGTCTTCGGTGTTAG	GCAGCCTTATCCTTGTCAGTGAAC

the manufacturer's instructions. Quantitative PCR (qPCR) primers were designed (amplicon size 130 bp) using Primer Express software (Table 1) followed by synthesis (M/s. Ocimum Biosolutions, Hyderabad). Quantitative gene expression analysis was eventually carried out using Light Cycler 480 II, Roche Real Time PCR System. qPCR was performed in a 20 µl reaction mixture containing 1 µl from 1/10 dilution of first-strand cDNA reaction, 125 nM of each primer and 10 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics Gmbh, Germany). qPCR was performed by incubation at 95º C for 7 min, followed by 40 cycles of 95ºC for 20 seconds and 60ºC for 30 seconds. This was followed by a melt curve analysis (95ºC for 20 seconds, 60ºC for one minute and 95ºC for 5 minutes). Each PCR with three biological replications was repeated twice or thrice in triplicates with null-template controls. Reaction efficiency of both the target genes and the endogenous control was calculated based on the formula, Efficiency $= 10^{(-1/slope)} - 1$. The primers were standardized based on serial dilution experiment and were ensured to have a slope value between -3.2 and -3.5 before proceeding for qPCR analysis. GAPDH was used as endogenous control. The relative quantification (RQ) values were analyzed (using Light Cycler 480 Software; release 1.5.0) and the expression rate is represented as fold change.

Data Analysis

The $2-\triangle\triangle Ct$ method was adopted to analyze the relative changes in gene expression from qPCR experiments (Livak and Schmittgen 2001). The data are presented as fold change in transcript level normalized to the endogenous control (GAPDH) gene, relative to that in irrigated plants. Statistical analysis was performed with the relative quantification data using ANOVA. The ratio with P-value < 0.05 was adopted as significant for either down or up-regulation.

Plant material and stress induction for copy number determination of *NAC tf*

Genomic DNA was isolated from leaf samples of RRII 105 and RRIM 600 as reported previously (Thomas et al. 2001). Optimum concentration of DNA and primers required for obtaining Ct value in the range of 20-25 was standardised. *COI1*, the coronatine insensitive gene (a single copy gene in *Hevea*) was used as reference (Peng et al. 2009) and GAPDH was used as internal control.

Results

Plants of both the clones (RRII 105 and RRIM 600) before imposing drought treatment had an CO_2 assimilation rate (A) of about 10 and 11 µ mol m^{-1} s^{-1}, respectively (Fig. 1). Upon undergoing water deficit stress for five days, the A reduced to about 2.7 µ mol m^{-1} s^{-1} in clone RRII 105 while RRIM 600 had 3.4 µ mol m^{-1} s^{-1}. Upon drought treatment for ten days, the A reduced further to about 0.8 µ mol m^{-1} s^{-1} in clone RRII 105 while RRIM 600 had exhibited 1.6 µ mol m^{-1} s^{-1}. Though A got reduced in both the clones, the clone RRIM 600 maintained better A than clone RRII 105. After ten days of withholding water, the plants were watered daily for five days. On the sixth day, A got improved to about 5.5 µ mol m^{-1} s^{-1} in RRII 105 and 7.3 µ mol m^{-1} s^{-1} in RRIM 600. When a second cycle of drought was imposed for five days on these plants, the A got reduced to

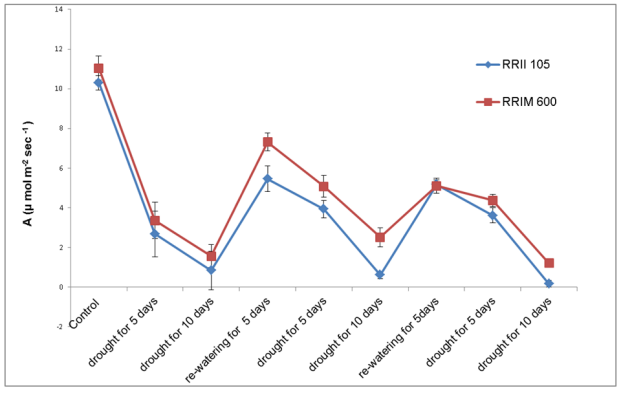

Fig. 1 CO_2 assimilation rate (A) measured in clones RRII 105 and RRIM 600 of *Hevea brasiliensis* under intermittent drought and watering cycles (during 5th and 10 day upon each drought and re-watered periods)

Table 2 Ct values of *NAC tf* in RRII 105 and RRIM 600

Gene	Ct value	
	RRII 105	RRIM 600
*HbCOI*1	21.53	21.18
HbDRT5b (*NAC tf*)	21.69	21.74

the levels of 3.3 μ mol m^{-1} s^{-1} in RRII 105 and 5 μ mol m^{-1} s^{-1} in RRIM 600 respectively. When compared to the drought for 5 days on the first cycle, the reduction in A was lesser in the second cycle of drought. But when the drought was extended for ten days during the second cycle of drought, the A reduced to 0.6 μ mol m^{-1} s^{-1} in RRII 105 and 2.5 μ mol m^{-1} s^{-1} RRIM 600. This indicates that the reduction in A in susceptible clone was much more than the tolerant clone. When this was followed by another round of re-watering, A improved to about 5.1 μ mol m^{-1} s^{-1} in both the clones. When a third round of drought stress was imposed, the plants exhibited about 3.6 and 4.4 μ mol m^{-1} s^{-1} on the fifth day and about 0.17 and 1.2 μ mol m^{-1} s^{-1} on the tenth day in RRII 105 and RRIM 600, respectively. Throughout the treatments (of intermittent watering and three rounds of drought treatment), clone RRIM 600 maintained better A indicating its drought tolerance nature. When A of clone RRII 105 during the third round of drought treatment reached near zero, RRIM 600 maintained A to the level of 1.2 μ mol m^{-1} s^{-1}. This indicates that RRIM 600 has the inherent capacity to perform well under drought stress by maintaining better A.

In order to find if the copy number of *NAC tf* in the genome of the two clones was same, a PCR was performed using specific primers of *NAC tf* and a single copy gene *COI1* (coronatine insensitive gene 1) as reference gene (Peng et al. 2009). The Ct value of *NAC tf* in both the clones was found similar indicating that the copy number of this particular gene in both the clones did not differ (Table 2). Gene expression analysis of *NAC tf* was performed in irrigated and drought treated leaf samples of the two clones (Fig. 2). After 5 days (during the first cycle of drought), its expression in clone RRIM 600 was about 5.5 fold higher while it was only 1.4 fold in RRII 105 than the respective watered controls. Interestingly, its expression got highly triggered to about 37 fold in RRIM 600 in drought treatment for 10 days while this was about only 2.7 fold in RRII 105. During the subsequent watering treatment, the expression level was much lower (between 0.3 and 0.2 fold) in both the clones. While no much change could be observed 5 days of drought in both the clones, it got about 9 and 1.5 fold up-regulated in clones RRIM 600 and RRII 105 respectively in the second cycle of drought (10 days). Again

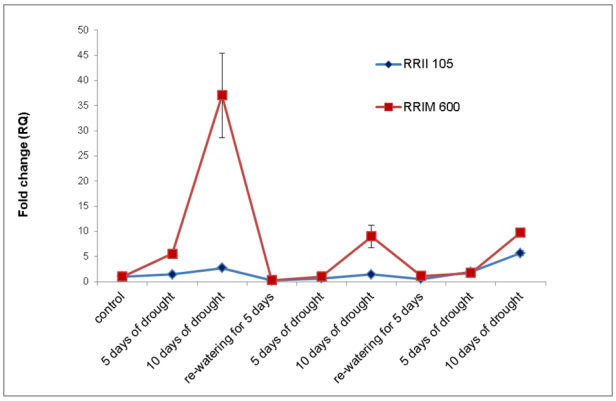

Fig. 2 Quantitative expression analysis of *NAC tf* in clones RRII 105 and RRIM 600 of *Hevea brasiliensis* under intermittent drought and watering cycles (during 5 and 10 days of drought treatment in each cycle). ± Error bars indicate standard error of three biological replicates

during the second cycle of re-watering, while there was no much change in RRIM 600, it got 0.5 fold down regulated in clone RRII 105. During the third round of drought cycle, there was slight increase in both the clones (1.7 and 1.9 fold increase in RRIM 600 and RRI 105, respectively) on the 5th day while there was about 9.8 and 5.7 fold increase in RRIM 600 and RRII 105, respectively on the 10th day. It was interesting to note that the levels of *NAC tf* expression shot up to about 37 fold in clone RRIM 600 after 10 days of continuous drought treatment while it was only at meagre levels in clone RRII 105. Up-regulation of *NAC tf* only in the relatively drought tolerant clone (RRIM 600) during the subsequent drought cycles indicates its stronger association with drought tolerance. The up-regulation of *NAC tf* in RRII 105 in the third cycle of drought (5.7 fold increase) indicates that its up-regulation in this clone is much slower than that of RRIM 600 under repeated drought/watering cycles.

Discussion

During summer season in India, the agroclimatic regions like North Konkan, Maharashtra, Madhya Pradesh, Orissa which are prone to drought do not get summer showers. But the traditional regions often get intermittent summer showers which come as a boon thus saving crop plants from acute drought stress. Though it could be presumed that the summer shower helps the plants to recover from the severity of the drought, very few reports are available on its impact on the physiological and molecular aspects of rubber plants. Hence, this experiment was designed to study the effect of alternate cycles of drought and watering

on photosynthesis (CO_2 assimilation rate) and expression of *NAC tf*. Our previous studies in *Hevea* also established *NAC tf* as stress responsive and to have much stronger association with stress tolerance (Thomas et al. 2011).

In the Indian rubber scenario, drought in both the traditional and non-traditional regions is severe during summer except for the fact that non-traditional regions are relatively warmer. The growth and productivity of *Hevea* plants are negatively influenced by drought (Sethuraj 1986, 1989; Chandrasekhar, 1990) while the photosynthetic mechanisms get severely affected under drought and high light thus leading to photoinhibition and photodamage (Devakumar et al. 2002, 1998; Jacob 1999). Irrigation during drought season resulted in better growth, leaf area index and photosynthesis (Vijayakumar et al. 1998, Devakumar et al., 1998, 1999). It is a common phenomenon that the leaves of *Hevea* under such prolonged water deficit conditions turn yellow and necrotic eventually leading to the death of the plants. Trees irrigated during drought season had resulted in better growth, leaf area index and photosynthesis (Vijayakumar et al. 1998, Devakumar et al. 1998, 1999).

Gas exchange parameters have been proven to be good indicators for evaluating the impact of stress on plants. But, the effect of long term drought with intermittent watering cycle on rubber had not been investigated earlier. In this study, both the clones maintained an optimum A at 10 ~ 11 μ mol m^{-1} s^{-1} under optimum soil moisture conditions. Though A got reduced in the first days of drought treatment to near 3 μ mol m^{-1} s^{-1} in both the clones, it went further down to less than 2 in RRIM 600 and below 1 in RRII 105. However, RRIM 600 maintained better tolerance than RRII 105 throughout the course of the treatment. Though A improved in both the clones during the subsequent irrigation cycles, it never regained its original level which indicates the severity of the damage inflicted upon the photosynthetic apparatus. Interestingly, the levels of *NAC tf* also showed the same trend.

Upon re-watering, many genes involved in growth, cell wall modification and lignin biosynthesis are up-regulated in addition to photosynthesis and re-hydration related genes (Zhou et al. 2007) while genes involved in stress protection mechanisms such as Early light inducible protein (ELIP) or LEA proteins and in detoxifying systems (thioredoxins) get repressed (Spiess et al. 2012). Transcription factors (tfs) for e.g. MYB, DREB, bZIP and WRKY have been found directly or indirectly involved in plant response to drought stress which generally get up-regulated under drought conditions and revert back to original levels under re-watered conditions (Golldack et al. 2014).

Prior to the selection of a candidate stress responsive gene, its copy number in genome should be ensured same in both the clones. Difference in copy number may end up with drastic change in their expression levels. For this purpose, a PCR was performed for *NAC tf* by using a single copy gene *COI1* (coronatine insensitive gene) as reference gene (Peng et al. 2009). The results indicated that the copy number of *NAC tf* was same in both the clones (Table 2). Hence, *NAC tf* was employed further in the drought and intermittent re-watering experiment as reference gene. When the expression pattern of *NAC tf* under drought stress and subsequent re-watering was evaluated, *NAC tf* was found up-regulated in the relatively tolerant clone RRIM 600 under drought stress and the expression was much higher after 10 days of drought imposition (37 fold) when compared to the irrigated plants whereas the level of expression was relatively lesser in RRII 105. In the second and third cycle of drought also, *NAC tf* got highly up-regulated (8.9 and 9.7 fold) after 10 days of drought imposition in clone RRIM 600. Thus, the significant up-regulation in the levels of *NAC tf* in RRIM 600 under drought stress might be associated with its inherent drought tolerance nature. Upon re-watering, the expression of *NAC tf* got repressed in both the clones followed by a gradual increase during the subsequent drought stress cycles. After second re-watering, the level of expression went back to levels similar to control in RRIM 600 and 0.5 fold in RRII 105 indicating the recovery to the normal levels.

Though both the clones exhibited a similar trend in expression of *NAC tf* under both drought stress and re-watered conditions, RRIM 600 exhibited relatively higher levels of expression thus conforming to our previous results as well as trend shown by physiological parameters in this study. From these results, it can be understood that the quantitative differences in the responses at the physiological and gene expression levels might have contributed to the increased drought tolerance observed in RRIM 600. The copy number of *NAC tf* when estimated did confirm the fact that higher levels of *NAC tf* found in clone RRIM 600 was not due to any difference in the copy number of the same in both the clones but due to the inherent mechanism of up-regulation existing in clone RRIM 600. Another interesting feature is the higher levels of *NAC tf* to drought in the first cycle when compared to its level in the subsequent drought cycles. This indicates that the rate of response decreases gradually over the subsequent cycles of drought and re-watering. But among the clones studied, the tolerant ones always displayed better response to the drought/re-watering cycle.

The physiological parameters indicated that drought stress leads to reduction in CO_2 assimilation rate as well as poor crop performance while sub-sequent watering cycles help the plants to recover from stress though there were differences in its response among the clones studied. This study also confirmed the similarity in copy number of *NAC tf* in both the clones. The quantitative expression studies performed after confirming the similarity in copy number of *NAC tf* in both the clones revealed that expression of *NAC tf* is triggered as a response to drought in both the clones though at different levels. The level of *NAC tf* in tolerant clone RRIM 600 was many folds higher than in the susceptible clone RRII 105. This study indicates the association between *NAC tf* and the drought tolerance trait. Above all, this study could establish influence of drought and subsequent re-watering cycles on photosynthesis and expression of *NAC tf*. The study reiterates the relevance of *NAC tf* in drought response and in drought tolerance while opening up the possibility of employing this particular transcription factor in crop improvement programmes of *Hevea brasiliensis*.

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