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ANALYSIS OF DROUGHT RESPONSIVE GENE EXPRESSION IN *HEVEA BRASILIENSIS*

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Cultivation of *Hevea brasiliensis* is being extended to drought-prone marginal areas and therefore, identification of suitable clones that can survive under adverse climatic conditions is highly essential for enhancing the production of natural rubber in India. Identification of genes/factors contributing to stress tolerance will help in understanding the molecular basis of drought tolerance. Relevance of seven drought responsive genes (identified earlier) was studied in drought sensitive/tolerant clones. Studies on net CO_2 assimilation rate (A) and stomatal conductance (g_s) indicated that clones such as RRIM 600 and RRII 430 were relatively more tolerant to drought than RRII 105 and RRII 414. The gene expression studied by quantitative PCR method demonstrated strong association of genes such as CRT/DRE binding factor and ABC transporter protein with drought tolerance, as evidenced by their up-regulation and down-regulation, respectively only in the drought-tolerant clones (RRIM 600 and RRII 430) when exposed to drought stress. Expression analysis of these genes in more *Hevea* clones would further strengthen the significance of their association with drought tolerance potential.

Keywords: Drought stress, Gene expression, *Hevea brasiliensis*, Stress responsive genes, qPCR analysis

INTRODUCTION

Cultivation of *Hevea brasiliensis,* the most important source of natural rubber, is being extended to marginal areas of western and central India where soil and atmospheric drought and high temperature are the major environmental factors limiting its growth and yield (Jacob *et al*., 1999). Identification of clones that can survive under extreme climatic conditions giving sustainable yield is essential to maximize productivity in rubber plantations in such stressful regions in India.

Tolerant clones develop several adaptive mechanisms to manage or escape the adverse impacts of environmental extremes. One of the major molecular responses that plants exhibit when exposed to drought stress is altered expression of genes related to different metabolic pathways associated with stress perception, signal transduction and regulation and synthesis of a number of compounds (Ramanjulu and Bartels, 2002; Sreenivasulu *et al*., 2007). Understanding the mechanisms involved in the plant's response to abiotic stresses such as drought, cold, *etc.* is required for selecting plants with better tolerance to environmental stresses.

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Several hundred genes that respond to drought stress at the transcriptional level have been identified in many plants (Shinozaki and Yamaguchi- Shinozaki, 2007; Govind *et al*., 2009; Wilkins *et al*., 2009). Studies on transcriptome-level changes in response to drought stress in two hybrid *Populus* clones indicated that although there are a number of conserved transcriptome-level changes between the genotypes, there are many more changes that appear to be specific to one or the other genotypes (Wilkins *et al*., 2009). To understand the molecular basis of drought tolerance in *H. brasiliensis*, genes/factors contributing to stress tolerance have to be identified. Although some mechanisms of stress tolerance are common to all *H. brasiliensis* clones, the degree of tolerance varies among clones. An attempt has been made here to assess the relevance of a few selected stress-related genes (from the *Hevea* EST library of drought - imposed plants) in some popular *H. brasiliensis* clones.

MATERIALS AND METHODS

Sample collection and mRNA isolation

Seven-month-old ploybag-grown plants of RRII 105 (drought sensitive), RRIM 600 (relatively drought tolerant), RRII 414 and RRII 430 (newly-released clones with unknown drought tolerance potential) were selected for this study at RRII. Drought treatment was imposed during February 2010. One set of plants (n=6) from each clone was subjected to water stress by withholding water for 15 days and another set of plants was irrigated to field capacity every alternate day. The impact of stress was assessed by measuring net CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI- 6400, LI-COR, USA) attached to a leaf chamber fluorometer (LCF-6400-40, LI-COR, USA). Gas exchange measurements were made at a leaf temperature of 30 ± 2.0 °C, light intensity 500 µmol/mol/s and a constant CO_2 concentration of 380 µmol/mol using a CO_2 injector (LI-6400-01, Li-COR, USA), between 8:30 a.m and 11:30 a.m. IST.

Leaf samples collected in liquid N_2 were ground and mixed with RNA extraction buffer containing 50 mM Tris (pH 8.0), 150 mM $LiCl_2$, 5 mM EDTA, 5% sodium dodecyl sulphate (SDS), 1% soluble polyvinylpyrrolidone (PVP) and 2% β-mercaptoethanol. After a brief spin to remove the debris, mRNA was isolated from the supernatant using magnetic beads (Dynabeads, Invitrogen). The quantity of mRNA was determined by using a Nanodrop spectrophotometer (USA). cDNA was prepared from 250 ng mRNA in a 20 µl reaction mixture using SuperScript III First-Strand synthesis system for RT-PCR (Invitrogen, USA) according to the manufacturer's recommendations.

Quantitative PCR (qPCR)

Quantitative PCR analyses were carried out to study the expression levels of seven stress responsive genes, *viz.* HbNRG 18 (*rps* 14), HbDRT 50 (unknown), peroxidase, ABC transporter protein, LEA 5 protein, CRT/ DRE binding factor and glutathione peroxidase (GPX). Two housekeeping genes, ADP ribosylation factor and polyubiquitin were used as the internal controls for normalization (Vandesompele *et al*., 2002). The primers were designed (determined on the 3' regions of the genes with an amplicon size of 75-150 bp) using the Primer Express software (Version 3.0) and were synthesized by Ocimum Biosolutions, Hyderabad. The

Table 1. **Nucleotide sequences of primers used for qPCR analysis**

Sl.No.	Gene	Forward primer	Reverse primer
1	HbNRG 18	GAGGATCGACGCATCAAATGA	AAAGTTTGATTCAGCGGGAGAA
2	HbDRT 50	TCGGAAGCTTCGACTGTGATG	TACTGCTAGGATTTCGACC TTAAACA
3	Peroxidase	AATTGGCACGAATTTCCC	CACGGATAAGAGAACAAGG
4	ABC transporter protein	ACCGGCGTCCTATGTTCTCA	TTTAATCCCAGCAATCCATATCG
5	LEA 5 protein	CTCGCTTTCCCTCCAATG	TTCCTCACCATACCACTCC
6	CRT/DRE binding factor	CCATAGTGACACATCAGCTGCAT	ATATGGACGAAGAGGCGGTTT
7	Glutathione peroxidase	GCCTGCGTTGTGTTCTTTGA	TCAACATCGTTTCCCCTAGCA
8	ADP Ribosylation factor	TGGTGGTCAAGACAAGATTCG	CGTCCTCATTCAACATTCTATGC
9	Polyubiquitin	CCGCCAGACCAGCAGAGG	CGAAGACGAAGAACCAAA TGAAGG

nucleotide sequences of primers used are given in Table 1. RT-PCR was performed in a 20 µl reaction mixture containing 1 µl of 1:20 times diluted first-strand cDNA reaction mixture, 6.5 nM of each primer and 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) on an Applied Biosystems 7500 Real Time PCR System. The PCR conditions were as follows: initial steps of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for one minute. This step was followed by a melt curve analysis (95 °C for 15 s, 60 °C for one min, 95 °C for 30 s and 60 °C for 15s). Each experiment was repeated two to three times and each PCR reaction was performed in triplicate with null-template controls. Reaction efficiency of both the target genes and the endogenous control was calculated based on the formula, $E = [10^{-1/slope}] - 1$. Primers with slope values between -3.2 and -3.5 only were employed for the qPCR analysis. The Relative Quantification (RQ) values were analyzed using the software of Applied Biosystems (SDS 7500, v. 2.0.3) to study the fold change in the expression levels between the treatments.

RESULTS AND DISCUSSION

The data on photosynthetic assimilation rate (A) and stomatal conductance (g_s) of the drought-imposed plants are given in Table 2. On the 15th day of drought imposition, the photosynthetic assimilation rate of RRII 105, RRIM 600, RRII 414 and RRII 430 were 1.5, 6.3, 0.0 and 3.5 µmol/m^2/s, respectively. Even on the 15th day of withholding the irrigation, RRIM 600 exhibited a high assimilation rate (6.3 µmol/m^2/s.) followed by RRII 430 (3.5 µmol/m^2/s.). A similar trend was also observed for g_s under drought stress. RRII

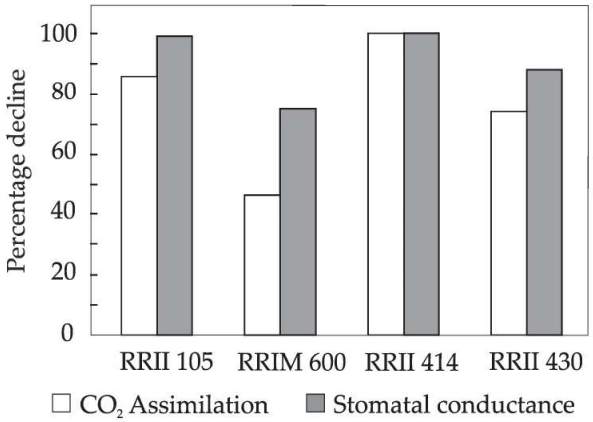

Fig. 1. Per cent decline in CO_2 assimilation rate and stomatal conductance of four *Hevea* clones after imposition of drought stress for 15 days

414 and RRII 105 recorded the lowest A and g_s under drought stress indicating the vulnerability of these clones to drought stress. The per cent decline in A (Fig. 1) was smaller in RRIM 600 (46%) than in RRII 430 (74%), RRII 105 (86%) and RRII 414 (100%). RRII 414 was found to be the most drought sensitive clone as 100% reduction in A was recorded after 15 days of drought imposition.

After 15 days of water stress, g_s came to near zero level in the clones RRII 105 and RRII 414 whereas it remained better in clones RRIM 600 and RRII 430 (Table 2). RRIM 600 recorded (Fig. 1) relatively lower reduction (75%) in g_s than RRII 430 (88%). This indicates the susceptibility of hydraulic factors in the stomatal apparatus to water deficit stress in RRII 105 and RRII 414, while RRIM 600 and RRII 430 remained relatively tolerant. It has been reported earlier that stomatal closure is one of the first responses to soil drying (Medrano *et al*., 1997). When water loss from leaves exceeds the uptake, stomatal conductance decreases and the limitation of CO_2 diffusion results in substantial reduction in photosynthesis (Lawlor and Tezara, 2009). RRIM 600 is a well-known drought tolerant clone (Jacob *et al*., 1999) and the data from the present study also indicate its drought tolerance potential in terms of stable photosynthetic rate and stomatal conductance.

The results of the qPCR analyses are given in (Table 3). The expression of C-repeat element/dehydration-responsive element (CRT/DRE) binding factor was found to be higher in both the drought-tolerant clones (RRIM 600 and RRII 430) and was remarkably high in the clone RRIM 600 when RRII 105 control was taken as the calibrator. CRT/DRE binding factors are known to be upstream transcription factors that bind to promoter *cis*-element CRT/DRE and play vital regulatory roles in abiotic stress responses in plants. The up-regulation of this factor may be associated with the drought tolerance in RRIM 600 and RRII 430. ABC transporter (ABCT) protein was found down-regulated in RRIM 600 and RRII 430

Table 2. **CO_2 assimilation rate (A) and stomatal conductance (g_s) of four clones after imposition of drought stress for 15 days**

Clone	A(μmol/m²/ s)		g_s (mol/m²/s)	
	Control	Drought	Control	Drought
RRII 105	10.5 ±0.45	1.5 ±0.19	0.19 ±0.01	0.002 ±0.00
RRII 414	9.8 ±0.26	0.0 ±0.02	0.20 ±0.01	0.001 ±0.00
RRIM 600	11.7 ±0.45	6.3 ±0.26	0.27 ±0.01	0.065 ±0.01
RRII 430	13.2 ±0.43	3.5 ±0.56	0.33 ±0.02	0.039 ±0.01
CD (P=0.05)	1.20	1.00	0.047	0.015

Table 3. **Relative quantification (RQ) of stress responsive genes in *Hevea* under drought stress with reference to irrigated plants of RRII 105 as calibrator**

Gene	RRII 105		RRII 414		RRIM 600		RRII 430	
	C	D	C	D	C	D	C	D
HbNRG18	1	1.57	1.97	1.89	1.61	1.84	2.71	7.10
HbDRT 50	1	0.65	1.68	1.09	1.18	1.52	2.55	0.98
Peroxidase	1	0.28	1.45	0.56	2.64	3.98	2.57	0.44
ABC transporter	1	1.25	1.06	1.52	0.59	0.18	0.66	0.42
LEA 5	1	4.95	1.12	20.38	0.80	1.03	0.77	1.47
CRT/ DRE	1	0.36	0.07	0.93	2.93	7.51	0.90	1.45
GPX	1	1.12	1.10	1.18	1.03	0.85	1.05	0.97

C- Control (irrigated); D- Drought stressed

(drought-tolerant clones) whereas it did not vary much in RRII 105 and RRII 414 (drought-susceptible clones). More than 120 genes encoding ABC proteins have been identified from *Arabidopsis* genome (Garcia *et al*., 2004), but their functional significance remains to be clarified in most cases. ATP-binding cassette (ABC) transporters are involved in the membrane transport of a wide range of structurally and functionally unrelated compounds such as ions, inorganic acids, peptides, secondary metabolites, toxins and drugs (Holland *et al*., 2003). Many molecules which require energy to be transported against a concentration gradient are transported by ABCT by means of ATP hydrolysis. Being immobile, plants have been forced to develop a sophisticated enzymatic machinery to cope with the environmental constraints and the ABCs appear to be an important part of this machinery (Jasinski *et al*., 2003). Down-regulation of ABC transporter implies that this protein might be contributing to drought stress tolerance in the clones RRIM 600 and RRII 430.

HbNRG 18 (rps 14 proteins) was found to be remarkably high in the drought-tolerant clone RRII 430. This indicates that the inherent level of expression of this particular gene was higher in this clone than in the susceptible clone RRII 105. Expression of LEA 5 was more in the drought-susceptible clones (RRII 105 and RRII 414) and was not much altered in the tolerant clones (RRIM 600 and RRII 430) after imposition of drought stress. Late Embryogenesis Abundant (LEA) proteins accumulate in higher levels during the last stage of seed maturation and during water deficit in vegetative organs playing a protective role during water limitation (Battaglia *et al*., 2008). Expression of peroxidase was relatively lesser under drought condition in all the clones studied except RRIM 600. Higher levels of peroxidase in clone RRIM 600 indicate the possibility of better scavenging of reactive oxygen species produced in the tree during stress situation, thereby imparting better drought tolerance. Expression of glutathione peroxidase and HbDRT 50 did not show any significant changes in the drought susceptible/tolerant clones.

The adaptive mechanisms under stresses are a net effect of altered cell metabolism resulting from regulated expression of stress responsive genes. An

understanding of gene regulation is particularly important in the case of multigenic traits like drought tolerance as different regulatory pathways determine the expression of a set of drought responsive genes. The results of the present study showed a strong association of genes such as CRT/DRE binding factor and ABC transporter with drought tolerance, as evidenced by their up-regulation and down-regulation respectively under drought stress, only in the tolerant clones (RRIM 600 and RRII 430). The expression analysis of these genes in more number of drought sensitive/ tolerant *Hevea* clones would further strengthen the significance of their association with drought tolerance potential.

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