IDENTIFICATION OF DROUGHT TOLERANT GENES BY QUANTITATIVE EXPRESSION ANALYSIS IN *HEVEA BRASILIENSIS*

Mohamed Sathik, M.B., Luke, L.P., Molly Thomas, Sumesh, K.V., Satheesh, P.R., Annamalainathan, K. and James Jacob

Rubber Research Institute of India, Rubber Board, Kottayam 686 009, Kerala, India

ABSTRACT

The global demand for natural rubber is on rise, continuously. In order to meet this increasing demand, it is necessary to extend the cultivation of *Hevea brasiliensis* to non-traditional areas which are experiencing extreme climatic conditions like drought. Drought, one among the abiotic stresses is a major cause of crop loss world wide. Hence, it is crucial to find out drought tolerant *Hevea* clones which can cope up with the drought stressed environmental conditions. In the present study an attempt was made to out understand the molecular basis of drought tolerance in selected *Hevea* clones. For this, quantitative expression analysis of ten genes was carried out in clones *viz.*, RRII 105 (susceptible) and RRIM 600, RRII 208 and RRII 430 (tolerant) using quantitative PCR (qPCR). Of these genes, peroxidase was found over expressed many fold in tolerant clones compared to the susceptible one. LEA 5 gene also showed a similar trend but with a lesser extent than peroxidase. The physiological role of these genes and their relationship with drought tolerance in *Hevea* clones are discussed in this paper.

Keywords: Drought, Gene expression, *Hevea brasiliensis*, qPCR

INTRODUCTION

Hevea brasiliensis, which is the commercial source of natural rubber (NR), is widely cultivated in Southeast Asian countries like Malaysia, Thailand, Indonesia, India, China, Sri Lanka, Vietnam, *etc.* A warm, humid, equable climate (21-35 °C) and a well distributed annual rainfall of not less than 200 cm is necessary for the optimum growth and productivity. In India, the traditional rubber growing region includes Kerala State and Kanniyakumari District of Tamil Nadu where the climatic conditions are more favourable for NR cultivation. The non-traditional region include drought prone regions like North Konkan, parts of Karnataka, Orissa, Madhya Pradesh and low temperature prevailing regions of North- Eastern States. To cope with the increasing global demand for NR, the area under traditional region is insufficient and hence the cultivation has to be extended to non-traditional regions which warrant identification of suitable clones that can perform better under such agroclimatic conditions.

The drought prone non-traditional regions during summer season, experiences soil and atmospheric drought, higher atmospheric temperatures combined with high light and low relative humidity severely affecting the performance of the crop (Chandrasekhar *et al.*, 1990; Jacob *et al.*, 1999; Devakumar *et al.*, 1998). Drought stress in concomitant with the high sunlight aggravates the damage to the green leaves resulting in severe inhibition of photosynthesis (Sathik *et al.*, 1998; Devakumar *et al.*, 2002). Drought tolerance capacity varies with different clones (Chandrasekhar, 1997; Alam *et al.*, 2006) and among the various clones evaluated in the field conditions, the clone RRIM 600 was found to have better growth and yield compared to other clones (Dey *et al.*, 1998). To identify or evolve a better tolerating clone, it is essential to understand the molecular mechanisms involved in drought tolerance in *Hevea*.

Plants respond to drought conditions by alterations in gene expression. With the introduction of new generation techniques such as DNA microarray technology and quantitative PCR, it is easy to identify and quantify genes if the database is available on the species specific mRNA population or the expressed sequence tags (ESTs). Many studies in this line indicate the expression of several hundreds of stress induced genes which have been identified as candidate genes for drought tolerance. Such identified genes are further utilized in engineering for drought tolerance in crops of huge economical importance (Chaves and Oliveira, 2004; Umezawa *et al.*, 2006). Large scale studies using microarray technologies revealed the regulation of several genes with diverse functions during various stresses (Shinozaki *et al.*, 2003; Bartels and Sunkar, 2005). Most of their gene products may function in stress response and tolerance at the cellular level. For *e.g.* gene expression of the hydrophobic late-embryogenesis-abundant (LEA) proteins are found to be rapidly induced under drought conditions both at transcriptional and protein levels. LEA proteins are generally associated with drought tolerance in stabilizing the proteins and preventing the protein aggregation (Battaglia *et al.*, 2008; Bhatnagar-Mathur *et al.*, 2008). Recent investigations revealed the involvement of several transcription factors (TFs) falling und families such as AP2/ERF, bZIP, NAC, MYB, MYC, zinc-finger and WRKY (Bartels a... Sunkar, 2005) apart from the above mentioned signalling factors.

Despite the availability of thousands of stress associated ESTs of *Hevea*, quantitative gene expression analysis of these genes is only recently being attempted for the identification of candidate genes/factors that are contributing to drought tolerance. With the advent of qPCR technique, it is easier to quantify each gene and establish their relevance under the given stress situations. In this study, attempts were made to identify the drought responsive transcripts by quantitative gene expression analysis with an objective to identify candidate genes/factors associated with drought tolerance. About ten transcripts identified as stress responsive (based on their function) were selected and their expression during drought stress in four *Hevea* clones with varying levels of stress tolerance was studied by qPCR. The most promising stress responsive genes were identified based on the quantitative expression analysis and the possibility of utilizing them in crop improvement is discussed in this paper.

METERIALS AND METHODS

Plant material and stress induction

Two year old field planted *Hevea* plants belonging to drought susceptible group (RRII 105) and drought tolerant group (RRIM 600, RRII 208 and RRII 430) grown in Regional Research Station, Dapchari, Maharashtra, India were selected for the present study. The control plants were irrigated on every third day, while the drought imposed plants were given life saving irrigation once in 15 days. Gas exchange parameters (A, g_s and transpiration rate) were measured using a portable photosynthesis system (Li-6400, Li-COR, USA) on intact, fully mature top leaves. The photosynthesis measurements were made at a constant CO_2 concentration of 380 µmol/mol using a CO_2 injector (Li-6400-01, Li-COR, USA) between 8.30 am and 11.00 am IST. Light intensity was 500 µmol/m^2/s obtained from the LED sources attached with a leaf chamber fluorometer (LCF-6400-40, Li-COR, USA). After confirming the impact of stress by gas exchange parameters, leaf samples were collected and frozen in liquid nitrogen before transporting in dry ice to RRII.

mRNA isolation and cDNA preparation

Leaf samples were ground in liquid nitrogen into fine powder. mRNA was isolated using magnetic beads (Dyna beads, Invitrogen, USA). The RNA quantity and quality were determined spectrophotometrically (Nanodrop, USA) and by agarose gel electrophoresis. cDNA was synthesized by using Superscript III reverse transcriptase (Invitrogen).

Quantitative real time PCR

A set of ten genes were selected for the quantitative expression study. Suitable primers were designed for these genes using Primer Express (Applied Biosystems, USA) as given in Table 2. Quantitative gene expression analysis was eventually carried out by using Applied Biosystems 7500 Real Time PCR System. RQ-PCR was performed in a 20 µl reaction mixture containing 1 µl from 1/20 dilution of first-strand cDNA reaction, 6.5 nM of each primer and 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, USA). PCR cycling was performed by incubating the mix at 95° C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for a minute. This was followed by a melt curve analysis (95 °C for 15 seconds, 60 °C for one minute, 95 °C for 30 seconds and 60 °C for 15 seconds). 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, Efficiency = $10^{(-1/\text{slope})} - 1$. Primers with slope values between -3.2 and -3.5 only were employed for these reactions. GAPDH was used as endogenous control for qPCR analysis. The Relative Quantification (RQ) values were analyzed using the software of Applied Biosystems (SDS 7500, v. 2.0.3).

Statistical analyses

For each treatment, three biological replications were included in the qPCR analysis. Statistical analysis was performed with the relative quantification data using ANOVA. The ratio with a P value ≤ 0.05 was adopted as significant for down or up-regulation.

RESULTS AND DISCUSSION

All the three tolerant clones (RRIM 600, RRII 208 and RRII 430) maintained significantly higher rate of photosynthesis than RRII 105 under water stress condition. Clone RRII 430 showed only a slight decline in photosynthetic rate under drought compared to its irrigated control (Fig. 1). Clones RRIM 600 and RRII 208 recorded nearly 30 per cent reduction in photosynthetic rate under drought condition while the susceptible clone RRII 105 showed more than 40 per cent reduction. Stomatal conductance was the lowest in clone RRIM 600 under drought while clone RRII 430 had the highest value (Fig. 2). Reduction in stomatal conductance (in percentage) was less in RRII 105 followed by RRII 430, RRII 208 and RRIM 600. Transpiration rate increased under drought in RRII 430 and RRII 105 (Fig. 3). There was no much change in the transpiration rate of the clone RRII 208 while it declined in clone RRIM 600. Instantaneous and intrinsic water use efficiency (WUE) analysis (Table 1) indicated clone RRIM 600 as most efficient, followed by RRII 430 and RRII 208 while RRII 105 was the least efficient.

The first effect of water stress will be on stomatal conductance where the stomatal closure occurs and as a consequence of limitation to CO_2 diffusion, assimilation also decreases substantially (Lawlor and Tezara, 2009). Drought induced reduction of photosynthetic rate in field grown *Hevea* plants was earlier reported by Devakumar *et al.* (2002) and Alam *et al.* (2006). The instantaneous and intrinsic water use efficiency values shows RRIM 600 as the most efficient among the clones tested, in terms of water use under drought conditions. The high water use efficiency of this clone at low g_s seems to reflect an ability to maintain a certain photosynthetic capacity under severe drought conditions. The relatively better performance exhibited by clone RRII 430 under drought in terms of carbon assimilation may be because of keeping their stomata open for a longer period compared to other clones. The studies made by Sangsing *et al.* (2004) also showed similar results. This stomatal behaviour is also supported by higher transpiration rates observed in this clone under drought condition. Other studies also indicate the role of high light intensity and warmer temperature on the overall performance of the clone in drought prone areas (Nair *et al.*, 1998

and Jacob *et al.*, 1999) in terms of declining gas exchange parameters rather than the shortage of water during the peak summer. The recent studies indicated that RRII 430 is on par with RRIM 600 displaying better photosynthetic rate and effective quantum yield compared to other clones under drought condition (Annamalainathan *et al.*, 2010).

The quantitative gene expression analysis made for ten genes (Table 2) revealed the existence of different trends of gene regulation under drought conditions in *Hevea* (Table 3 and Fig. 4). Among all the genes studied, expression of peroxidase gene was higher in droughted plants of clones RRIM 600 (95.74 fold) and RRII 430 (26.65 fold) and to a lesser extent RRII 208 (12.84 fold) though not significant when compared to irrigated plants of RRII 105 (susceptible). The control plants of RRIM 600 too had significantly higher levels of peroxidase. This shows the inherent capacity of RRIM 600 in combating the stress effect. Expression of LEA 5 protein in droughted plants of clones RRIM 600 (5.0 fold), RRII 208 (3.2 fold) and RRII 430 (2.97) was also significantly higher when compared to irrigated plants of RRII 105. The control plants of RRII 208 also displayed significantly higher levels of LEA 5 protein (as peroxidase in control plants of RRIM 600). Expression of glutathione peroxidase (GPX) was interesting that it was significantly higher in both control and treated plants of all tolerant clones studied when compared to irrigated plants of RRII 105. AT binding cassette protein (ABCT) was also up-regulated significantly in control and treated plants of RRIM 600 and RRII 208 and droughted plants of RRII 430 when compared to control plants of RRII 105. Expression of HbHP20 was also found significantly up-regulated in all the stress exposed tolerant clones and in the control plants of RRII 208. In the case of HbHP22, it was significantly up-regulated (at 0.05 %) only in droughted plants of RRIM 600 and in both droughted and control plants of RRII 208.

Tf MBF got up-regulated significantly in both control and drought exposed plants of RRIM 600 and in droughted plants of clone RRII 430 while it was not significant in droughted plants of RRII 208 though there was up-regulation. There are other genes which got up-regulated only in one or two clones and not in all stress tolerant clones. For *e.g.* WRKY tf got significantly up-regulated only in the drought exposed plants of clone RRII 430. The transcript of HbHP33 got significantly up-regulated only in drought treated plants of RRII 208. CRT/DRE bf is the only one transcript that displayed significant level of up-regulation in droughted plants of susceptible clone RRII 105. Tf MBF1 is a transcriptional co-activator that mediates transcriptional activation by bridging a sequence-specific activator and TATA-box–binding protein (TBP). In *Arabidopsis*, MBF1c has been reported to produced more in response to pathogen infection, salinity, drought, heat, hydrogen peroxide and plant hormones such as abscisic acid or salicylic acid (Rizhsky *et al.*, 2004). Tf MBF1 expression had also been shown to enhance the tolerance to heat and osmotic stress by partially activating, or perturbing the ethylene-response signal transduction pathway in transgenic plants (Suzuki *et al.*, 2005).

The higher levels of peroxidase found in droughted plants of RRIM 600 indicate its relevance in stress amelioration through detoxification of reactive oxygen species (ROS) which are produced in large quantity when plants are subjected to drought stress combined with higher temperature and sun light conditions. The reactive oxygen species which can disrupt normal metabolism through oxidative damage of lipids, proteins and nucleic acids in the absence of any protective scavenging mechanism. The up-regulation of peroxidase found under drought conditions in this study indicates the existence of oxidative stress in all the clones studied. The comparatively much higher levels of peroxidase in clone RRIM 600 and RRII 430 indicate the existence of better ROS scavenging system which could possibly be imparting better tolerance during the adverse field conditions experienced during severe summer.

Similarly, the higher levels of GPX in both the control and the droughted plants of all the three tolerant clones indicate that GPX is directly involved in drought tolerance. GPX

catalyzes the glutathione-dependent reduction of hydrogen peroxide and diverse alkylhydroperoxides to water or the corresponding alcohol. Glutathione has been shown to play antioxidant roles in cell compartments other than chloroplasts such as mitochondria, cytosol, peroxisomes (Noctor *et al.*, 2002) and in nuclei. Glutathione is the substrate of GPX reactions and the presence of higher levels of GPX in all the tolerant clones indicate its relevance in ROS scavenging during drought stress. Surprisingly GPX was found significantly higher in control plants of tolerant clones compared to the control plants of RRII 105. In a similar study in wheat by Loggini *et al.*, (1999), found that the quantity of GPX in tolerant verities was not much altered when droughted and the inherent levels of GPX were sufficient enough to impart stress resistance.

Another protein identified as contributing for the drought tolerance from this study is the late embryogenesis abundant (LEA) proteins which accumulate in higher levels during the last stage of seed maturation and during water deficit in vegetative organs (Battaglia *et al.*, 2008). LEA 5 proteins are generally hydrophobic proteins that contain a significantly higher proportion of hydrophobic residues and are assumed to be sequestering ions during water loss (Bhatnagar-Mathur *et al.*, 2008). Although little is known about this set of proteins, it is understood that these proteins accumulate during the late stage of seed development and also as a response to stress conditions, such as drought, UV light, salinity, cold and wounding. Higher levels of LEA 5 protein found in the droughted plants of all the three drought tolerant clones studied indicate that this might be associated with the drought tolerant nature of these clones.

Table 1. Instantaneous and intrinsic water use efficiency under irrigated and drought conditions in different clones

Clone	Instantaneous WUE (µmol/mmol)		Intrinsic WUE (µmol/mol)	
	Control	Drought	Control	Drought
RRIM 600	3.0	3.5	39.0	86.4
RRII 430	3.1	2.1	39.1	50.4
RRII 208	3.1	2.1	35.1	42.1
RRII 105	2.7	1.4	46.3	35.8

Table 2. List of genes and the corresponding primers used for qPCR analysis

Sl. No.	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
1	LEA5	CTCGCTTTCCCTCCAATG	TTCCTCACCATACCACTCC
2	Peroxidase	AATTGGCACGAATTTCCC	CACGGATAAGAGAACAAGG
3	HbHP 33	GGCCGTGCAATACGTGAGA	GCCATTTTCTTCGCGTAAGG
4	ABCT	ACCGGCGTCCTATGTTCTCA	TTTAATCCCAGCAATCCATATCG
5	WRKY tf	AGGGAATGGAGATGAGGGAAA	GGGACATAACCAGGTGGCTAGA
6	TF MBF1	GTTGGTAGGCACTCTCACTTGAC	AGAAGCGTGGACATAAGAAGAAGG
7	GPX	GCCTGCGTTGTGTTCTTTGA	TCAACATCGTTTCCCCTAGCA
8	CRT/DRE BF	CCATAGTGACACATCAGCTGCAT	ATATGGACGAAGAGGCGGTTT
9	HbHP 20	CTCGACATCCCTTCGTTCCA	TTGGTGGCCTTGTAGGTGTTC
10	HbHP 22	CACCCCAACGAGTGACAACA	TGCTCAGAAGGTGGACTTTGC

Table 3. Relative quantification of ten genes under drought conditions with reference to irrigated plants of RRII 105 (RQ va

Genes	RRII 105 C	RRII 105 D	RRIM 600 C	RRIM 600 D	RR11 208 C	RRII 208
Peroxidase	1	2.195	42.31**	95.74**	1.441	12.84
LEA 5	1	0.856	1.150	5.001**	2.583**	3.219**
CRT/DRE bf	1	1.922**	0.827	0.845	0.639	1.404
WRKY tf	1	0.897	2.063	3.778	4.524	3.729
Tf MBF	1	1.075	2.448**	1.582**	0.957	1.435
GPX	1	1.204	2.237**	2.756**	3.198**	4.418**
ABCT	1	1.672	2.813**	4.865**	3.034**	3.175**
Hb 33 HP	1	0.631	0.835	1.149	0.694	1.897**
Hb 22HP	1	1.54	2.28	4.54*	3.39*	3.16*
Hb 20 HP	1	1.27	1.65	3.86**	3.34**	3.060**

Figure 1. CO_2 assimilation rates (A) in different *Hevea* clones under irrigated and drought conditions in Dapchari

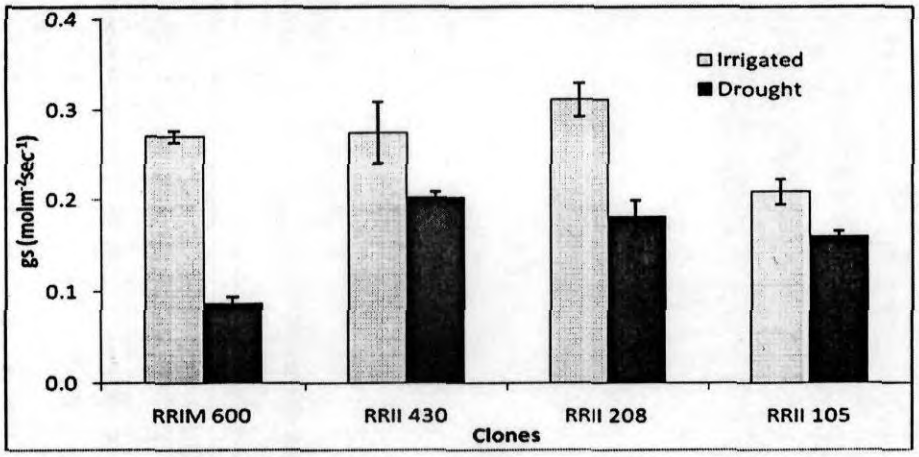


Figure 2. Stomatal conductance (gs) in different *Hevea* clones under irrigated and drought conditions in Dapchari

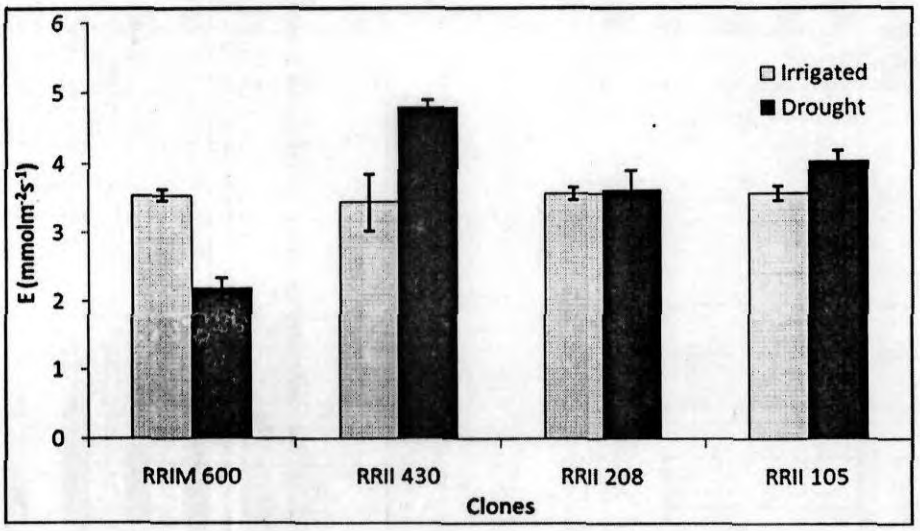
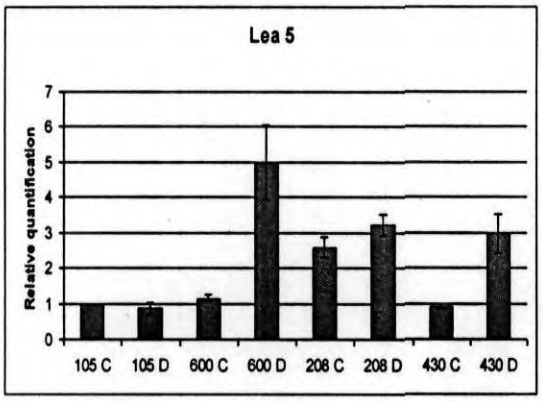
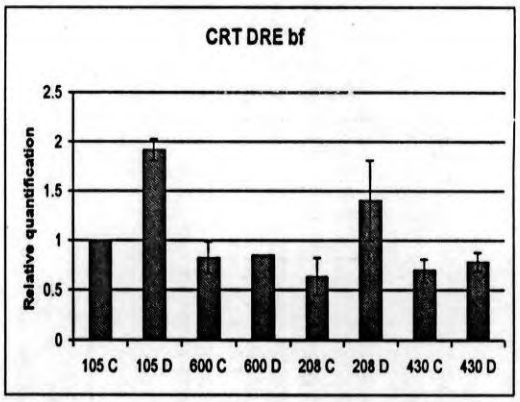
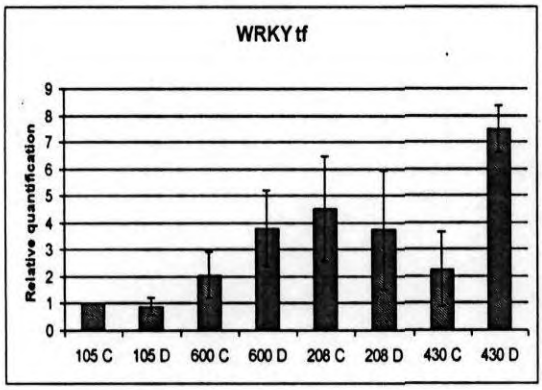
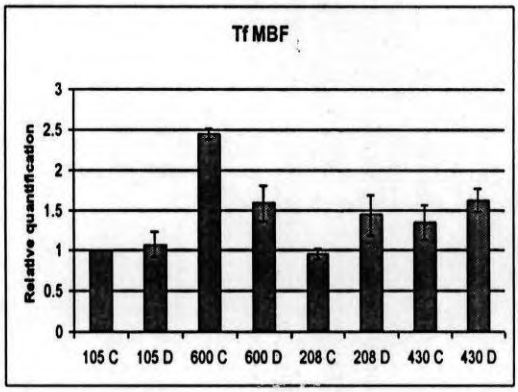


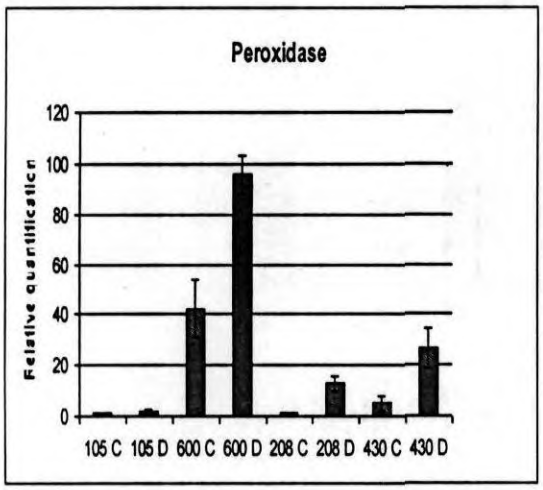
Figure 3. Transpiration rate (E) in different *Hevea* clones under irrigated and drought conditions in Dapchari


Lea 5
Relative quantification
0
1
2
3
4
5
6
7
105 C
105 D
600 C
600 D
208 C
208 D
430 C
430 D


CRT DRE bf
Relative quantification
0
0.5
1
1.5
2
2.5
105 C
105 D
600 C
600 D
208 C
208 D
430 C
430 D


WRKY tf
Relative quantification
0
1
2
3
4
5
6
7
8
9
105 C
105 D
600 C
600 D
208 C
208 D
430 C
430 D


Tf MBF
Relative quantification
0
0.5
1
1.5
2
2.5
3
105 C
105 D
600 C
600 D
208 C
208 D
430 C
430 D


Peroxidase
Relative quantification
0
20
40
60
80
100
120
105 C
105 D
600 C
600 D
208 C
208 D
430 C
430 D

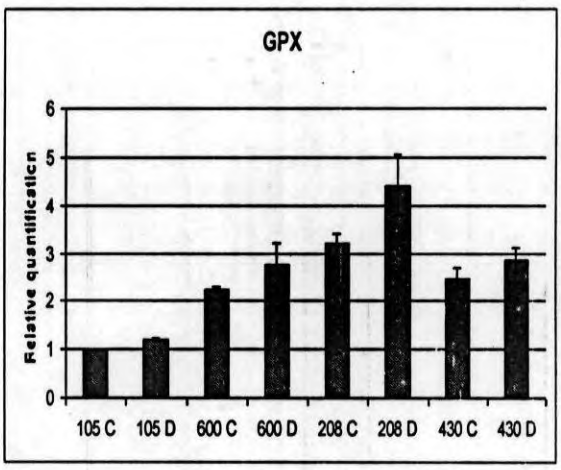

GPX
Relative quantification
0
1
2
3
4
5
6
105 C
105 D
600 C
600 D
208 C
208 D
430 C
430 D

Figure 4A. Graphical representation of quantitative expression analysis of six genes (LEA5, CRTDRE bf, WRKY tf, Tf MBF, Peroxidase, GPX). The values of relative fold change are expressed in log 10 values. Treatments are given in X axis and the log 10 value of fold change is given in the Y axis.
105C – RRII 105 Control; 105D – RRII 105 drought stressed; 600C – RRIM 600 Control; 600D- RRIM 600 drought stressed; 208C– RRII 208Control; 208 D – RRII 208 Drought stressed; 430 C – RRII 430 control; 430D– RRII 430 Drought stressed.

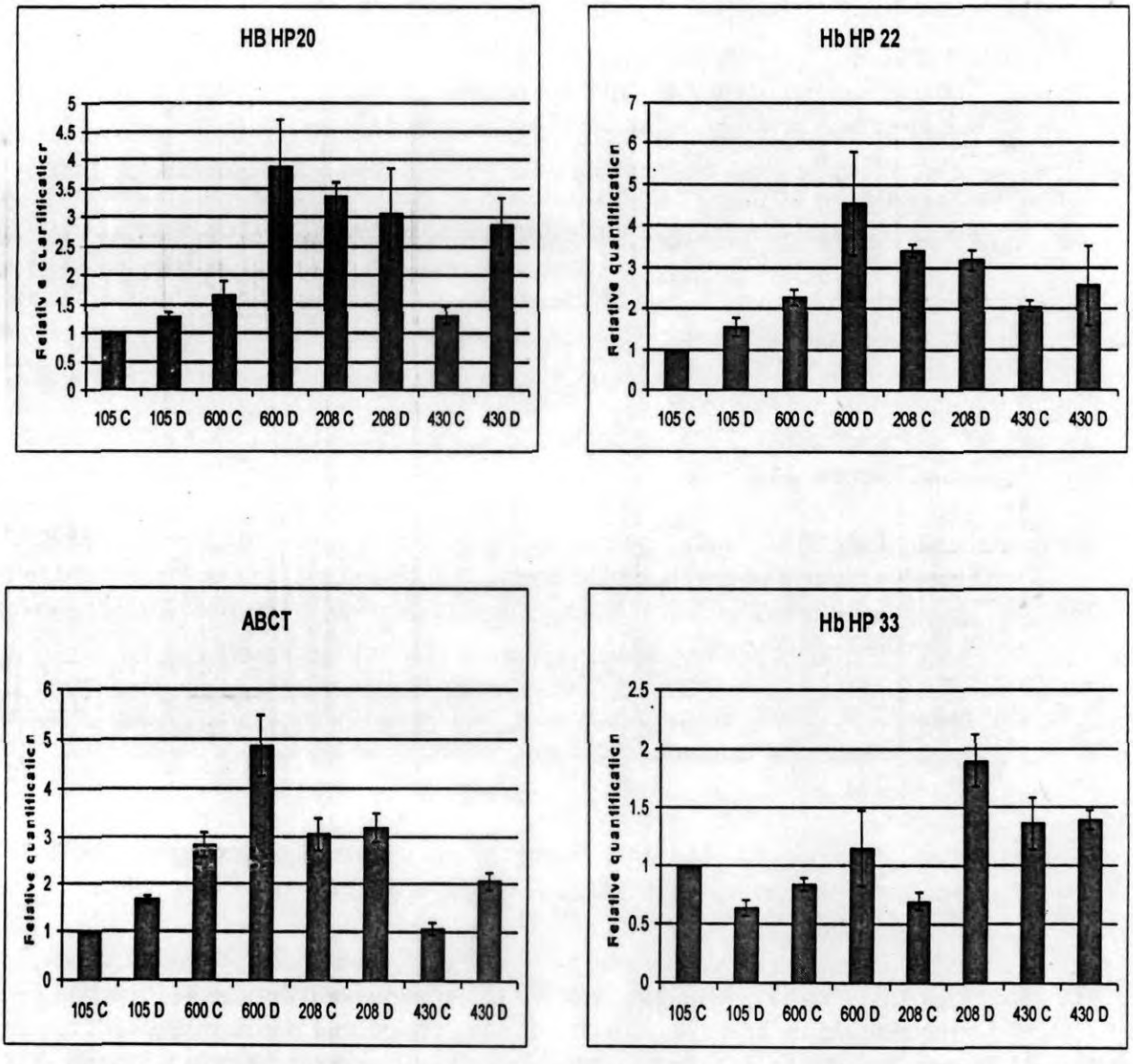


Figure 4B. Graphical representation of quantitative expression analysis of four genes (HbHP20, HbHP22, ABCT protein and HbHP33). The values of relative fold change are expressed in log 10 values. Treatments are given in X axis and the log 10 value of fold change is given in the Y axis.

CONCLUSIONS

The results indicate that among the genes studied, three genes such as peroxidase, LEA 5 and glutathione peroxidase are associated with drought tolerance in *Hevea brasiliensis.*

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