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QUANTITATIVE EXPRESSION ANALYSIS OF STRESS RESPONSIVE GENES UNDER COLD STRESS IN *HEVEA BRASILIENSIS*

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Tolerance to extreme climatic conditions being experienced in non-traditional rubber growing regions in India involves genes/regulatory factors associated with this trait in *Hevea brasiliensis*. Identification of such genes/factors is necessary for evolving suitable clones for such regions. For this purpose, gene expression analysis was carried out on leaf samples of *Hevea* clones with varying tolerance levels *viz*. RRII 105 and RRIM 600 (relatively cold susceptible and tolerant respectively) exposed to low temperature stress. Among the 21 *Hevea* specific genes analyzed, expression of LEA 5 protein, ETR1, NAC transcription factor, annexin, ABC transporter protein, WRKY transcription factor, GPX, ACO2, HbDRT50 and peroxidase responded to low temperature stress. Among these genes, LEA 5 protein, peroxidase, ETR1, ETR2 and NAC transcription factor were found associated with tolerance. Genes such as NAC transcription factor, LEA 5 protein and peroxidase showed high magnitude level of expression under cold conditions indicating its stronger association with cold tolerance. This need to be further validated with more such tolerant clones to identify the best candidate genes/regulatory factors associated with cold tolerance.

Keywords: Cold stress, Gene expression, *Hevea brasiliensis*, qPCR analysis

INTRODUCTION

To meet the ever increasing demand for natural rubber (NR) both domestically and globally, cultivation of *Hevea brasiliensis*, in India is being extended to marginal areas like north-eastern regions where the temperature during winter is too low for its survival and optimum productivity. As *H. brasiliensis* is originated from the tropical regions of Amazonian forest where the mean temperature ranges between 25 and 28 °C (Strahler, 1969), it is very sensitive to low temperature conditions. While the required optimal growth temperature for successful cultivation of NR lies between 20 and 34 °C (Webster and Baulkwill, 1989), the mean temperature in North East India is less than 20 °C for five months with minimum temperature going as low as 2 °C occasionally in winter nights (Meenattoor *et al*., 1989). In

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general, growth retardation has been observed in North East India, China and other sub-tropical rubber growing regions during winter. Clonal variation in cold tolerance has also been reported (Polhamus, 1962).

Cold damage to rubber trees is a complex phenomenon as it involves the differential response of clones, age and vigour status of the plant. In general, cold injury is seen by the end of the cold season (late February) during which the plants display symptoms like wilting of leaves followed by withering without abscission, occasional inter-venal chlorosis, black discolouration of green bark and its drying off extending downward, occasional oozing of latex from green bark and dieback of shoots (Meti *et al*., 2003). Clones such as RRII 118, RRII 300 and RRIM 600 were reported to be performing well in Tripura conditions (Sethuraj *et al*., 1989). In a study by Meti *et al*. (2003), clones such as RRIM 600 and RRII 429 displayed no cold injury while clones RRII 422 and RRIC 100 displayed severe cold injury after the winter in Nagrakata, the cold prone region of North East India. In another study, Reju *et al*. (2003) reported that clones such as RRIM 600, RRII 118, PB 235 and RRII 203 had better growth during immature phase in Meghalaya which is again a cold prone and high elevated region. Clonal difference is low temperature tolerance has been reported in *Hevea* based on physiological trait like loss of membrane stability (Sathik *et al*., 1998a). When leaf discs from 13 clones were studied for its membrane permeability after imposing cold stress, the clone RRII 105 showed higher rate of membrane permeability under cold stress conditions (Sathik *et al*., 1998b). In another study by Ray *et al*. (2004), while RRII 105 showed early wintering with high membrane damage, chloroplyll degradation and low Late Embryogenesis Abundant (LEA) protein levels, clone RRIM 600, a late wintering clone displayed minimum chlorophyll degradation and higher levels of LEA protein under cold stress conditions. High light during day time combined with cold stress in the previous nights during winter seasons leads to severe inhibition of photosynthesis and chloroplyll bleaching (Powles, 1984; Jacob *et al*., 1999; Devakumar *et al*., 2002 and Ray *et al*., 2004) in cold susceptible clones like RRII 105 whereas the cold tolerant clones like RRIM 600 showed better photosynthesis and lesser membrane permeability. Concomitant occurrence of high light and low temperature inhibited PSII activity in cold stressed leaves of *H. brasiliensis* as evidenced by the reductions in the maximum and the effective quantum yield of PSII (Sathik *et al*., 1998b; Jacob *et al*., 1999; Devakumar *et al*., 2002). Cold induced symptoms such as reduction in photosynthesis and quantum yield and increased chlorophyll bleaching and membrane permeability are directly associated with reduction in growth and yield (Shuogang and Yagang, 1990).

Alam *et al*. (2003) reported that the percentage reduction in yield due to winter was lesser (8%) in clone RRIM 600 than PB 235 (40%). Thiol content in RRIM 600 was at the maximum during winter season which is evident from its increased free radical scavenging capacity during this season. Higher levels of antioxidant enzymes such as superoxide dismutase, peroxidase, ascorbate peroxidase, catalase and antioxidants like glutathione found in the cold exposed leaves indicate the occurrence of oxidative stress under such extreme condition (Devakumar *et al*., 2002). The ability of the photosynthetic apparatus to

repair the photoinhibitory damages and the capacity of the antioxidant systems to protect the cells from oxidative stress determine the survival and productivity of *H. brasiliensis* in cold stress prone areas. Though a lot of investigations have been made on physiological and biochemical parameters, no attempt has been made so far to identify the cold responsive gene expression profile which would rather provide information on genes associated with cold stress tolerance in *H. brasiliensis.*

The recent approaches on gene expression studies like microarray analysis has revealed a list of genes that are induced by various abiotic stresses (Kawasaki *et al*., 2001; Seki *et al*., 2001). Such genes apart from protecting the cells by producing important proteins, they also play a role in regulating genes for signal transduction. Two groups of gene products have been reported to be involved in stress response (Fowler and Thomashow, 2002; Seki *et al*., 2002). First group includes proteins such as chaperones, LEA proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. The second group involves proteins mainly involved in regulation of signal transduction and stress responsive gene expression.

Though a lot of information is being generated on cold response in tropical plants and about the genes/regulatory factors associated with triggering of cold response and cold stress amelioration proteins, no molecular level information is available on cold response and cold tolerance in *H. brasiliensis.* As a preliminary study, an attempt was made to identify genes that are associated with low temperature tolerance by estimating the expression levels of selected genes in two *H. brasiliensis* clones with varying levels of cold tolerance. For this purpose, quantitative PCR (qPCR) was performed for 21 selected genes and their association with cold tolerance was evaluated. The results and the findings are discussed in this paper.

MATERIALS AND METHODS

Plant material and stress induction

Six-months-old polybag plants of clone RRII 105 (cold susceptible) and RRIM 600 (cold tolerant) of *H. brasiliensis* were acclimatized in a growth chamber for three days with a minimum temperature of 15 °C during night (for 3 h) and a gradual rise in maximum temperature up to 25 °C in the day time. Fourth day onwards, cold treatment was imposed by reducing the temperature to 8 °C for 3 h between 2 and 5 am followed by a gradual increase in maximum temperature up to 16 °C in the day time for five consecutive days mimicking ambient conditions of winter season in north eastern region of India. Light intensity regime was ranging between a minimum of 400 to a maximum of 800 μ mol/m^2/s. Control plants were allowed to grow at stress free and ambient weather conditions of RRII during August 2010.

Gas exchange measurements

Leaf samples were harvested at about 10 h after assessing the stress response of the plants by measuring the net CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI-6400),

LI-COR, U.S.A. Gas exchange measurements were made at a leaf temperature of 30 ± 1 °C and leaf-air VPD of 1.5 – 2 k Pa. All the gas exchange parameters were measured at a constant CO_2 concentration of 360 ppm using a CO_2 injector (LI-6400-01, LI-COR, U.S.A) and at 400 µmol/m^2/s of light intensity using red LED source (with 10% of blue light) attached with the leaf chamber of LI-6400.

mRNA isolation and cDNA synthesis

The 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 by magnetically capturing them using dynabeads (Invitrogen, USA) and its quality and quantity was confirmed on agarose gel as well as by absorption at 260 and 280 nm (Nanodrop, USA). cDNA was prepared from 250 ng mRNA in a 20 µL reaction mixture using Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to the manufacturer's recommendations.

Quantitative PCR (qPCR) analysis

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 synthesized by Ocimum Biosolutions, Hyderabad. Glutaraldehyde 3-phospate dehydrogenase (GAPDH) gene was selected as internal control for normalization from a set of four genes (cyclophilin, GAPDH, ADP ribosylation factor and polyubiquitin) after performing qPCR and calculating their gene stability (*M* value) using GeNorm software (Vandesompele *et al*., 2002). qPCR was performed in a 20 µL reaction mixture containing 1 µL of 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) on a Real Time PCR System (Applied Biosystems 7500). PCR cycling was performed by incubating at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for one minute. This was followed by a melt curve analysis (95 °C for 15 seconds, 60 °C for one minute, 95 °C for 15 seconds and 60 °C for one minute). Each experiment was repeated twice 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 these reactions. The relative quantification (RQ) values were analyzed using the software (Applied Biosystems, SDS 7500, v. 2.0.3). One set of quantitative expression analysis was performed with control plants of RRII 105 as calibrator and the other set with respective control plants of each clone as calibrator. The list of genes analyzed and their primer sequences are given in Table 1.

RESULTS AND DISCUSSION

Plants of both the cold susceptible (RRII 105) and tolerant (RRIM 600) clones when exposed to low temperature with light levels ranging between 400 and 800 µ mol m^2/s in the day time for five consecutive days showed photobleaching in the leaves. Among the control plants, RRIM 600 exhibited (Fig.1) slightly better (12.1 µ mol/

m^2/s) photosynthetic rate (A) than RRII 105 (10.7 µmol/m^2/s). However, stomatal conductance (g_s) was almost same in both the clones (Fig. 2). After the imposition of cold stress for five days, 'A' was drastically inhibited (90-98 %) in both the clones. At the same time 'g_s' was completely inhibited in both the clones.

There are many reports to prove that repeated and prolonged exposure to abiotic stresses may make the repair mechanisms inadequate resulting in irreversible damage to the photosynthetic machinery leading to photobleaching and senescence of leaves affecting the dry matter production (Long *et al*., 1994). The growth rate of *H. brasiliensis* plant is lesser during winter than the stress-free post-monsoon season in Agartala. It is known that the inadequacy of biochemical mechanisms to scavenge the reactive oxygen species such as superoxide, hydroxyl radical *etc*. which are produced during photochemical reactions in the thylakoid membranes causes serious damage to plants experiencing

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

Sl. No.	Gene	Forward primer 5′-3′	Reverse primer 5′-3′
1	WRKY tf	AGGGAATGGAGATGAGGGAAA	GGGACATAACCAGGTGGCTAGA
2	ABCT	ACCGGCGTCCTATGTTCTCA	TTTAATCCCAGCAATCCATATCG
3	Tf MBF	GTTGGTAGGCACTCTCACTTGAC	AGAAGCGTGGACATA AGAAGAAGG
4	LEA 5	CTCGCTTTCCCTCCAATG	TTCCTCACCATACCACTCC
5	CRT/DRE bf	CCATAGTGACACATCAGCTGCAT	ATATGGACGAAGAGGCGGTTT
6	GPX	GCCTGCGTTGTGTTCTTTGA	TCAACATCGTTTCCCCTAGCA
7	*HbHP33*	GGCCGTGCAATACGTGAGA	GCCATTTTCTTCGCGTAAGG
8	DnaJ protein	AGCATCCTAAGTTCAAGCG	CCTCTGATACATTGGCCATCC
9	Peroxidase	AATTGGCACGAATTTCCC	CACGGATAAGAGAACAAGG
10	ETR1	GGAAGGACATTGGCATTGGAA	CGAAGAGTGTAGGAAA GTTGAAGC
11	ETR2	AACCTCCCAGAATACAGCGT	GCCTCATGGTTGAGACAATC
12	ACO2	ATGGACACAGTTGAGAGGATGAC	AGGTGGCGGAGGAAGAAGG
13	*HbHP20*	CTCGACATCCCTTCGTTCCA	TTGGTGGCCTTGTAGGTGTTC
14	*HbHP22*	CACCCCAACGAGTGACAACA	TGCTCAGAAGGTGGACTTTGC
15	Cystein protease	ACGCTTAGTCGGATGATG	CGGTTGATACGGAGAAGG
16	Chitinase	AAATCTTCTTCCCGTCCCTTAG	CTTGCCTTGCTTGCTATATGC
17	Annexin	GGCTTACCATGCTCGCTACAA	TCAGCTTCAGATTTGGCCAAT
18	SUMO ap	ACAGAAACCCGAACCATTGG	GATGATCGTGCTGTGACTGAAGA
19	*HbDRT5b*	TCAAACACTGTCATGTCCAAGAAA	GAATCAGGGCAACCTTTTAAACC
20	*HbTPD24*	TCAGGGCAACCTTTTAAACCA	ACCAGGCAAATCATTGAAATTCA
21	*HbDRT50*	TCGGAAGCTTCGACTGTGATG	TACTGCTAGGATTTCGA CCTTAAACA
22	GAPDH	GCCTGTGATAGTCTTCGGTGTTAG	GCAGCCTTATCCTTG TCAGTGAAC

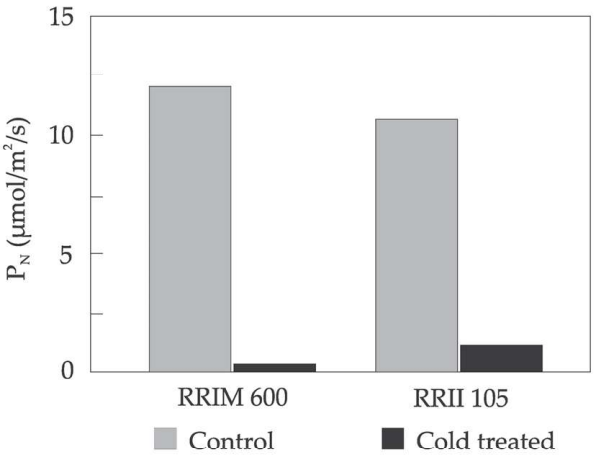

Fig. 1. CO_2 assimilation rate (A) of clone RRIM 600 and RRII 105 under control and low temperature conditions

abiotic stresses. In general, glutathione and phenol concentrations increased and specific activities of scavenging enzymes such as polyphenol oxidase (PPO) and ascorbate peroxidase (ASPX) decreased under drought and cold stresse conditions in *H. brasiliensis* (Devakumar *et al*., 2002). They also found increased activity of scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD) in cold exposed leaves of *H. brasiliensis* thus indicating the occurrence of higher levels of oxidative stress.

In this context, a set of 21 stress related genes identified from *H. brasiliensis* were analyzed for their expression under cold stress conditions. The list of genes studied and their possible functions are furnished in Table 2. The results of investigations made to estimate the level of gene expression by relative quantitative expression analysis are furnished in Fig.3. A few genes *viz*. NAC transcription factors [(NAC tf), (*HbDRT5b* and *HbTPD24*)], annexin protein, peroxidase and ABCT protein showed remarkable fold change (over control) under cold conditions in both the clones. Four genes such as LEA 5 protein, peroxidase, NAC tf and ethylene receptor protein (ETR 1) were found up-regulated in the tolerant clone (RRIM 600). Expression of LEA5 protein was about 10 fold higher in RRIM 600 under cold conditions when compared to its own control and about 59 times higher when compared to RRII 105 control plants (Table 3). Expression of peroxidase was >150 fold higher in low temperature treated plants of RRIM 600 when compared to control plants of RRII 105 though it was up-regulated by more than 8 fold in low temperature stressed plants of both the clones (RRII 105 and RRIM 600) when each of its control plants were taken as calibrator (Table 3). Similarly, two transcripts representing NAC transcription factor (*HbTPD 24* and *HbDRT 5b*) were also found up-regulated to an extent of about five and four fold in RRII 105 and about 19 and 26 fold in RRIM 600 plants when their respective control values were taken as calibrator and about 10 to 15 fold in RRIM 600 when RRII 105 control was taken as calibrator (Table 2). ETR1 was

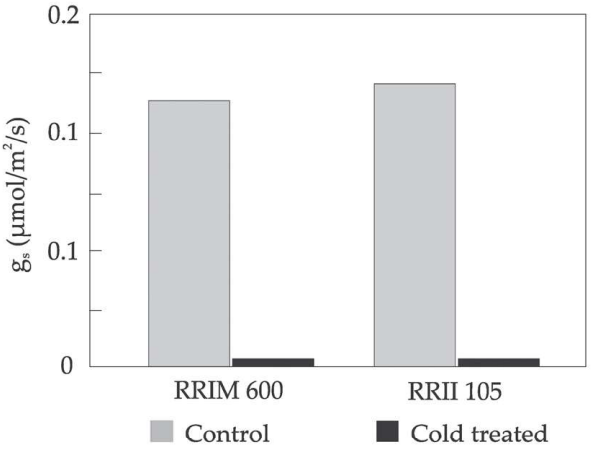

Fig. 2. Stomatal conductance (g_s) of clone RRIM 600 and RRII 105 under control and low temperature conditions

Table 2. **List of genes used and their possible function**

Sl. No.	Gene	Function
1	WRKY transcription factor (WRKY tf)	Involved in cold adaptation and cold tolerance, highly up-regulated during drought, senescence and wounding stress situations.
2	ATP Binding Cassette protein (ABCT)	Involved in active transport of substances like alkaloids, aminoacids, heavy metal chelates, inorganic ions, lipids, peptides and sugars.
3	Transcription factor MBF (Tf MBF)	tolerance to various stresses including salt, heat, osmotic, high light and disease conditions.
4	Late Embryogenesis Abundant (LEA5) protein	Protective role during water desiccation stress
5	CRT/DRE binding factor (CRT/DRE bf)	*cis*-acting element involved in ABA-independent cold and salinity responsive gene expression system; enhances tolerance to freeze, salt and drought stresses.
6	Glutathion peroxidase (GPX)	Protection from oxidative damage (antioxidant) and a signaling enzyme.
7	*HbHP33* (hypothetical protein)	Hypothetical protein; function unknown
8	DnaJ protein	molecular co-chaperones of Hsp70, role in protein folding, unfolding, and assembly
9	Peroxidase	Scavenging of free radicals (H_2O_2) and protection against oxidative species (OS).
10	ETR 1 (ethylene receptor protein 1)	Mediates ethylene perception
11	ETR 2 (ethylene receptor protein 2)	Mediates ethylene perception
12	ACC oxidase 2 (ACO2)	Ethylene biosynthesis
13	*HbHP20* (hypothetical protein)	Hypothetical protein; function unknown
14	*HbHP22* (hypothetical protein)	Hypothetical protein; function unknown
15	Cystein protease	Associated with programmed cell death, senescence, wounding cold and drought stresses.
16	Chitinase	Anti-fungal activity, biotic and abiotic stress inducible protein.
17	Annexin	Involved in maintaining membrane structure and transport.
18	SUMO activating protein (SUMO ap)	Small ubiquitin like Modifier protein; involved in protein stability, transcriptional regulation and stress response.
19	*HbDRT5b* (NAC transcription factor)	similar to NAC transcription factor; transcriptional activator, stress inducible.
20	*HbTPD24* (NAC transcription factor)	similar to NAC transcription factor; transcriptional activator, stress inducible.
21	*HbDRT50* (drought responsive transcript)	Drought responsive transcript from *Hevea brasiliensis.*

Table 3. **Relative quantification of 21 genes in low temperature stressed plants (LT) of RRII 105 and RRIM 600.**

Gene	RRII 105 (C)	RRII 105 (LT)	RRIM 600 (C)	RRIM 600 (LT)	RRIM 600 (C)	RRIM 600 (LT)
1	2	3	4	5	6	7
WRKY tf	1	164.3	64.3	5.00	1	0.07
ABCT	1	3.19	3.65	9.77	1	2.67
Tf MBF	1	6.13	0.64	0.89	1	1.37
LEA5	1	1.48	5.98	58.9	1	9.85
CRT/DRE bf	1	0.57	0.44	0.03	1	0.08
GPX	1	4.51	3.16	1.90	1	0.60
HbHP33	1	10.48	2.26	0.97	1	0.42
DnaJ protein	1	1.36	1.53	0.85	1	0.5
Peroxidase	1	9.48	19.87	158.3	1	7.96
ETR 1	1	1.38	4.00	15.97	1	3.98
ETR 2	1	1.18	2.13	5.13	1	2.4
ACO2	1	2.37	17.85	12.86	1	0.72
HbHP20	1	1.88	1.02	1.14	1	1.11
HbHP22	1	2.82	1.58	2.06	1	1.3
Cystein protease	1	0.82	4.46	3.2	1	0.71
Chitinase	1	2.13	0.57	0.72	1	1.26
Annexin	1	3.1	1.31	4.18	1	3.18
SUMO ap	1	2.03	0.84	1.49	1	1.76
HbDRT5b	1	5.2	0.6	15.48	1	25.59
HbTPD24	1	4.34	0.53	9.86	1	18.5
HbDRT50	1	16.03	12.13	9.87	1	0.81

Column 3 to 5 indicates fold change over RRII 105 control (Column 2). Column 7 indicates fold change over RRIM 600 control (column 6).

about four fold higher in cold stressed plants of RRIM 600 against its control and about 16 fold more when compared to RRII 105 control (Table 3).

Out of all the transcripts analyzed, eight transcripts were found up-regulated in RRII 105 plants. Among those up-regulated transcripts, genes such as WRKY transcription factor (WRKY tf), *HbDRT50, HbHP33* (hypothetical protein), transcription factor MBF (tf MBF) and glutathione peroxidase (GPX) were over-expressed 164, 16, 10, 6 and 4.5 fold higher than control, respectively. Transcripts such as *HbHP22* (hypothetical protein), ACO2 and chitinase were up-regulated moderately by 2.8, 2.4 and 2.1 fold, respectively. Few transcripts such as ATP binding cassette (ABCT) protein, annexin and SUMO activating protein were found moderately up-regulated in both the clones (Table 3).

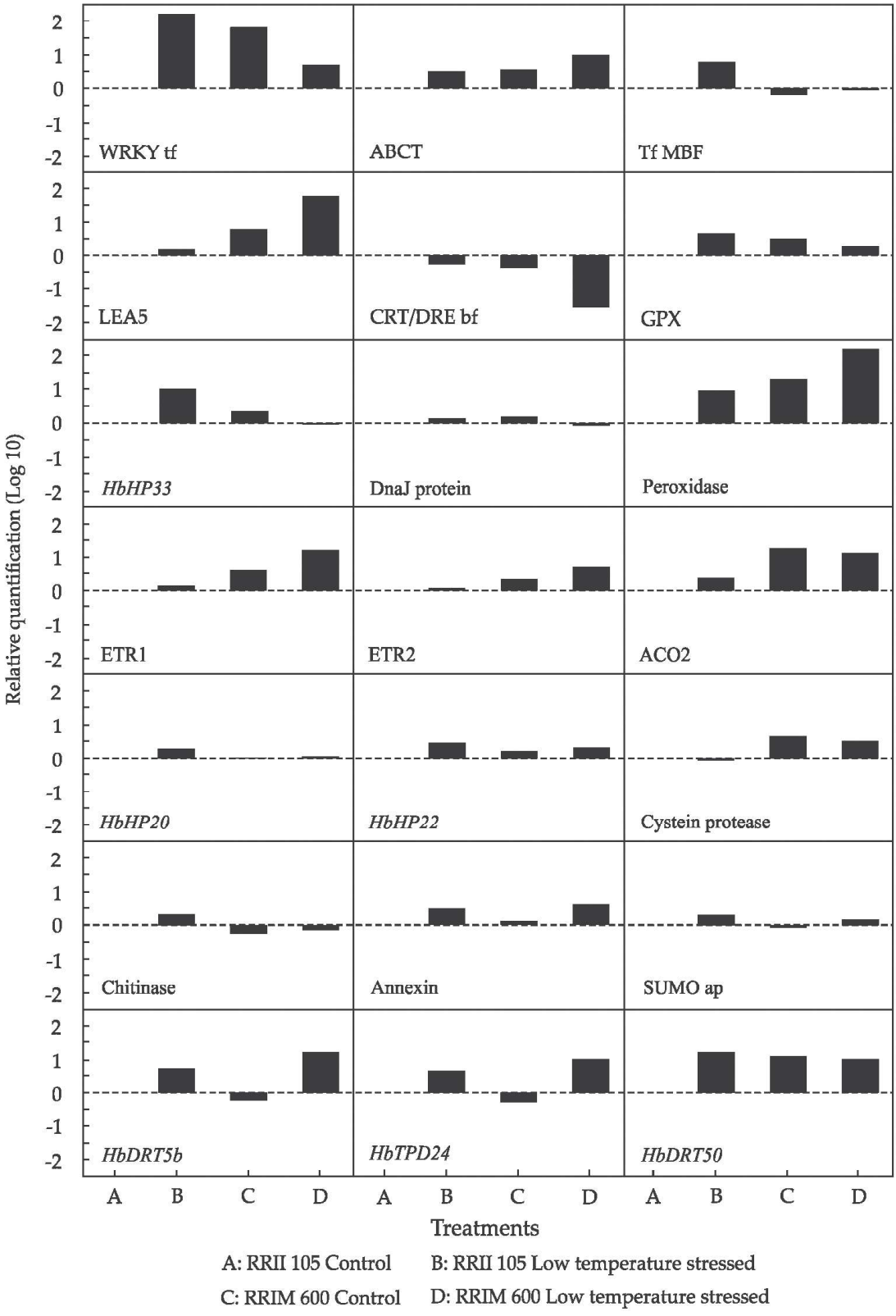

Fig. 3. Quantitative expression analysis of 21 genes in *H. brasiliensis*

Expression of ABCT protein in cold exposed plants of clone RRIM 600 was about 10 fold higher than control plants of clone RRII 105. While expression of both the cystein protease and CRT/DRE binding factor (CRT/DRE bf) transcripts showed a declining trend in the low temperature stressed plants, cystein protease remained higher in RRIM 600 than RRII 105 plants when values of RRII105 control plants were used as calibrator irrespective of the treatment. When expression of *HbHP20* (hypothetical protein) increased in RRII105, it did not vary much under low temperature stress conditions in RRIM600.

When the data was analyzed by keeping RRII 105 control plants as calibrator (Table 3), about 20 and 158 fold increases in peroxidase expression was found in control and cold treated plants of RRIM 600, respectively. This high level of expression in RRIM 600 may be attributed to its inherent cold tolerance behaviour observed in the field conditions at NE regions. Similarly, expression level of LEA5 was also found to be several folds higher in RRIM 600 (about 6 and 59 fold in control and cold treated plants, respectively). Expression of NAC tfs such as HbDRT 5b and HbTPD 24 was about 15 and 10 fold higher in RRIM 600 respectively than RRII 105 control plants. Similarly the expression level of ABCT protein was ten fold higher in RRIM 600 than the RRII 105 control plants. These results indicate the relevance of LEA5 and peroxidase in contributing for the enhanced cold tolerance in RRIM 600 as evidenced from its field performance. Expression of ABCT was found higher only in RRIM 600 while it was repressed in RRII 105. It needs to be studied further in other cold tolerant clones to understand the role of ABCT imparting cold tolerance. Expression of WRKY tf, HbDRT 50, HbHP 33, TfMBF and GPX was higher only in RRII 105 cold treated plants. Though these transcripts were up-regulated in RRII 105 plants to a higher level, their relevance and functional role in a susceptible clone need to be ascertained further.

LEA proteins may play a protective role during water limitation in vegetative organs (Battaglia *et al*., 2008). They are generally hydrophilic, whereas LEA 5 proteins are hydrophobic which contain a significantly higher proportion of hydrophobic residues and are assumed to be sequestering ions during water loss (Bhatnagar-Mathur *et al*., 2008). Higher levels of LEA5 protein found under cold stress conditions especially in clone RRIM 600 indicate that this might be contributing for the cold tolerance trait exhibited by this clone.

Similarly, higher level of peroxidase expressed in RRIM 600 indicate its relevance in the context of stress amelioration through detoxification of reactive oxygen species (ROS) which are likely produced in large scale when plants are subjected to low temperature stress. ROS which are highly reactive can disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids in the absence of any protective scavenging mechanism (Allen, 1995). Enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (PX) are the important scavenging enzymes of ROS. Hence, up-regulation of peroxidase under low temperature conditions in clone RRIM 600 indicate the existence of better ROS scavenging system which could possibly be imparting tolerance. Apart from the above genes, NAC tf (Hb DRT 5b and Hb TPD 24) were up-regulated in low

temperature exposed plants of RRIM 600 clone than RRII 105 plants. This is also interesting to note that the expression levels are more only in cold tolerant clone (RRIM 600). While ETR1 was found up-regulated remarkably about 16 fold, ETR2 was up-regulated only about 5 fold in RRIM 600.

Three genes such as ABCT, annexin 1 and SUMO activating protein were up-regulated under cold conditions in both the *H. brasiliensis* clones. The ATP-binding cassette transporter proteins (ABCT) are known to be involved in membrane transport of wide range of compounds such as glutathione conjugates, lipids, inorganic acids, peptides, secondary metabolites, toxins and drugs (Holland *et al*., 2003) which are active metabolic processes that require energy. Up-regulation of ABCT gene in the cold tolerant clone (RRIM 600) implied that this might be associated with stress tolerance mechanism at membrane level. Up-regulation of annexin protein in both the clones suggests that it might be cold stress responsive in *H. brasiliensis.* They are multi-functional proteins that bind membranes in a calcium-dependent manner. They are produced as a response to various stresses such as drought, cold, ABA, osmotic stress *etc.* (Kovacs *et al*., 1998; Kreps *et al*., 2002). Annexin genes have also been reported to be up-regulated by reactive oxygen species (Apel and Hirt, 2004). Their expression has been reported to be increased under cold temperature conditions in poplar leaves (Renaut *et al*., 2006). Konopka *et al*., (2009) reported that over-expression of *AnnAt1*, an annexin of *Arabidopsis* had a protective effect on plant survival under drought conditions and its lack of expression or under expression resulted in stress sensitivity. SUMO proteins are involved in post-translational modifications and are known to be key regulators of a range of biological functions such as DNA repair, subcellular localization, stress response and chromatin structural maintenance. In particular, SUMO modification of transcription factors is necessary for various physiological processes such as heat response, phosphate deficiency, ABA signaling and low temperature (Miura and Hasegawa, 2008). SUMO activating protein induced moderately under cold conditions in both the *H. brasiliensis* clones in this study.

Genes such as WRKY tf, tf MBF, GPX, HbHP 33, HbDRT 50, ACO2, HbHP 22 and chitinase were up-regulated only in cold susceptible clone. *WRKY* genes are involved in many different biological processes such as response to wounding, senescence, water deficit, hormone signaling pathways and cold (Mare *et al*., 2004). It enhances transcriptional activation mainly by bridging a basic region/leucine zipper (bZIP) type transcriptional activator and a TATA box binding protein (Tsuda *et al*., 2004). Generally, GPX uses glutathione to reduce H_2O_2 and organic and lipid hydro-peroxides, thereby protecting cells against oxidative damage. *HbDRT50* which is a drought responsive transcript from *H. brasiliensis* (identity not known) had also been up-regulated remarkably (about 16 fold) in RRII 105, its level was about 10 fold in low temperature treated plants of clone RRIM 600 (Table 3). Multi-protein bridging factor 1 (MBF1) is a highly conserved transcriptional co-activator involved in the regulation of tolerance to various stresses, including salt, heat, osmotic, high light and disease (Suzuki *et al*., 2008) by activating ethylene mediated responses (Kim *et al*., 2007a; Suzuki *et al*., 2005). Its up-regulation

only in susceptible clone probably indicates that the plants are trying to cope up with the stress situation. In the case of ACO2 which is involved in ethylene synthesis, though it was shown to be up-regulated moderately (2.4 fold) in RRII 105, the levels were still higher in RRIM 600 where it was slightly reduced from 18 fold to 13 fold than RRII 105 control under cold stress.

Two genes such as CRT/DRE binding factor and cystein protease were down-regulated in both the clones under cold stress conditions. CRT/DRE bf (C-repeat element/dehydration-responsive element-binding protein) are upstream transcription factors known to bind to promoter *cis* element CRT/DRE and activate the expression of stress induced genes (Seki *et al*., 2003; Shinozaki *et al*., 2003). The down-regulation of CRT/DRE bf observed in this study indicates the necessity to explore the exact isoforms contributing for the cold tolerance. Similarly, cystein protease was also down-regulated in both the clones. Though it showed a slight decrease under low temperature, its intrinsic levels were relatively higher under control and cold conditions in RRIM 600 (4.5 and 3.2 fold, respectively) than in RRII 105 (1 and 0.8 fold, respectively). Cysteine proteases are involved in signaling pathways in response to biotic and abiotic stresses apart from their role in post-translational modification of proteins and enzymes. They also play a major role in senescence and cell death (Schaller, 2004).

The results indicated that among the genes studied, the level of expression of genes such as LEA5 protein, peroxidase and NAC tf (both the transcripts studied) was remarkably higher in RRIM 600 than RRII 105. The ethylene responsive transcripts such as ETR1 and ETR2 also showed up-regulation in RRIM 600. However, more such cold responsive genes have to be validated for their association with cold tolerance in *Hevea*. Among the twenty one genes analyzed, about a dozen genes were identified as cold responsive and only a few genes were found specific to cold tolerant clone. Probably, more investigations are needed for the validation of genes such as LEA5, peroxidase and NAC tf with cold tolerance by extending this study to more number of clones with varried levels of stress tolerance. Identification of more genes associated with cold tolerance gains importance in the context of evolving clones suitable for regions experiencing severe low temperature stress in the winter. Further, this study needs to be taken in two major directions. One is identification of more stress related genes by employing techniques like transcriptome sequencing or microarray and the other is to extend the quantitative expression study to more stress related genes in different clones and wild accessions with varying levels of stress tolerance as a measure to evaluate them as markers for cold tolerance. Moreover, identification of all isoforms of various genes through new generation sequencing technologies and validation of these genes by multilevel gene expression analysis such as microarray and quantitiative PCR, it would be possible to understand genes/ factors/markers associated with cold tolerance.

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