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De novo transcriptome analysis of abiotic stress-responsive transcripts of *Hevea brasiliensis*

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Abstract Cultivation of *Hevea brasiliensis*, which is being expanded to non-traditional regions, is constrained due to the adverse environmental conditions like extreme drought with high light and low humidity during summer and low temperature with high light during winter, prevailing in these regions. Many attempts are being made to develop drought and low-temperature tolerant varieties of *Hevea brasiliensis* by both conventional and modern methods of breeding. For this purpose, identification of candidate genes/markers associated with drought/cold tolerance is essential. In this attempt, transcriptome sequencing was performed in leaf samples of *H. brasiliensis* exposed to drought as well as cold stresses using Illumina sequencing technology (RNA-Seq) in order to generate functional genomic resource data which might eventually provide details on molecular mechanisms underlying drought/cold responses in *H. brasiliensis* Annotated transcriptome data of both drought and cold stress-responsive transcripts were analyzed and further validated by quantitative gene expression analyses. The digital gene expression analysis indicated an upregulation of 268 transcripts and downregulation of 566 transcripts under drought stress while 961 and 109 transcripts were found up- and down regulated respectively, under cold stress. Quantitative gene expression analysis of selected drought-responsive transcripts revealed the association between transcripts such as ferritin, DNA-binding protein, NAC tf and aquaporin with drought tolerance, and ethylene-responsive transcription factor (ERF) with cold tolerance. These results will enrich the available transcriptome data on *H. brasiliensis* and would enable the discovery of more genes/markers associated with drought or cold tolerance which can be employed in breeding for drought/cold tolerance in *H. brasiliensis*

Keywords Abiotic stress · *Hevea brasiliensis* · Drought · Low temperature · NGS · Quantitative gene expression · Stress tolerance markers · Transcriptome sequencing

Abbreviations

NR	Natural rubber
PS II	Photosystem II
TPD	Tapping panel dryness
ORF	Open reading frame
GO	Gene ontology
DE	Differentially expressed
DGE	Differential gene expression

Introduction

Constraints in cultivable land, competition from other crops, and ever-increasing demand for natural rubber (NR) led to the extension of rubber cultivation to even

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marginal and subtropical environments. In India, cultivation of *H. brasiliensis* is being extended to non-traditional regions with adverse climatic conditions which limit the growth, yield, and performance of the crop. These regions include North Konkan, where the summer is very severe, and northeastern states, where the temperature during winter is too low. The drought-prone non-traditional regions experience soil and atmospheric drought, warm atmospheric temperature concomitant with high light, and low relative humidity during extreme summer season, which is detrimental to the trees resulting in poor performance of the crop (Chandrasekhar et al. 1990; Jacob et al. 1999; Devakumar et al. 1998). In *H. brasiliensis*, several reports have confirmed the adverse effect of drought stress on yield and general performance of the crop (Sethuraj et al. 1984; Huang and Pan 1992; Sreelatha et al. 2007, 2011).

In South Central China and North Eastern states of India, low temperature stress (between 0 to 10 °C) strongly affects the cultivation of rubber trees and latex production (Priyadarshan et al. 2005). In addition to growth reduction, low temperature is responsible for loss in yield due to a tapping rest for a period of 1 to 3 months every year in North Eastern (NE) regions of India (Jacob et al. 1999). Young rubber plants are more susceptible to abiotic stress conditions (Jacob et al. 1999). Low temperature stress in concomitant with high light is reported to damage PS II and photosynthetic apparatus from its normal functioning (Annamalainathan et al. 2010). Clonal variation in cold tolerance (Polhamus 1962; Das et al. 2013) and response of various genotypes to cold under controlled environmental conditions have been reported earlier (Sarkar et al. 2013). During winter, high light during day time combined with cold stress in the previous nights led to severe inhibition in photosynthesis and chlorophyll bleaching (Powles 1984; Ray et al. 2004) and PS II activity as evidenced by the reductions in the maximum and the effective quantum yield of PS II (Sathik et al. 1998b; Jacob et al. 1999; Devakumar et al. 2002).

Molecular studies in *H. brasiliensis* have also reported the existence of altered level of expression of various transcripts/factors in different genotypes with varying levels of abiotic stress tolerance (Thomas et al. 2011, 2012; Luke et al. 2015). These studies have identified specific transcripts having strong association with drought stress tolerance. Differential gene expression was also reported in *H. brasiliensis* genotypes with varying levels of tolerance to cold stress (Sathik et al. 2012). Silva et al. (2014) identified low temperature stress influenced transcripts through cDNA library approach and also reported the EST-SSR and SNP markers from these transcripts. In order to obtain maximum growth and yield, it is necessary to identify genotypes with better tolerance to withstand suboptimal conditions prevailing in different agro-climatic zones. However, lack of availability of suitable methods for early evaluation of cold and drought tolerance and the long time required to develop stress-tolerant genotypes by conventional breeding programs in rubber are challenging. In *H. brasiliensis*, lots of attempts are being made to breed for drought- and cold-tolerant genotypes.

New generation sequencing (RNA-Seq) technology is an emerging tool employed for profiling of expressed genes to identify key genes/factors associated with specific situations like abiotic/biotic stresses or diseases in plants and other organisms (Schuster 2008; Wang et al. 2009; Van Verk et al. 2013). It has many advantages such as enabling large-scale functional assignment of genes, thorough qualitative and quantitative analysis of gene expression, improved sensitivity, and accurate profiling of eukaryotic transcriptomes including non-model organisms (Ekblom and Galindo 2011; Tang et al. 2011; Xia et al. 2011). Approximately, 53,000 EST sequences of *H. brasiliensis* have been deposited (as on March 2017) in the National Center of Biotechnology Information (NCBI), and it is amazing to see the progress made over the past two decades on data acquisition with regard to gene expression studies in rubber (Kush 1990; Chye 1991; Ko et al. 2003). In *H. brasiliensis*, transcriptional profiles of different tissues and organs such as latex, leaves, bark, and shoot apical meristem were generated using new generation sequencing (NGS) technology (Triwitayakom et al. 2011; Xia et al. 2011; Li et al. 2012; Salgado et al. 2014; Mantello et al. 2014).

De novo sequencing and comparative analysis of transcriptomes of healthy trees with tapping panel dryness (TPD) syndrome were carried out to identify genes and pathways associated with TPD syndrome (Liu et al. 2015; Li et al. 2016). In order to understand the molecular mechanisms involved in regulation of latex regeneration and duration of latex flow, Chao et al. (2015) analyzed latex from genotype CATAS8-79 and PR107 at the transcriptome level. Li et al. (2015) studied latex transcriptome and molecular mechanisms in connection with yield in rubber tree. Recently, Fang et al. (2016) reported about the distinct defense mechanisms existing in young and mature leaves of *H.*

brasiliensis by de novo transcriptome analysis. The recent report by Tang et al. (2016) on rubber tree genome reveals new insights into rubber production and species adaptation. The most recent report by Luke et al. (2017) indicated the existence of strong association of NAC transcription factor (NAC tf) with drought stress tolerance in *H. brasiliensis*. This study also indicated its up- and downregulation under intermittent drought stress and irrigated conditions. All these studies and reports have enriched the transcriptomic and genomic literature of *H. brasiliensis* to a considerable level.

In the present study, transcriptome sequencing was performed in leaf samples of *H. brasiliensis* exposed independently to drought and cold stress using Illumina RNA-Seq protocol in order to generate functional genomic resource data which might eventually provide details on molecular mechanisms underlying drought/cold responses in *H. brasiliensis*. Annotated transcriptome data of both drought and cold stress-responsive transcripts was analyzed, and selected transcripts were further validated using quantitative gene expression analysis. The results and the findings are discussed.

Materials and methods

Plant material and stress induction

The clonal plants (RRIM 600, RRII 430, RRII 208, RRII 105, and RRII 414) were developed by budgrafting of specific clonal buds on 10-month old seedlings of *H. brasiliensis.* With regard to drought stress, one set of six plants (6 months old) grown in poly bags was subjected to water stress by withholding irrigation for 10 days while the other set of control (six) plants was watered on alternate days. 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). All the gas exchange measurements were made as described earlier (Luke et al. 2015), and leaf samples from these plants were collected in liquid N_2 and stored at − 80 °C for further proceeding with NGS. For NGS protocol, total RNA samples were collected from drought-stressed and irrigated plants of the genotype RRIM 600 only. For the purpose of subsequent validation of drought-responsive transcripts, RNA samples collected from genotypes RRIM 600, RRII 430, RRII 208, RRII 105, and RRII 414 were used.

With regard to cold stress, six numbers of 6-month-old poly bag grown plants (genotype RRIM 600) were acclimatized in a growth chamber for 3 days with a minimum temperature of 15 °C during night (for 3 h) and a gradual rise in maximum temperature (T max) up to 25 °C in the day time. Fourth day onwards, cold treatment was imposed by reducing the minimum temperature to 8 °C during night followed by a gradual increase in T max up to 16 °C in the day time for five consecutive days after which leaf samples were collected and stored as described above. Light intensity regime in the growth chamber was provided between 400 and 800 μmol m^{-2} s^{-1}, and the relative humidity (RH) was maintained between 60 and 70%. Control plants were maintained at stress-free ambient weather conditions. For NGS purpose, plants of genotype RRIM 600 only were used. For validation of cold stress-responsive transcripts, cold susceptible genotype RRII 105 and cold-tolerant genotype RRIM 600 were used.

Physiological parameters

Fully matured leaves from the top most whorl were chosen for gas exchange measurements. Measurements were taken in two leaves each from four plants selected from each treatment ($n = 8$). Net CO_2 assimilation rate (A) and stomatal conductance (g_s) were measured using a portable photosynthesis system (LI-6400 XT), LI-COR, USA. 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 of LI-6400XT. Maximum potential quantum yield (F_v/F_m) was measured by using a fluorescence monitoring system (Hansatech, UK). The leaves were dark adapted for 20 min to determine minimal fluorescence (Fo), after which a saturating flash was applied to determine maximum fluorescence (Fm). The difference between Fm and Fo is referred to as variable fluorescence (Fv), using which maximum quantum yield was obtained (F_v/F_m).

Total RNA isolation and cDNA synthesis

Total RNA was extracted from leaf samples using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). Quality and quantity of the isolated RNA were checked by nanodrop and gel electrophoresis. RNA integrity was

evaluated using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and samples with a RNA Index Number (RIN) value of above 7.0 only were taken up further for downstream processes (Supplementary data 1). Total RNA samples of three plants from each treatment were mixed for the sequencing of drought- and cold-responsive transcripts. Poly RNA from total RNA was isolated with oligoDT beads using TruSeq RNA sample preparation kit, Illumina. The purified mRNA were then fragmented with divalent cations at an elevated temperature (94 °C, 4 min) and reverse transcribed with superscript II reverse transcriptase by priming with random hexamers. DNA polymerase I was used for second-strand synthesis in the presence of RNase H.

Library construction and sequencing

Transcriptome library was constructed by employing Illumina TruSeq RNA library protocol. Illumina adapters were ligated to the cDNA followed by PCR amplification (11 cycles) for enrichment. Sequencing was carried out using Illumina Hi-Seq 2000 system by outsourcing with Genotypic Technology Pvt. Ltd., Bangalore, India.

De novo assembly

Raw sequencing reads were subjected to quality filtering using SeqQC V2.1. Reads with adapter contamination were trimmed and low-quality bases were discarded. Further, reads with ≥ 50 bases were filtered for assembly. Contamination screening was carried out using FastQ Screen (Andrews 2011) tool against human, mouse, *E. coli*, yeast, PhiX, adapters, vectors, and *H. brasiliensis* sequences. No potential cross-species contamination was found in the data (Supplementary Figs. 1, 2, and 3). The sequencing reads were submitted to NCBI Sequence Read Archive (SRA) under the accession numbers SRR620233, SRR620234, and SRR620235. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFZB00000000. The version described in this paper is the first version, GFZB01000000. The filtered sequencing reads were assembled using Oases 0.1.21 (Schultz et al. 2012) assembler tool. Oases assembler is a robust transcriptome assembly program which generates contigs using Velvet 1.2.07 (Zerbino and Birney 2008) algorithm. Further, the transcripts were constructed using contigs with the help of paired-end sequencing information.

Open reading frames and Pfam domain detection

The likely coding regions from transcripts were identified using TransDecoder program (http://transdecoder.sf.net). To support the open reading frame (ORF) prediction, Pfam domain database search methods were set up using Pfam-A database (Finn 2014). Hmmscan3 program was used for Pfam detection. Further, the sequence data were subjected to multi-level filtration to obtain complete ORF sequences using in-house perl scripts.

Differential gene expression and gene ontology enrichment analysis

In order to construct the reference sequence for read mapping, the transcripts from control and treated samples were clustered using CD-HIT tool with 90% sequence identity. Read mapping was carried out using Bowtie2 tool. DESeq (Anders and Huber 2010) program was used for finding differentially expressed (DE) transcripts from mapping data. The DE transcripts were annotated using blast program against *Ricinus communis* protein sequence from UniProt. The aligned transcripts were further annotated using gene ontology (GO) terms (Ashburner et al. 2000). Gene ontology enrichment analysis was carried out in differentially expressed transcripts. The enrichment analysis was performed using agriGO tool (Du et al. 2010). AgriGO uses hypergeometric test with Benjamini-Hochberg FDR correction, and a "*P*" value ≤ 0.05 was employed for finding significantly enriched GO terms. After successful completion of enrichment analysis, the transcripts which were enriched under various biological processes were extracted for further analyses.

Validation by quantitative PCR

The *H. brasiliensis* genotypes viz., RRIM 600, RRII 430, RRII 208, RRII 105, and RRII 414 were included for validation of selected transcripts by quantitative PCR (qPCR) analyses in order to identify marker gene/s for drought tolerance while genotypes RRII 105 and RRIM 600 were chosen for cold tolerance. Imposition of stress to the plants and leaf sample collection was carried out as described earlier. Total RNA was extracted from the leaf

samples (three biological replicates for each treatment) using Spectrum Plant Total RNA Kit (Sigma-Aldrich), and cDNA was synthesized using superscript III reverse transcriptase (Invitrogen) following manufacturer's instructions. Levels of expression of selected genes in plants under stress and control conditions were determined by qPCR. Attempts were made to validate about two dozen genes from the DGE libraries of both drought- and cold-treated plants by qPCR. Suitable primers were designed with Primer 3 Express (Applied Biosystems, USA) and were synthesized by M/s. Sigma-Aldrich, India (Table 1).

Further, quantitative gene expression analyses were carried out using Light Cycler 480 II, Roche Real Time PCR System as reported earlier (Luke et al. 2015). qPCR was performed in a 20-μl reaction mixture containing 1-μl template DNA from 1/10 dilution of first-strand cDNA reaction, 125 nM of each primer, and 10 μl of Light Cycler 480 SYBR Green I Master (Roche Diagnostics Gmbh, Germany). The mixture was first incubated at 95 °C for 7 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. Subsequently, a melt curve analysis (95 °C for 20 s, 60 °C for 1 min, and 95 °C for about 5 min) was conducted. Each experiment was repeated twice or thrice, and each PCR reaction was performed in triplicate with no template controls (NTC). Efficiency of the primers was calculated based on the formula, Efficiency = [10 (−1/slope)] − 1. The primers with slope values between − 3.2 and − 3.5 only were further employed for qPCR analysis in which GAPDH gene was used as endogenous control. The Light Cycler 480 Software (release 1.5.0) was used for relative quantification analysis, and the expression rate of each gene was represented as fold change. Statistical analysis was performed with single factor ANOVA using normalized expression data.

Results

De novo assembly and annotation

RNA-Seq libraries were paired-end sequenced using Illumina Hi-Seq 2000 system. A total of 63 million sequencing reads generated from control sample, and more than 70 million reads were generated in both cold- and drought-stressed samples. Adequate amounts of sequencing reads were generated as suggested by Chow et al. (2014) in order to cover maximum transcripts from *H. brasiliensis*. The filtered sequencing reads were assembled using Velvet/Oases transcriptome assembly program. Finding optimum kmer for RNA-Seq data is crucial for de novo assembly. This was achieved by performing multiple assemblies using different kmer sizes. The expected coverage and coverage cutoff parameters were set as "auto" for the assembly runs. Kmer of 49, 51, and 49 was considered as optimum for control, drought, and cold samples, respectively. Around 88% reads were assembled in control, and about 90% reads were assembled in drought- and cold-treated samples. In total, 90,735 transcripts were generated in control, whereas as 104,071 and 100,120 transcripts were generated in drought- and cold-treated samples, respectively. The N50 value was above 2 kb in both control and treated samples. The complete assembly statistics is given in Supplementary Table 1. The boxplot of transcript length comparison is shown in Supplementary Fig. 4. Sequence clustering was performed to obtain non-redundant transcript sequences. The sequence clustering reduced the total count of transcripts to 58,581, 67,482, and 66,019 in control and drought- and cold-treated samples, respectively. The N50 statistics was still around 2 kb in these samples. The main aim of the sequence clustering was to avoid multiple hits in open reading frame (ORF) and Pfam domain detection analyses. The maximum transcript lengths remained unchanged in all samples as expected of good clustering.

Open reading frames and Pfam domain detection

Multi-level filtering (extraction of complete ORFs and filtering sequences with partial ORFs, discarding sequences with ambiguities (N), and filtering of sequences containing more than one ORF) was applied on the predicted likely coding regions from sequences of transcripts. After multi-level ORF filtration, 18,789 ORFs were obtained in the control sample. Similar filtration was applied on drought and cold samples which yielded 21,308 and 21,077 ORFs, respectively. Full-length ORFs from control and drought- and cold-stressed samples were aligned against protein datasets of *Ricinus communis* from Uniprot protein resources. In order to identify the common and unique genes, the annotated ORF sequences were compared with each other. In total, 6881 genes were identified as common among all the treatments. Similarly, 715, 958 and 1210 genes were identified as unique in control and drought- and cold-stressed plants, respectively (Supplementary Fig. 5).

Table 1 List of genes used in qPCR analysis

ID No	Gene/transcript	Gene abbreviation	Forward primer	Reverse primer
B_23441	Protein phosphatase 2c	pp2c	AAACGAAACAGAAG GAGAGATTAC	AGAGACGATGAAGGAGAG
B_5266	Ferritin	ferritin	CTAAACGAGAATAGAAAGCC CAAA	CAGAGCCACCATCCTTCAT
A_29734	Mitochondrial dicarboxylate carrier protein	MDCP	ACAAATTACAGAAGGAGAGA TTAC	CAGCAACAATGGAA GCAAT
B_30709	Glutaredoxin	GRX	GGTGAGTTGGTGCCTATT	GAAAGAAAACCCAT CCATCAAA
B_19881	21 kDA protein	21 KDa	CTTGGCTCTTATCAATAACT ATGC	CAACGAGAAAGGAG ACATACAA
A_25234	ATP-binding cassette transporter	ABCTP	AGGACTGTTATTGCTTCA	AATAGACTGTTCTGCCATA
B_11741	DNA-binding protein RAV1	DNA bp	CTACGAGAAGAGAACAGA	AATGGATATGGAGTCACTA
A_7332	Heat shock protein-binding protein	HSP bp	CAAGAGGCTTATTCAGTGTT ATCA	CTTCATCATCATCA TCTCCAA
A_5921	NAC domain-containing protein	NAC tf	TTCAATGGTGGCTTACTCT	CAAGACTGACTGGA TTATGC
B_31589	Mitogen-activated protein kinase kinase kinase	MAP3K	TAACAAGTCCTCCAATGATT	AGAGAAGACCGTGATACA
B_324	Ethylene-responsive transcription factor	ERF	AGGATTATAGAGTCTTTGAG ATTGA	GATAGTTCTTGTGG CTTGTAG
A_15463	Malate dehydrogenase	MDH	ATAAGCACACCACCTCTC	CACTCAACCACTCAGGAT
A_28725	Serine carboxypeptidase	SCP	AAGGTTGACAGATGATGAAC	GGACTTCTACAGGCATTG
A_9060	Aquaporin Pip2	PIP 2.1	GCGGGCTTTCCGTTTAGG	TGAGATCATCGCCA CTTTCG
A_22946	Caspase	Caspase	GTATGGAAAGGAACAAATGG T	GAAGTGTCAGCAGAGGTT
B_ 24,758	Tonoplast intrinsic protein	TIP	CCCACTGGTCTCATGCCATTA	TGCCGCTATTGTGG CTTCTC
B_32804	Chaperone protein DnaJ 11	DnaJ 11	CAAGTTGCCATGAGATAA	CAGACAAGGTAGAA TAAGC
A_18065	Heat shock protein	HSP	TGAGAACATCGCGTTTGAGA A	TGGTACTGGCTTTA GGGATGGT
A_27682	Oxygen-evolving enhancer protein 1	OEP	GAAGAAGGCGAGGA AAGTGAAA	TGGTGGGTTGCATG AGTGTT
A_20,212	Ethylene-responsive transcription factor	ERF D	TTAGGCAACATTGATTGATTG	CATTCTTCAGTGGG TTACAA
A_16967	Dehydration-responsive element-binding protein	DRE bp	CCTGTATTGATGTGGATGTT	AACCAAGACGAGAA TATGC
C_591	Ethylene-responsive transcription factor	ERF U	TGGTGTTGAAGAGAAAAGGC ATTAC	GATCTCCGCTGCGT ATTTGC
C_1073	Auxin/hydrogen symporter	AUX/H Sym	CCCATCTGTGCTACATTTTC TGA	GTCCTGATTGCAGC TCTATGCA
C_9761	Transcription factor	TF	CGTCGGTGTCATTAACTTC	CTATCAGTATGCGGTGGAA

Around 8000 genes were detected as common among drought and cold transcriptomes. Multiple putative uncharacterized proteins were the largest common group followed by pentatricopeptide repeat-containing proteins. A good number of DNA-binding protein, ATP-binding protein, transcription factor, protein-binding protein, zinc finger protein, and nucleic acid binding proteins were also found common among these two

groups. The protein sequences obtained from ORF pipeline were searched against Pfam-A database for domain detection. In total, 32,050, 35,691 and 36,313 transcript sequences were found to have at least one Pfam domain hit in control, drought, and cold samples, respectively. The top 30 Pfam domain hits from all three samples are given in Supplementary Fig. 6. High abundance of pentatricopeptide repeat (PPR) motif was found in all three samples which had been found commonly present across the plant kingdom (Mingler 2006).

Differential gene expression and gene ontology enrichment analysis

In total, 834 transcripts were identified as differentially expressed (DE) between control- and drought-stressed samples. Among the differentially expressed transcripts, 268 and 566 transcripts were found up- and downregulated, respectively. The differentially expressed transcripts were annotated against *Ricinus communis* protein database using homology search. Gene ontology (GO) term mapping was carried out for DE transcripts in order to classify them into biological process, molecular function, and cellular component categories. A total of 292 GO terms were assigned to DE transcripts. Further, GO term enrichment analysis was performed using *Ricinus communis* as background reference. Significantly enriched DE transcripts were extracted, and their expression values were tabulated for further analyses. The majority of the DE transcripts were enriched under metabolic process (GO: 0008152) and cellular process (GO: 0009987) categories. In total, 130 downregulated transcripts were grouped under metabolic process, whereas 59 upregulated transcripts were assigned to the same process. A total of 180 DE transcripts were enriched under cellular process. Other biological processes such as carbohydrate metabolic process (GO: 0005975), primary metabolic process (GO: 0044238), and cellular amino acid and derivative metabolic process (GO: 0006519) were also found significantly enriched.

Similarly, 961 upregulated and 109 downregulated transcripts from control and cold-stressed samples, respectively, were mapped to a total of 353 GO terms. These transcripts were further subjected to GO enrichment analysis. Biological processes such as response to stress (GO:0006950), response to stimulus (GO:0050896), protein folding (GO:0006457), photosynthesis (GO:0015979), response to oxidative stress (GO:0006979), response to chemical stimulus (GO:0042221), protein metabolic process (GO:0019538), proteolysis (GO:0006508), cellular process (GO:0009987), metabolic process (GO:0008152), cellular metabolic process (GO:0044237), primary metabolic process (GO:0044238), and macromolecule metabolic process (GO:0043170) were found enriched in downregulated transcripts. A high percentage of upregulated transcripts were enriched under metabolic process (GO: 0008152), cellular process (GO: 0009987), primary metabolic process (GO: 0044238), and cellular metabolic process (GO: 0044237) categories (Supplementary data 2).

Expression analysis of drought-responsive transcripts in *H. brasiliensis* genotypes with varying levels of drought tolerance

In order to determine the expression levels of drought-specific transcripts from transcriptome sequencing, five genotypes of *H. brasiliensis* viz., RRII 105, RRIM 600, RRII 414, RRII 208, and RRII 430 with varying levels of drought tolerance were exposed to drought and further were subjected to gene expression analysis by qPCR. Prior to sample collection for qPCR, physiological parameters were measured in drought-exposed leaves of genotypes mentioned above to confirm the impact of drought stress on these plants. Reduction in stomatal conductance was noticed in all the genotypes under drought stress, while reduction was maximum in RRII 414 and minimum in RRIM 600 (Supplementary Fig. 7a). Similarly, reduction in CO_2 assimilation rate was found in all the genotypes, while it was maximum in genotype RRII 414 (0.7 μmol m^{-2} s^{-1}) and minimum in genotype RRIM 600 (1.9 μmol m^{-2} s^{-1}; Supplementary Fig. 7b). Previous studies in genotype RRIM 600 and RRII 414 also reported lesser inhibition of gas exchange parameters in genotype RRIM 600 under drought condition than in genotype RRII 414 and confirmed the higher levels of tolerance in genotype RRIM 600 (Sumesh et al. 2011). Expression analysis was carried out in the above *H. brasiliensis* genotypes with qPCR being confined to 17 transcripts shortlisted based on primer standardization (Fig. 1; Supplementary Table 2). Among the transcripts investigated, ferritin, DNA-binding protein RAV1, and NAC tf were found significantly upregulated in tolerant genotypes viz., RRIM 600, RRII 208, and RRII 430 when compared to RRII 105 and RRII 414 which is moderately tolerant and susceptible genotype, respectively. MAP kinase

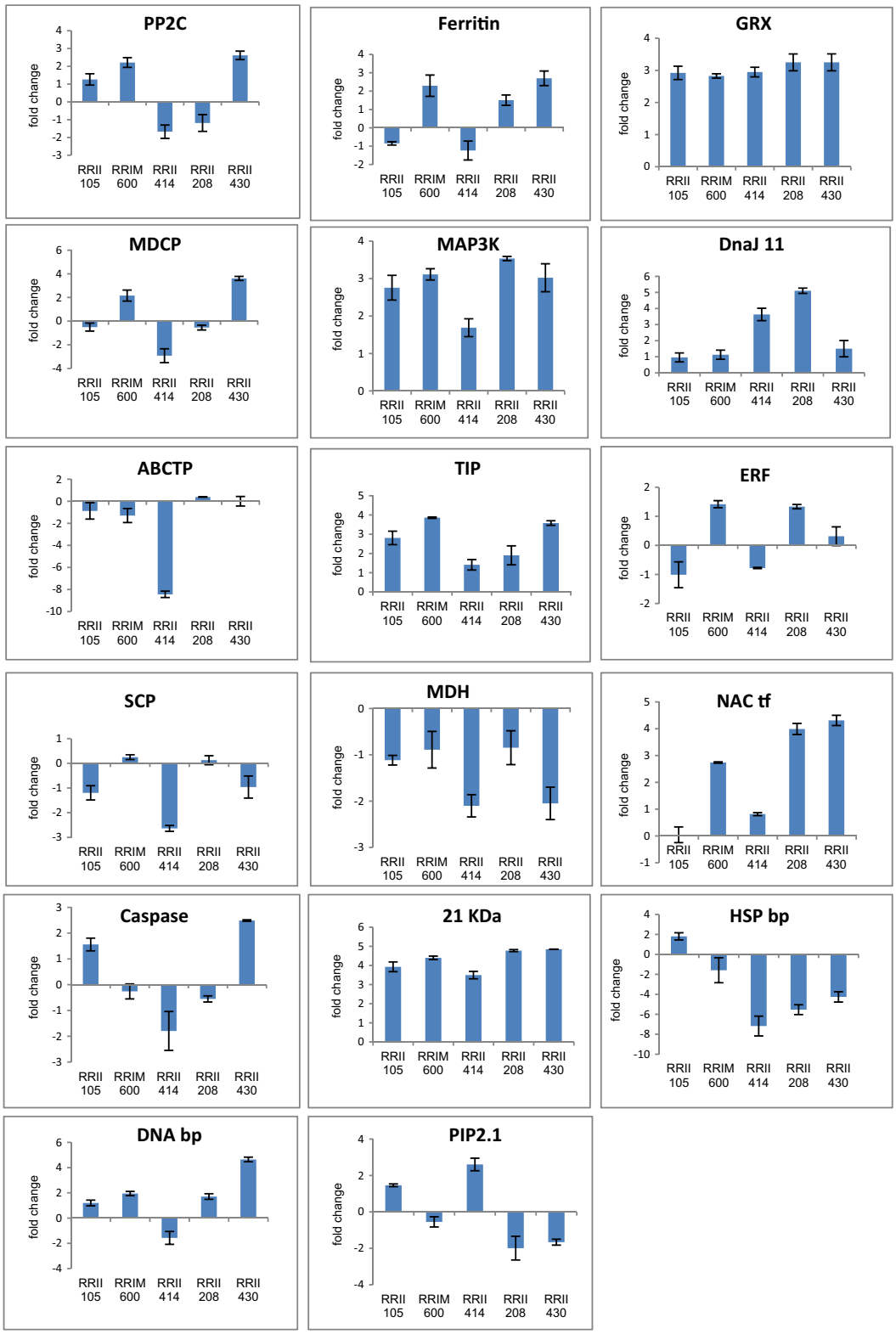

Fig. 1 Expression analysis of 17 genes in five drought-imposed clones of *H. brasiliensis* with their own control as calibrator (fold change in log2 ratio). Log 2 ratio ≥ 0 upregulated; ≤ 0 downregulated. ± Error bars indicate standard error of three biological replicates

kinase kinase (MAP3K) and tonoplast intrinsic protein (TIP) were found upregulated in all the genotypes studied, while their expression was significantly higher in tolerant genotypes. Interestingly, expression of aquaporin (Pip2.1) was found significantly lesser in tolerant genotypes. The expression of glutaredoxin, 21 kDa protein, and Dna J11 protein was found significantly higher in all the genotypes suggesting their drought responsiveness.

The accuracy and the reproducibility of the Illumina RNA-Seq results were confirmed by performing quantitative PCR (qPCR) on 17 selected transcripts in genotype RRIM 600. The correlation between RNA-Seq and qPCR was made using the log2 fold change value determined between RNA-Seq and qPCR data by comparative Ct method ($2^{-\Delta\Delta Ct}$). Findings of qPCR results revealed the existence of similar trend in expression of these transcripts ($R^2 = 0.712$) with the RNA-Seq results as depicted in Fig. 2a, b confirming the accuracy and reproducibility of RNA-Seq results. Even though the exact fold change of the selected transcripts varied between RNA-Seq and qPCR analyses, all the 17 transcripts exhibited similar trends in expression pattern with RNA-Seq data. Heat map generated of relative quantification values of 17 genes under drought stress indicates the association of transcripts like NAC tf, DNA bp, and Dnaj 11 with drought tolerance among which NAC tf was found to be more appropriate as a marker for drought tolerance (Fig. 3).

Expression analysis of cold-responsive transcripts in *H. brasiliensis* genotypes

The plants (genotypes RRIM 600 and RRII 105) grown under growth chamber conditions showed cold stress-responsive syndrome after cold treatment at 8 °C during night and at 16 °C during day time for 5 days. Physiological parameters such as stomatal

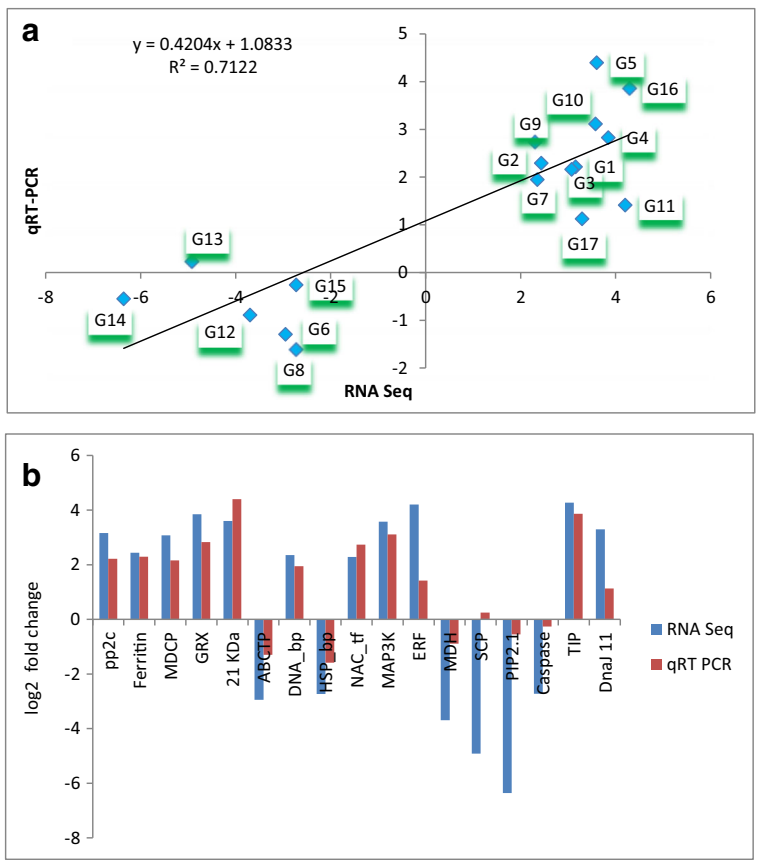

Fig. 2 qPCR validation of differentially expressed genes in RRIM 600. **a** Correlation between fold change (log 2) analyzed by RNA-Seq (*x*-axis) and data obtained using qPCR (*y*-axis). **b** Expression analysis of selected transcripts between RNA Seq and qPCR

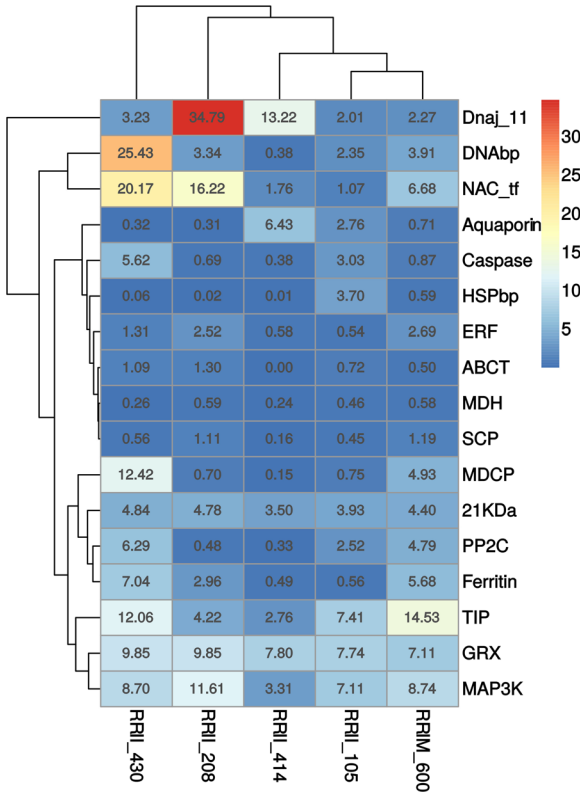

Fig. 3 Heat map of relative quantification (RQ) values of 17 genes in five genotypes of *Hevea* under drought stress with its own control as calibrator

conductance (g_s), net CO_2 assimilation rate (A), and quantum efficiency of PS II indicated the impact of stress in both the genotypes. Stomatal conductance in susceptible genotype RRII 105 came down drastically from about 0.11 mol m^{-2} s^{-1} to near zero under cold stress while the tolerant genotype RRIM 600 could maintain at about 0.04 mol m^{-2} s^{-1} (from 0.13 mol m^{-2} s^{-1}) in control condition (Supplementary Fig. 8a). While both the genotypes maintained A at about 6 to 7 μmol m^{-2} s^{-1} in control conditions, RRIM 600 maintained better A (3 μmol m^{-2} s^{-1}) than RRII 105 (near 0) upon exposure to cold stress (Supplementary Fig. 8b). Similarly, the F_v/F_m ratio also was found better in RRIM 600 (0.6) than that in RRII 105 (0.3) under low-temperature condition (Supplementary Fig. 8c).

Expression analysis was carried out for seven transcripts in the above *H. brasiliensis* genotypes which vary in their cold tolerance levels (Fig. 4; Supplementary Table 3). Among the transcripts investigated, an ethylene-responsive transcription factor was found significantly upregulated in RRIM 600, than that in RRII 105. Other transcripts like heat shock protein, drought-responsive element-binding protein, and auxin/hydrogen symporter were found upregulated in both the genotypes. The transcripts like oxygen-evolving enhancer protein 1 and a transcription factor exhibited a downward trend in both the genotypes.

Discussion

In this study, gene expression analysis of *H. brasiliensis* (genotype RRIM 600) under irrigated and drought-stressed condition was made using RNA-Seq data from Illumina high-throughput sequencing platform. Whole transcriptome de novo assembly is a complex process which requires high-quality reads with adequate sequencing depth. Therefore, more than 60 million high-quality sequencing reads from Illumina sequencing platform were generated. On an average, over 89% of these sequencing reads were successfully assembled using de Bruijn graph based de novo assembler. The transcriptome assembly statistics suggests effective assembly of the sequencing reads. Around 2 kb of N50 statistics and more than 1 kb of average transcript length indicated higher standard of assembled transcripts. The functional annotation of transcripts was carried out using blast homology search methods with *Ricinus communis* as reference. Protein domain was identified using Pfam database. Over 30,000 sequences were found to have at least one pfam domain hit in assembled transcripts. Protein domains such as pentatricopeptide repeat, leucine-rich repeats, tetratricopeptide repeat, WD domain, and protein kinase were highly abundant in Pfam results. Full-length open reading frames were predicted, and over 18,000 complete ORFs were filtered using in-house methods.

In order to carry out further comparative analysis among the samples, two sets (control with drought stressed and control with cold/low temperature stressed) of clustered references were generated. The clustered transcripts were subsequently used for differential gene expression (DGE) analysis. Comparison of transcriptome profile of control and drought-stressed plants resulted in 834 differentially expressed (DE) transcripts. Similarly, 1070 DE

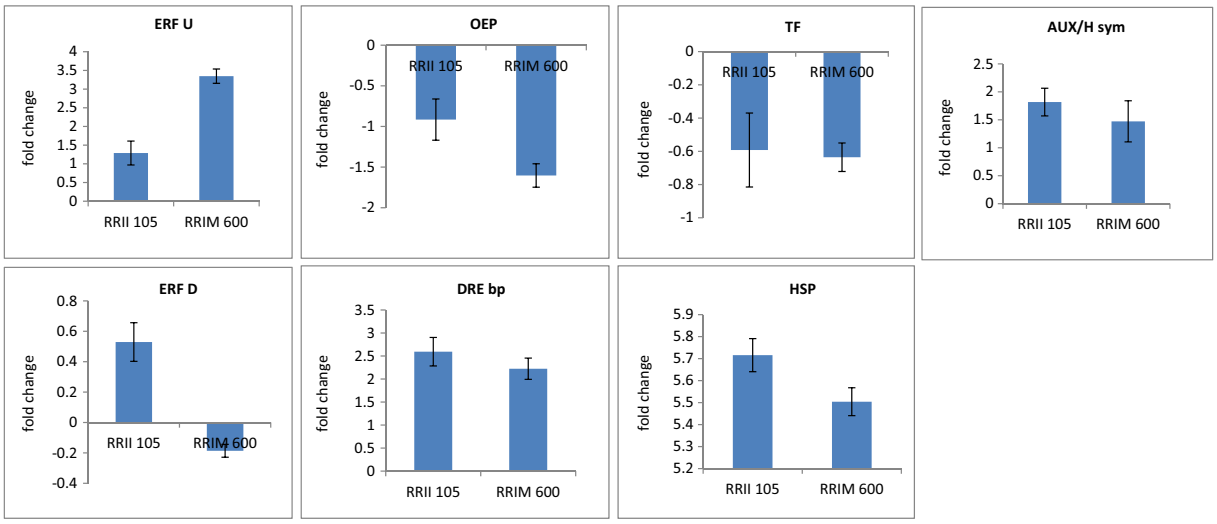

Fig. 4 Relative quantification of cold-responsive genes in two genotypes of *H. brasiliensis* exposed to cold stress (fold change in log2 ratio). Log 2 ratio ≥ 0 upregulated; ≤ 0 downregulated. ± Error bars indicate standard error of three biological replicates

transcripts were identified between control and cold-stressed samples. The DE transcripts were annotated and classified based on GO terms. Further, GO term enrichment analysis was carried out for screening transcripts that were involved in important biological processes during drought stress in *H. brasiliensis*. Metabolic process, cellular process, and carbohydrate metabolic process were found significantly enriched among the biological processes in the GO term enrichment analysis. Transcripts enriched under various GO terms were extracted and used for further analysis. Since, the GO term enrichment analysis was carried out from pooled sample data, the results could not be evaluated statistically. But it served the purpose of initial screening of abiotic stress-responsive transcripts of *H. brasiliensis*.

Based on the results of differential gene expression (DGE) analysis, the transcripts responsive to drought and cold stresses were identified (Supplementary data 3), and transcripts with much higher fold change (both up and down regulation), specifically expressed only in one treatment (like either drought/cold or control) and having important functions, were shortlisted for further analysis. For validation, five genotypes with different levels of drought tolerance were included in this study. Based on field performance, the genotypes RRII 430, RRIM 600, and RRII 208 have been known as drought-tolerant genotypes whereas, RRII 105 and RRII 414 are known as moderately tolerant and drought susceptible genotype, respectively. The gas exchange parameters (CO_2 assimilation rate and stomatal conductance) measured in drought exposed plants also asserted the findings of previous reports on these genotypes (Sumesh et al., 2011). The DGE results also confirmed the drought susceptibility/tolerance of the genotypes under investigation.

Drought-responsive transcripts

Based on the expression analysis data, transcripts such as ferritin, DNA-binding protein RAV1, and NAC tf were found significantly upregulated in the tolerant genotypes viz., RRIM 600, RRII 208, and RRII 430 when compared to RRII 105 and RRII 414. In general, expression of transcripts such as MAP3K and TIP has been found triggered in all the genotypes while it was much higher in tolerant genotypes. In the case of ferritin, significant upregulation was observed in genotypes RRIM 600, RRII 430, and RRII 208 when compared to their respective control plants. While it got downregulated in RRII 105 which is a moderately tolerant genotype, in genotype RRII 414, a known drought susceptible genotype, it got significantly downregulated. Ferritin family of genes is drought responsive which is known to be upregulated strongly and uniformly under drought. They play crucial role in removal of free Fe, which catalyzes

the Fenton reaction that produces highly reactive hydroxyl radicals (Becana et al. 1998; Briat et al. 2010; Kang and Udvardi 2012). tpiUnder stress-free conditions, ferritin level is maintained at lower levels, but it shoots up to higher levels when exposed to water-stressed conditions. Several reports indicate that ferritin expression in plants is induced by many environmental factors such as drought, salinity, cold, light intensity, pathogen attack, NO, and ozone (De Laat et al. 2014) that stimulate ROS production. Similarly, the DNA-binding protein RAV1 also exhibited the same trend of ferritin. RAV (related to ABI3/VP1) protein is known to positively regulate leaf maturation and senescence, control flowering, and cold response. The significant up-regulation of putative RAV1 DNA-binding protein in tolerant genotypes indicates its strong association with drought tolerance.

NAC transcription factors which belong to one of the largest families of plant-specific transcriptional regulators (NAC gene family) play important roles in the regulation of transcriptional reprogramming associated with plant stress responses. Genes in the NAC family have been shown to regulate a wide range of developmental processes of plants (Duval et al. 2002; Guo and Gan 2006; Kim et al. 2006; Kim et al. 2007; Ko et al. 2007; Sperotto et al. 2009). The overexpression of *ANAC019*, *ANAC055*, and *RD26* (*ANAC072*) in *Arabidopsis* resulted in upregulation of stress-inducible genes which enhanced drought and salt tolerance in plants. *ATAF1* and *ATAF2* in *Arabidopsis* and *HvNAC6* in barley have been found to play important roles in response to drought and pathogen stresses (Mao et al. 2014).

The other two transcripts which got triggered in all the genotypes irrespective of drought tolerance/susceptibility are MAP3K and putative TIP. Mitogen-activated protein kinases play essential roles in plant growth and development and in signaling response to various stresses such as pathogen infection, wounding, drought, salinity, UV irradiation, ozone, and free radicals (Wang 2015). In plants, MAPKs are rapidly activated when exposed to multiple abiotic stress stimuli (Hirt 2000). MAPK cascades relay and amplify signals through the phosphorylation of substrate proteins, mediating a wide array of responses, including changes in gene expression. Higher levels of H_2O_2 which is generated during severe stress conditions in plants act as messengers in activating the expression of MAPK cascades that in turn trigger expression of transcription factors to cope up with the stress. The level of expression of MAP3K in the genotypes studied indicates its association with drought stress irrespective of whether the plants are stress tolerant or susceptible. But the levels were much higher in tolerant genotypes when compared to moderate and susceptible genotypes.

The other transcript, TIP was found drought stress responsive and was found expressed at higher levels in tolerant genotypes. TIP is a subfamily of aquaporin which regulates water movement across vacuolar membranes, and TIPs are reported to be associated with drought tolerance in many plants (Wang et al. 2011). Expression analysis indicated TIP as drought responsive though not strictly associated with only tolerant genotypes. In contrast to this, aquaporin displayed a negative trend. Its expression was significantly higher in susceptible and moderate genotypes when compared to tolerant genotypes. Aquaporins are integral membrane proteins that are involved in regulating the rapid movement of water as well as molecules like CO_2, H_2O_2, urea, ammonia, silicic acid, and arsenite and wide range of small uncharged solutes. They have also been reported to modulate abiotic stress-induced signaling in plants (Srivastava et al. 2014). In this study, aquaporin was found upregulated in susceptible/moderate genotypes indicating its relevance as marker for drought susceptibility.

Interestingly, two transcripts viz., glutaredoxins (GRX) and 21 kDa protein were found upregulated in drought-stressed samples of all the genotypes irrespective of their tolerance/susceptibility. GRXs are small, heat-stable disulfide oxidoreductases that play important role in plant development and in response to oxidative stress. In response to oxidative stress, GRXs are known to limit ROS production, participate in redox signaling, and play antioxidant role, thereby protecting plants from cellular oxidative damage. The transcript homologous to ethylene response factor (ERF) was found upregulated in tolerant genotypes viz., RRIM 600 and RRII 208 and was found lesser in RRII 105 and RRII 414 while there was no significant change in RRII 430. ERFs belonging to the transcription factor family APETALA2/ERF are associated with plant development and are reported to express in response to biotic and abiotic stresses (Mizoi et al. 2012). Several ERFs also bind to dehydration-responsive elements (DREs) and play regulatory role in plants under abiotic stress situations (Cheng et al. 2013). In this attempt, though ERF was found expressed at significantly higher levels

in the tolerant genotypes RRIM 600 and RRII 208, it was not so in the other tolerant genotype RRII 430. Hence, this particular transcript cannot be considered as marker for drought tolerance.

Transcripts like malate dehydrogenase (MDH), serine carboxypeptidase, and ABC transporter protein got either downregulated or did not show any change across the genotypes investigated. MDH catalyzes a reversible NAD^+-dependent dehydrogenase reaction (oxaloacetate to malate conversion) involved in central metabolism and redox homeostasis between organelle compartments using either NAD/H or NADP/H as oxidant/reductant, respectively (Scheibe et al. 2005; Hebbelmann et al. 2012). Serine carboxypeptidases (SCPs) are members of the α/β hydrolase family of proteins that play roles in multiple cellular processes by making use of a Ser-Asp-His catalytic triad to cleave the carboxy terminal peptide bonds of their protein or peptide substrates. *OsBISCPL1*, a serine carboxypeptidase-like gene, is reported to be involved in regulation of defense responses against biotic and oxidative stress in rice (Liu et al. 2008). ABC transporters are one of the largest protein families which are present in all organisms ranging from bacteria to humans (Henikoff et al. 1997), and in most cases, functional ABC transporters act as ATP-driven pumps. In plants, apart from detoxification, ABC transporters have been reported to be involved in diverse processes such as pathogen response, surface lipid deposition, and phytate accumulation in seeds, and in transport of the hormones like auxin and abscisic acid (Martinoia et al. 1993). They are also involved in overall plant growth and development, response to abiotic stress, and interaction of the plant with its environment (Kang et al. 2011). Owing to downregulation or no change in expression of these three transcripts in the experiment, they are ruled out as candidate genes for stress tolerance. Transcripts such as DnaJ 11 (involved in protein folding/unfolding), caspase (which cleave aspartate residues), mitochondrial dicarboxylate carrier protein (transports molecules across membranes and ATP/ADP and Pi), and protein phosphatase 2c (mediates abiotic stress triggered signals), did not show any trend in its expression pattern in any of the genotypes studied.

Cold-responsive transcripts

In the gene expression study of cold-responsive transcripts, seven transcripts were analyzed among which only one transcript coding for ERF1 was found significantly upregulated in cold-tolerant genotype RRIM 600 while it was not much induced in genotype RRII 105. There were two transcripts viz., HSP and DRE bp which got significantly upregulated in both the genotypes while transcripts viz., OEC, ERF (2), and TF (putative) were found significantly downregulated in both the genotypes. The transcript-encoding AUX/H symporter displayed an upward trend in both the genotypes. ERF was the only transcript which displayed a significant upregulation in genotype RRIM 600 which is known as cold-tolerant genotype in the North East regions of India. ERFs are known to function as regulators of stress-responsive genes by binding to sequences containing AGCCGCC motifs (GCC box) (Solano et al., 1998) and also by binding to DREs (Cheng et al. 2013). ERF genes are known to get induced by various abiotic stresses, such as drought, high salinity, osmotic stress, and cold (Xu et al. 2008), and its overexpression was found to enhance salt, drought, light stress, cold and heat tolerance, and disease resistance in many plants (Muller and Bosch 2015). Upregulation of ERF (transcript id: C_591) in the cold-tolerant genotype indicates its possible involvement in stress regulation. In contrast to this, another ERF (transcript id: 20212) was found downregulated in both the genotypes indicating its non-association with cold tolerance. As per the literature available, there are as high as 120 and 140 ERFs reported in *Arabidopsis* and rice respectively which are involved in stress regulation and signaling pathways of ethylene and jasmonic acid. They are also known to regulate ROS-responsive gene expression (You and Chan 2015; Muller and Munne-Bosch 2015; Zhang et al. 2016).

Two transcripts which were found upregulated in both RRII 105 and RRIM 600 were DREbp1A and HSP. Dehydration-responsive element-binding protein 1A which was found upregulated in both the genotypes is a transcriptional activator that binds specifically to the DNA sequence 5′-AGCCGAC-3′ and bind to the C-repeat/DRE element that mediates cold-inducible transcription. Similarly, a cold-induced *DREB1/CBF* (*C-repeat binding factor*)-like gene, belonging to the A-1 subgroup, was identified by Sakuma et al. (2002). CBF/DREB1 factors play key role in freezing tolerance and cold acclimation in many plants (Nakashima and Yamaguchi-Shinozaki 2006). Recent study on cold stress influenced expression of C-repeat binding factor 1 (isolated from *H. brasiliensis*) indicated that it is involved in enhancing cold tolerance in *Arabidopsis* (Cheng et al. 2015). Heat shock protein (HSP) was also

found upregulated in both the genotypes. HSP or stress proteins are a family of proteins that are expressed in response to stressful conditions (Morimoto et al. 1994; Gupta et al. 2010) and are induced by all types of stresses (Feige et al. 1996; De Maio 1999). In *Arabidopsis* and some other plant species, various stresses like low temperature, osmotic, salinity, oxidative, desiccation, high intensity irradiations, wounding, and heavy metal stresses were found to induce production of HSPs (Swindell et al. 2007). In the context of cold stress, HSPs are involved in cryoprotection of membrane by facilitating refolding of denatured proteins and by preventing aggregation of proteins (Renaut et al. 2006).

Transcript of auxin/H symporter was found upregulated in both the genotypes. Cold stress affects plant growth and development, and its regulation is closely related to the intracellular auxin gradient controlled by polar localization and intracellular trafficking of auxin transporters (Rahman 2013). Many auxin-related genes affected by cold stress had been reported in *Arabidopsis* including auxin transporter genes (Lee et al. 2005). Oxygen-evolving enhancer protein (OEP) was found downregulated under cold stress in both the genotypes. According to Peng et al. (2015), chloroplast and photosynthesis are affected by low temperature stress resulting in reduction in the expression levels of oxygen enhancer protein in paper mulberry. Gao et al. (2009) reported differential regulation of many photosynthesis-related proteins including oxygen-evolving enhancer protein under cold stress in *Thellungiella halophila*, a chilling-tolerant plant. Downregulation of oxygen enhancer protein in both the genotypes indicates reduction in photosynthetic activity under cold stress in *H. brasiliensis* which is quite common. Another transcript found downregulated was a putative transcription factor (TF). As its identity is not known, it may be presumed that this cold-responsive transcription factor may be involved in suppression of its associated genes to prevent the plants from cold injury. This study indicates the association of genes like ferritin, DNA-binding protein, NAC tf and aquaporin with drought tolerance, and ERF with cold tolerance.

Conclusion

In this study, using RNA-Seq technology, abiotic stress-responsive transcripts of *H. brasiliensis* were identified. The digital gene expression analysis indicated the upregulation of 268 transcripts and downregulation of 566 transcripts under drought stress while between control and cold-stressed samples, 961 and 109 transcripts were found up- and downregulated, respectively. Further, quantitative gene expression analysis of 17 prominent drought-responsive transcripts identified from transcriptome sequencing data revealed the existence of association between transcripts such as ferritin, DNA-binding protein, NAC tf, and aquaporin with drought tolerance. An ethylene-responsive transcription factor was found associated with cold tolerance. Further experimental validation of more number of transcripts in *H. brasiliensis* would reveal more number of candidate genes associated with drought/cold tolerance.

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