Quantitative expression analysis of drought responsive genes in clones of *Hevea* with varying levels of drought tolerance

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Received: 19 January 2015 / Revised: 27 February 2015 / Accepted: 3 March 2015 / Published online: 15 March 2015
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Abstract In order to meet the ever rising global demand for natural rubber, cultivation of *Hevea* is being extended to non-traditional regions of India where extreme climatic conditions like drought and low temperature negatively influence the crop performance. In order to ensure maximum productivity, identification of drought tolerant clones of *Hevea* which can cope up with stress and give better crop yield is essential. Several attempts have been made previously to identify genes that are associated with drought tolerance in *Hevea*. In the present study, quantitative expression analysis was made using quantitative PCR for seven drought associated transcripts in four clones of *Hevea* with varying levels of drought tolerance. Among the seven genes studied, Mitogen Activated Protein (MAP) kinase, Myeloblastosis (Myb) transcription factor, C-repeat responsive element/Dehydration Responsive Element (CRT/DRE) binding factor and Nuclear Factor Y subunit A (NFYA) showed a positive association with drought tolerance. Transcripts of ascorbate peroxidase and heat shock protein 70 (HSP 70) did not show any correlation with drought tolerance. Interestingly, catalase gene was found down regulated in all the clones under drought condition. The possible role of these genes based on their level of gene expression in four different clones of *Hevea* with varying levels of drought tolerance is discussed.

Keywords *Hevea brasiliensis* · Drought tolerance · Gene expression analysis · qPCR

Abbreviations

ROS	Reactive oxygen species
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
qPCR	quantitative PCR
APX	Ascorbate peroxidase
CAT	Catalase

Introduction

In India, cultivation of natural rubber (*Hevea brasiliensis*) is being extended to non-traditional regions like North Konkan, parts of Karnataka, Orissa, Madhya Pradesh which are drought prone during summer and north eastern states which are low temperature prone during winter. The drought prone areas experience soil and atmospheric drought, higher atmospheric temperatures combined with high solar light and low relative humidity (RH) during summer which affect the performance of the crop severely (Chandrasekhar et al. 1990; Devakumar et al. 1998). Drought tolerance capacity of *Hevea* has been reported to vary with different clones (Chandrasekhar 1997) and among the clones evaluated in the field conditions, clone RRIM 600 exhibited better growth and yield (Dey et al. 1998). In order to obtain maximum growth and yield performance in such non traditional regions, clones with better drought tolerance capacity have to be identified/developed, for which, it is essential to understand the molecular mechanisms involved in drought tolerance.

Plants undergo various alterations at the physiological, biochemical and molecular levels during environmental constraints like drought. To mitigate the effect of drought stress, plants respond inherently by altering their gene expression. An assortment of genes with diverse functions are induced or repressed by abiotic stresses (Shinozaki et al. 2003; Bartels and Sunkar 2005). Drought tolerance is a complex trait

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that is being influenced by multiple genes, interactions among genes and environmental cues (Tardieu and Tuberosa 2010). Perception of stress signals and activation of complex signaling pathways bring about drastic changes in the cellular gene expression which is a prerequisite for plants to acclimatize under extreme conditions (Kasuga et al. 1999; Tong et al. 2007). It is well established that almost all biotic and abiotic stresses induce oxidative stress by producing higher levels of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\cdot-}$), hydroxyl radical ($\cdot\text{OH}$), singlet oxygen ($^1\text{O}_2$) and nitric oxide (NO). Increased ROS levels under stress conditions induces oxidation of cellular components thus becoming extremely deleterious which damages cellular membranes eventually leading to cell death (Mittler 2002; Dat et al. 2000; Kar 2011).

Studies on the molecular basis of plant responses to drought are critical in furthering our understanding of the regulatory mechanisms for tolerating environmental stresses which can be ultimately utilized to enhance stress tolerance through genetic manipulation (Xiong et al. 2002; Umezawa et al. 2006) or through molecular breeding. Many drought-inducible genes with varying roles have been identified through molecular and genomic analyses of *Arabidopsis*, rice and other species. Recently, the signal transduction and gene networks involved in the drought response and tolerance have been extensively reviewed (Golldack et al. 2014; Nakashima et al. 2014) in which signaling mechanism, transcriptional regulatory network and their cross-talk in various abiotic stress responses are discussed. Both the reviews highlight the central role of transcription factors (TFs) in abiotic stress response and tolerance mechanisms.

In *Hevea*, drought stress has been reported to influence its yield and general performance (Buttery and Boatman 1976; Sethuraj et al. 1984; Sreelatha et al. 2007, 2011). The metabolic activity of clone RR11 105 has been found to be severely affected during drought stress. The biochemical investigations on antioxidant system like super oxide dismutase (SOD) and peroxidase, C-serum invertase activity, C-serum thiols, sucrose and ATP levels also indicated its drought susceptibility (Sreelatha et al. 2007). The gas exchange parameters (CO_2 assimilation rate and stomatal conductance) measured under drought stress indicated that clone RR11 600 had least inhibition during summer proving that it is a drought tolerant clone while the clone RR11 414 was severely affected (Sumesh et al. 2011). The clones RR11 600 and RR11 208 were reported to be surviving well under dry conditions (Singh et al. 2012) and also better yielding (Mydin 2014). All these reports indicate clones RR11 600 and RR11 208 as drought tolerant and RR11 105 as a moderate clone while RR11 414 as drought susceptible clone.

In rubber, various studies were conducted previously to quantify the level of gene expression of several abiotic stress

responsive transcripts (Thomas et al. 2011, 2012 and Sathik et al. 2011, 2012). Thomas et al. (2011) indicated the association of CRT/DRE binding factor (CRT/DRE bf) and ABC transporter protein with drought tolerance by comparing their expression in four different clones of *Hevea*. Genes such as peroxidase, WRKY transcription factor (WRKY) and Late Embryogenesis Abundant 5 (LEA 5) protein were reported to have strong association with drought tolerance in *Hevea* (Sathik et al. 2011; Thomas et al. 2012). Even though tolerance/susceptibility of a particular clone is attributed to many factors, genes/factors that are directly involved in contributing for the drought tolerance have not been identified in *Hevea*. Hence in the present study, attempts were made to quantify the expression of selected genes specifically with regard to drought tolerance using quantitative real time PCR in four *Hevea* clones with varying levels of drought tolerance and to identify the candidate gene for drought tolerance.

Materials and methods

Plant material and stress induction

Four *Hevea* clones, RR11 105 (moderately drought tolerant), RR11 600 and RR11 208 (drought tolerant) and RR11 414 (drought susceptible) were employed for the present study. The clones were generated by budding of seedlings raised from *Hevea* seeds at Rubber Research Institute of India (RR11) farm with clonal buds collected from *Hevea* budwood nursery maintained at RR11. The budded stumps were grown in polythene bags (65×35 cm) in open field conditions of RR11 nursery. After growing for 6 months (two to three whorl stage) in open field conditions, the plants were transferred to glass house before imposing drought treatment. One set of plants from each clone was subjected to water stress by withholding irrigation for 10 days and the other set of plants was watered on alternate days.

Physiological parameters

The degree of impact of drought stress on young plants was assessed by measuring the net CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI-6400 XT), LI-COR, U.S.A. All the gas exchange measurements were made at a constant CO_2 concentration of 400 ppm using a CO_2 injector (LI-6400-01, LI-COR, USA) and at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light intensity using red LED source (with 10 % blue light) attached with the leaf chamber of LI-6400. Leaf samples from these plants were collected in liquid N_2 and stored at -80°C .

Gene expression analysis

Total RNA was extracted from the leaf samples using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Suitable primers were designed with Primer 3 Express (Applied Biosystems, USA) and got synthesized by Sigma. The list of primer pairs used in this study is given in Table 1. Quantitative gene expression analysis was eventually carried out using Light Cycler 480 II, Roche Real Time PCR System. qPCR was performed in a 20 µl reaction mixture containing 1 µl from 1/10 dilution of first-strand cDNA reaction, 125 nM of each primer and 10 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Germany). qPCR was performed by incubating the mixture at 95 °C for 7 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. This was followed by a melt curve analysis (95 °C for 20 s, 60 °C for 1 min and 95 °C for about 5 min). Each experiment was repeated two to three times and each PCR reaction was performed in triplicate with no template controls (NTC). Reaction efficiency of both the target genes and the endogenous control was calculated based on the formula, Efficiency = $10^{(-1/\text{slope})} - 1$. The slope values of the primers were between -3.2 and -3.5. GAPDH was used as endogenous control for the qPCR analysis. The relative quantification (RQ) values were analyzed (using Light Cycler 480 Software; release 1.5.0) and the expression rate of these genes is represented as fold change.

Table 1 List of genes and the corresponding primers used for qPCR analysis

Sl. no.	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
1	NFYA1	TTGGTGAAGGA TGTGTTTGC	ACTCCTCTGGTGGT CAGCTAGAA
2	MAPK	CTGTTGTGTGCA AGCAGGTTTT	CCCTATGTATGACA TGTCGCTCAT
3	CRT/DRE bf	AGTCCCGGCATTG CAAAA	GAGTCAGCGCCGG AGGAT
4	HSP 70	ATGCCTGAGAGCT CAAATTTGC	GGAAACTGCTGGT GGTGTC
5	MYB tf	TGTGACCACTAGA ACACCAACTCA	TCCTGTGCTCTGCC TGATAAAA
6	APX	GTAGAGGATGGTG CCGACAAC	CCAAACTTCCCCAG TTACAAGAAA
7	CATALASE	TCTCCTTTTCAA TGACGCACTTC	GCTGAGAGAACCCC ACTTCCT
8	GAPDH	GCCTGTGATAGTC TTCGGTGTTAG	GCAGCCTTATCCTT GTCAGTGAAC

Data analysis

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

Results and discussion

In order to quantify the magnitude of expression of the genes, four clones of *Hevea* viz. RR11 414 (drought susceptible), RR11 105 (moderately tolerant clone), RRIM 600 and RR11 208 (drought tolerant) were exposed to water deficit stress by withholding irrigation. Stomatal conductance was found declining significantly in all the clones under drought stress measured on 10th day of withdrawing water while it was almost nil in RR11 414 (Fig. 1). Among the clones studied, RRIM 600 had better stomatal conductance under drought stress indicating its drought tolerance capacity. Similarly, A was also found significantly inhibited in all the drought imposed plants of all the clones (Fig. 2). While A was severely affected in relatively susceptible clone RR11 414, it was stable in both the tolerant clones (RRIM 600 and RR11 208). Interestingly, the moderate clone RR11 105 also was found to have photosynthetic activity on par with RRIM 600. These results confirmed the drought effect on these plants.

In order to determine the association of the selected genes to drought tolerance/susceptibility and to evaluate their differential expression under stress conditions in various clones of

Fig. 1 Stomatal conductance (g_s) in irrigated and drought imposed plants of four *Hevea* clones

Fig. 2 CO₂ assimilation rate (A) in irrigated and drought imposed plants of four *Hevea* clones

Hevea, quantitative expression analyses were made by qPCR and the results are given in Fig. 3. Our previous investigations on quantitative gene expression analysis with different set of genes in *Hevea* had confirmed the existence of differential expression under drought stress (Thomas et al. 2012). Among the seven genes investigated in this study, APX was found significantly up regulated in the relatively tolerant clone RRIM 600 and slightly higher in RRII 105 whereas it was found down regulated in RRII 414 and RRII 208. Under drought stress conditions, ROS such as, singlet oxygen (¹O₂), superoxide (O₂^{•−}), hydrogen peroxide (H₂O₂), and hydroxyl radical (•OH) are produced in higher quantities and only the cultivars/genotypes having an improved ROS detoxification process can protect the cells and their organelles from the toxic effect of these species (Apel and Hirt 2004; Mittler 2002; Caverzan et al. 2012). APX is the key enzyme that is involved in detoxification of hydrogen peroxide in plant chloroplasts, cytosol, mitochondria and peroxisomes (Asada 1992). APX activity has been reported to shoot up with other ROS scavenging enzymes such as catalase, SOD and glutathione reductase (GR) (Shigeoka et al. 2002) under stress conditions. The cytosolic *APX1* (Koussevitzky et al. 2008), *Populus* peroxisomal ascorbate peroxidase (*PpAPX*) (Li et al. 2009) and tomato (*Solanum lycopersicum*) thylakoid-bound *APX* (*StAPX*) genes (Sun et al. 2010) have all shown to improve abiotic stress tolerance. Reduction of its expression in drought tolerant clone like RRII 208 needs to be revisited and involvement of other isoforms of this gene need to be investigated further.

The results of gene expression analysis for CAT indicated down regulation in all the four clones analyzed. Catalases have high specificity for H₂O₂ and are unique that they do not require cellular reducing equivalent. CATs have a very fast turnover rate, but a much lower affinity for H₂O₂ than APX. They are known to remove H₂O₂ produced in the peroxisomes

by photorespiration (Noctor et al. 2000), β-oxidation of fatty acids and other enzyme systems such as xanthine oxidase (XOD) coupled to SOD (Sharma et al. 2012). CAT activity has also been found to decrease under conditions that suppress photorespiration, such as elevated CO₂ (Azevedo et al. 1998) and high light intensities (Shang and Feierabend 1999). The reduction in catalase expression found in all the clones in this study need to be further investigated.

Expression of MYB transcription factor (Myb) was found significantly higher in both the tolerant clones (RRII 208 and in RRIM 600), whereas it was found moderately up regulated in RRII 105 which is a moderately tolerant clone. But in clone RRII 414, it got significantly down regulated. This trend coincides with the in vivo performance of clones studied and surely indicates the existence of strong correlation between the Myb tf and stress tolerance. The Myb tfs are known to be induced by various abiotic stress factors such as dehydration, osmotic or salinity stress, ABA, wound signalling pathways, cold stress, salt stress, etc. Myb tfs interact with *cis*-elements in the promoter regions of several stress-related genes through ABA-dependent pathway of stress signalling for the upregulation of abiotic stress responsive genes. Overexpression of Myb tf results in improved drought and salt tolerance in *Arabidopsis* (Ding et al. 2009), osmotic tolerance (Abe et al. 2003), drought stress in tomato (Vannini et al. 2007) and drought and cold tolerance in the apple transgenic plants (Pasquali et al. 2008). Overexpression of a *StMYB1R-1* transgene in potato plants improved tolerance to drought stress while not influencing significant effects on other agronomical traits (Shin et al. 2011). Higher levels of Myb tf in tolerant clones might be attributed to better tolerance found in tolerant clones.

MAP Kinase was found significantly upregulated in RRIM 600 followed by RRII 208 and RRII 105 where it got moderately upregulated when compared to RRII 414 in which no much change was noticed. MAP kinases play pivotal role in signal transduction pathways. Protein phosphorylation is the most important mechanism for controlling many fundamental cellular processes in all living organisms including plants. A specific class of serine/threonine protein kinases, MAP kinases plays a central role in the transduction of various extra- and intracellular signals (Rodriguez et al. 2010). In a cascade of signalling event, MAPK is phosphorylated and activated by MAPK kinase (MAPKK), which itself is activated by MAPKK kinase (MAPKKK). MAPK signaling triggers expression of a cascade of genes that are involved in cellular protection, desiccation tolerance, ROS scavenging enzymes and DNA damage repair by activating corresponding transcription factors (Neill et al. 2002a, b; Hung et al. 2005; Desikan et al. 2000). Involvement of MAPKs in abiotic stress adaptation has been widely reported in many plant species (Yu et al. 2010; Ning et al. 2010). The distinct higher level of expression found in RRIM 600 and RRII 208, moderate up

Fig. 3 Expression analysis of seven genes in four clones of *Hevea* under drought condition. (Fold change in log2 ratio). Log 2 ratio >0 up regulated; <0 down regulated. $\pm$ Error bars indicate standard error of three biological replicates

regulation in RRII 105 and no change in RRII 414 clearly indicate its strong association with drought tolerance. Probably this particular transcript can be employed for further use after proper validation in large number of clones with wide range of tolerance/susceptibility.

The transcript abundance of Nuclear Factor Y subunit A (NFYA) was found more in RRII 600 and RRII 105 while it was moderate in RRII 208. But it got slightly down regulated

in RRII 414. Nuclear Factor Ys are sequence-specific transcription factors with histone-like subunits and they activate transcription by recognizing the CCAAT-box *cis*-acting element present in about 30 % of eukaryotic promoters in a highly specific manner *via* the DNA binding domain present in the NFYA subunit. In the case of plants, the CCAAT-box is present mainly in the promoters of genes involved in photosynthesis. In *Arabidopsis thaliana* NFYA5 transcript is

strongly induced by drought stress in an abscisic acid (ABA)-dependent manner (Li et al. 2008). They bind DNA at CCAAT sites as heterotrimeric complexes composed of single subunits and are reported to be regulating drought responses in many plant species (Stephenson et al. 2007; Hackenberg et al. 2012). NFYA's expression is regulated at transcriptional and post transcriptional level by miR169 (Petroni et al. 2012) and has positive and negative effects on gene expression (González et al. 2012). Upregulation of NFYA in tolerant clones of *Hevea* in this study indicates its strong association with drought tolerance.

Heat shock protein 70 (HSP 70) was found significantly upregulated in both RRIM 105 and in RRIM 600 while it got significantly down regulated in RRIM 414 and RRIM 208. HSPs perform fundamental role in protecting plant against abiotic stresses (Ahuja et al. 2010; Timperio et al. 2008). They act as molecular chaperones to prevent denaturation or aggregation of target proteins as well as facilitate protein refolding. Many studies have established the influence of Hsp 70 genes in enhancing the plant's tolerance to environmental stresses (Ono et al. 2001; Rouch et al. 2004; Zhang et al. 2008; Al-Wahaibi 2011). Expression of HSP 70 in this study did not exhibit any trend with tolerance or susceptibility of clones. HSP being a strong contributing gene for drought tolerance, its down regulation seen in RRIM 208 does not support its relevance as candidate gene for drought tolerance.

Expression of CRT/DRE bf got up regulated in RRIM 105, RRIM 600 and RRIM 208 whereas it got down regulated in RRIM 414. CRT/DRE bf has been reported to be upstream transcription factors that play vital regulatory roles in abiotic stress responses in plants. They bind to promoter *cis*-element CRT/DRE and activate the expression of downstream genes. Among the DRE binding proteins, the DREB2 subfamily has been reported to be induced by drought and high salinity stress. Transgenic *Arabidopsis* plants expressing DREB1 B/CBF1 or DREB1 A/CBF3 under the control of CaMV 35S promoter showed strong tolerance to freezing, drought and high salinity stresses suggesting that DREBs/CBFs target multiple genes (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999). Microarray analyses have indicated that these factors target stress inducible genes containing the conserved DRE or DRE-related core motifs in their promoter regions (Maruyama et al. 2004). Significant upregulation of this particular transcript in the tolerant clones as well as moderate clone and significant down regulation in the susceptible clone indicate its strong association with drought tolerance in young plants of *Hevea*.

In this study, seven stress responsive transcripts selected specifically from the data of *Hevea brasiliensis* were validated for their association with drought stress tolerance by quantifying its expression in four different clones among which two are drought tolerant, one clone moderate and one drought susceptible clone. The data of gas exchange parameters also

supported the tolerance level found in field conditions. Previous reports on *Hevea* also indicated RRIM 600 and RRIM 208 as tolerant clones that are suitable for drought and cold prone regions (Priyadarshan et al. 2000; Sumesh et al. 2011; Mydin 2014). From the gene expression analysis data, four transcripts were identified to have stronger association with drought tolerance in clones of *Hevea* with different levels of drought tolerance.

The clone suitable for drought prone regions should have a better ROS scavenging system. As it is known that increased levels of ROS under drought conditions can induce oxidation of cellular components that damage cellular membranes eventually leading to cell death, only a clone with better ROS scavenging system can withstand the severity of summer in such drought prone regions. Among the various free radicals, H_2O_2 has been known as a potential ROS which is highly damaging especially in the presence of transition metal like Fe^{2+} . H_2O_2 triggers MAPK signaling cascade that modulates expression of corresponding genes *via* activation of transcription factors (Neill et al. 2002a, b; Hung et al. 2005). These genes are basically involved in cell wall protection, desiccation tolerance, production of ROS scavenging enzymes and DNA damage repair (Desikan et al. 2000). Among the four transcripts having stronger association with drought tolerance, transcript of MAPK exhibited a perfect correlation with drought tolerance according to the level of drought tolerance they exhibit in the field condition. The level of MAPK expression was found 13, 7.06 and 3.6 fold increased in RRIM 600, RRIM 208 and RRIM 105 respectively and no change was noticed in clone RRIM 414 conforming with our previous findings that RRIM 600 and RRIM 208 are drought tolerant (Priyadarshan 2003) and RRIM 105, a moderate performer.

In the case of MYB tf, 12, 2 and 1.6 fold upregulation was found in RRIM 208, RRIM 600 and RRIM 105 respectively while there was down regulation in RRIM 414. The upregulation of Myb tf in clone RRIM 600 does not seem to be in proportion to its level in tolerant clone RRIM 208, necessitating further investigation in more number of clones with varying levels of drought tolerance. In the case of CRT/DRE bf, higher magnitude of upregulation was found in RRIM 600 (7.4 fold) and RRIM 105 (7.3 fold) while it got only 3.2 fold increase in RRIM 208. But its expression got down regulated in the drought susceptible clone RRIM 414 (0.2 fold times). In the case of transcript NFYA also, there was 6.6, 1.8 and 3.9 fold upregulation in clones RRIM 600, RRIM 208 and RRIM 105 respectively while there was 0.8 fold reduction in clone RRIM 414. Among the four transcripts, though all of them got up regulated in tolerant clones and moderate clone, only MAPK exhibited a stronger correlation with the drought tolerance potential of the clones studied. In the case of APX and HSP 70, no definite trend could be observed while catalase got fully down regulated in all the clones. Catalase activity has been reported to be suppressed under elevated CO_2 and high

light intensity conditions. The overall down regulation of catalase in all the clones also necessitates further investigation.

This study focuses on expression pattern of seven drought responsive transcripts in young *Hevea* plants experiencing drought stress among which MAPK exhibited a strong correlation with drought tolerance. But to identify more drought tolerant genes, it is imperative to use a tool by which more number of genes could be screened at one go. The recent technological innovations in new generation sequencing methods such as Illumina column sequencing and larger screening method like microarray can be employed to identify the overall drought responsive transcripts. Moreover, more number of clones with varying levels of drought response/tolerance has to be incorporated in future experiments to narrow down on to the genes contributing for drought tolerance. It would also be possible to identify various isoforms of genes through new generation sequencing technology and subsequent validation of each isoform by microarray and quantitative PCR in order to establish their association with drought tolerance. More such modern approaches are necessary to arrive at the candidate gene/marker for drought tolerance to accomplish the target of identifying/developing drought tolerant clones suitable for non-traditional rubber growing regions with adverse climatic conditions.

Acknowledgments The authors wish to thank Dr. R. Krishnakumar (Joint Director, RRII) and Dr. James Jacob (Director of Research, RRII) for their constant support and encouragement throughout the course of the work. Ms. Lisha P Luke is grateful to RRII, Rubber Board for the Senior Research Fellowship and financial assistance.

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