Molecular investigations on abiotic stress tolerance and development of transgenic plants of *Hevea brasiliensis*

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To meet the increasing demand for natural rubber, cultivation of *Hevea brasiliensis* is being extended to non-traditional areas where the plants are exposed to various environmental constraints such as drought, extremes of temperature etc. which limit the growth and productivity of the crop. To overcome this, it is imperative to identify or evolve suitable clones that can tolerate these adverse conditions. To achieve this, it is essential to identify genes/regulatory factors that are responsible for stress tolerance and to evolve suitable region-specific clones by transgenic approach.

Plants have the ability to respond to environmental changes by altering the expression of complex gene networks through sensing environmental cues, which result in successful adaptations to such adverse conditions. To understand stress adaptation processes, transcript profiling of stress response in both tolerant and sensitive clones under different abiotic stresses and quantification of gene expression were attempted. Simultaneously, transgenic approach was also adopted to over-express MnSOD gene which would contribute to detoxify the effect of reactive oxygen species (ROS).

In this report, we present data of the work carried out with regard to transcriptome profiling using Differential Display-RT PCR, Suppressive Subtraction Hybridization, microarray and quantitative PCR in drought as well as low temperature stressed young plants and about the performance of young transgenic plants under *in vitro* stress conditions. From differential display reverse transcript PCR (DDRT-PCR) study on drought imposed polybag plants of RRII 105 (drought sensitive) and RRIM 600 (relatively drought tolerant), out of 111 differentially expressed bands obtained, 37 transcripts showed significant homology with reported genes, while 74 transcripts were found to be novel. NAC transcription factor, Ferredoxin III, uvr ABC system protein A, zinc finger protein, yrdc family protein, general secretory pathway protein, cation channel family protein, peroxisomal biogenesis factor 3-1, peroxin 3 family

proteins, etc. were some of the known drought responsive transcripts identified from this study.

The macroarray hybridization analysis performed with selected 84 gene transcripts (drought and non drought responsive transcripts) indicated the down regulation of gene transcripts such as HbDRT 50, HbNRG 6 and HbNRG 7 and up-regulation of HbDRT 73 and HbTPD 29 in drought sensitive clone. In contrast, while HbNRG 33 was down-regulated in drought tolerant *Hevea* clones, HbNRG18 was upregulated. The quantitative expression analysis performed with real time PCR indicated the upregulation of HbNRG18 and HbDRT5b in both irrigated and droughted samples of RRIM 600 and RRII 208. This confirms the association of HbDRT5b and HbNRG18 with drought tolerance as both RRIM 600 and RRII 208 are drought tolerant clones.

Low temperature induced transcript profiling was performed with two relatively stress tolerant *Hevea* clones (PR 261 and RRII 208). Sequencing of 131 cDNA clones (59 down-regulated and 72 up-regulated cDNAs) revealed the existence of 110 unique sequences comprising of 13 clusters/contigs and 97 singletons. Several differentially expressed genes *i.e.*, catalase, phosphatidylinositol/ phosphatidylcholine transfer protein, NADH dehydrogenase, Myb transcription factor, downward leaf curling protein, epimerase/dehydratase, Na^+/H^+ antiporter, chloroplast Ycf2 and chloroplast FtsH protease involved in cold adaptation process were identified along with unique transcripts.

Sequencing of the cold stressed (leaf samples) subtracted library produced 156 cDNA clones (ScDNA) which upon contig analysis, yielded 31 contigs containing 90 clones (2 to 8 clones per contig) and 66 singletons. The ESTs obtained were grouped into (1) osmoprotection/detoxification, (2) oxidoreductases, (3) cell wall and polysaccharide metabolism, (4) protein/aminoacid metabolism (5) transport and secretion and (6) transcription factors. The reverse northern analysis of 96 clones revealed over-expression of genes such as carbonic anhydrase, glutathione peroxidase, metallothionein, chloroplastic Cu/Zn SOD, serine/threonine protein kinase, transcription factor, DNA binding protein etc. As the representation of the metallothionein gene (Hev-MT) (which acts as a reactive oxygen species scavenger) was found to be much higher in the cold stressed leaf samples, attempts were made to

clone it into an expression vector and to successfully express the protein under the control of T7 promoter in a bacterial system.

In another attempt, stress associated genes such as WRKY transcription factor (WRKY tf), ABC transporter protein (ABCT), transcription factor MBF (TfMBF), LEA 5 protein, CRT/DRE binding factor (CRT/DRE bf), glutathione peroxidase (GPX), a hypothetical protein (33HP), Dna J protein and peroxidase were subjected to qPCR analysis in cDNA derived from mRNA of cold treated leaf samples. Out of the nine genes analyzed, both LEA5 protein and peroxidase were found to be up-regulated by 8.14 and 5.8 fold in the low temperature treated plants of RRII 105 and RRIM 600 respectively. Though the expression of transcription factor MBF and hypothetical protein (HP33) were 2.66 and 3.26 fold higher in low temperature treated plants of RRII 105, they were down-regulated in RRIM 600. In contrast, ABC Transporter protein (ABCT) was 3.67 fold higher in cold treated plants of RRIM 600 and was lesser in RRII 105. These results indicate that both peroxidase and LEA5 protein were upregulated under cold conditions in both the clones. However, ABCT expression was more only in RRIM 600 indicating the association of ABCT in rendering cold tolerance.

Transgenic plants integrated with a gene coding for mitochondrial targeting MnSOD were developed in *H. brasiliensis* (clone RRII 105) through *Agrobacterium* mediated gene transfer and multiplied by bud grafting. Experiments were performed to assess the MnSOD transcript abundance and intrinsic drought tolerance traits of the six month old bud grafted plants of clone RRII 105. The transgenic plant, L1 maintained relatively better PS II activity upon exposure to drought than L2, bud grafted wild and the somatic plants of RRII 105. Though the maximum potential photochemical efficiency of PS II was not altered under drought stress, the effective PS II quantum yield and mid–day leaf water potential were less inhibited in L1 than in the other plants. The drought mediated reduction in photosynthetic oxygen evolution rate was smaller in L1 than the others. The SOD activity was 35% higher under normal condition and 31% higher under drought condition in L1 than the bud grafted wild plants of clone RRII 105. Though there was an increase in SOD activity and H_2O_2 content in L1, corresponding increase was not observed in peroxidase activity. RT-PCR and northern analysis indicated a higher MnSOD transcript level in the stressed transgenic plants. The level was higher in L1 than L2, whereas, the

expression was very less in the bud grafted control plants and the non-transgenic somatic plants of clone RRII105. The results indicated the significant reduction in oxidative stress in transgenic plants over-expressing SOD. The efficient ROS scavenging activities in L1 imparted better photosynthetic efficiency and thus protected the plants from stress induced photo-oxidative damage.