Novel insights into cold resistance in *Hevea brasiliensis* through coexpression networks

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Abstract

Hevea brasiliensis, a tropical tree species from the Amazon rainforest, is the main world source of natural rubber. Due to the high pressure of fungal diseases in hot humid regions, rubber plantations have been moved to “escape areas”, which are dryer and present lower temperatures during the winter. We carried out a study with a primary (GT1) and a secondary (RRIM 600) young rubber tree clones, which present different cold tolerance strategies, to analyze gene expression regulation while under 24 hours of cold exposure (10°C). Together with traditional differential expression approaches, a RNA-seq gene co-expression network (GCN) was established with 27,220 genes that were grouped into 205 gene clusters. Through the GCN, we could relate the predominance of rubber tree molecular responses on cold stress to 31 clusters, which were divided in three GCN modules: a down-regulated group with 16 clusters and two up-regulated groups with twelve and

three clusters, respectively. The hub genes of the cold-responsive modules were identified and analyzed as well. Considering the three modules and hub genes identified, the general *Hevea* response to short term cold exposure involves a complex regulation of Jasmonic Acid (JA) stress response and programmed cell death (PCD), up-regulation of Ethylene responsive genes and relaxation on the inhibition of florigen genes. As a result of the GCN strategy applied in this study, we could not only access single DEGs related to *Hevea* cold responses, but also provide insights into a deeper cascade of associated mechanisms involved in the response to cold stress in young rubber trees. Our results may represent the species genetic stress responses developed during its evolution since the varieties chosen for this work are genotypes selected during the early years of rubber tree domestication. The understanding of *H. brasiliensis* cold response mechanisms can greatly improve the breeding strategies for this crop that has such a narrow genetic base, is being impacted by climate change and is the only source for large scale rubber production.

1 Introduction

Hevea brasiliensis, mainly known as rubber tree, is an allogamous perennial tree species native from the Amazon Rainforest, belonging to the Euphorbiaceae family (Priyandarshan and Clément-Demange, 2004). Although rubber trees are the main world source of natural rubber (Pootakham et al., 2017; Gonçalves and Fontes, 2009; De Faÿ and Jacob, 1989), these tree species are a recent crop, being still in domestication (Priyadarshan and Clément-Demange, 2004). The dispersal and domestication of rubber trees around the world was based on only about 20 seedlings that were introduced in Southeast Asia in the late 19th century. These seedlings were the only survivors of a collection of thousands of seeds from the Amazon Basin, originating the selection of elite trees and controlled hybridization for reproduction in Hevea. Thus, until the present day, almost all commercial varieties of *H. brasiliensis* planted are derived from these seedlings, therefore, their genetic variability is quite narrow (Gonçalves and Fontes, 2009; Souza et al., 2015).

Although the Amazon Basin offers ideal conditions for rubber tree cultivation, the occurrence of the fungal South American Leaf Blight (SALB) disease in the region hinders the establishment of rubber tree plantations in the area. To overcome this situation, Brazilian rubber tree plantations were moved to sub-optimum or escape areas such as the Brazilian Center and South East regions, which are dryer and present lower temperatures during the winter (Priyadarshan et al., 2009). The South East Asian countries, which have similar Amazonian

climate conditions and successfully avoided the introduction of SALB, are the major rubber producers worldwide. Nevertheless, these Asian plantations have already suffered heavy losses because of other fungal disease outbreaks (Priyadarshan and Gonçalves, 2003; IRCo, 2019; Pornsuriya et al., 2020), being expanded to suboptimal areas as well, such as Southern China and Northeast India (Priyadarshan et al., 2009). Thus, rubber tree varieties adapted to areas with new edaphoclimatic conditions are of key interest.

Among the stressing factors characteristic of the escape areas, low temperatures heavily affect *H. brasiliensis* development, causing damage to leaves and latex production (Priyadarshan et al., 2005). For a rubber tree plantation, a prolonged low temperature period or a frost occurrence may cause the plants' death (Priyadarshan et al., 2009). Cold stress triggers a massive reprogramming of plants' gene expression to adjust their metabolic processes to cope with the cold environment (Theocharis et al., 2012). In order to promote breeding towards cold tolerance in *H. brasiliensis*, a deeper understanding of the species cold response mechanisms is necessary.

In the last years, due to the impact of low temperatures in rubber tree plantations and rubber production, there is an increase of studies about *H. brasiliensis* molecular cold stress responses, especially through comparative transcriptomics (Deng et al., 2018, Gong et al., 2018, Mantello et al., 2019). The modification of Hevea gene expression under cold treatments has already enabled the identification of thousands of differentially expressed genes (DEGs) (Mohamed Sathik et al, 2018; Deng et al., 2018; Cheng et al., 2018). However, despite these comparative transcriptome analysis between resistant and cold-sensitive clones show that cold is a key factor in latex production, more precise information on the main biological pathways and metabolic processes involved in the cold response has not yet been elucidated in rubber trees. Recently, Ding et al. (2020) modeled a gene coexpression network (GCN) and associated a network module with cold treatment. Although the cold response mechanisms were not assessed, the GCN created represented a rich source of data for investigating cold response.

Here we carried out a study based on a GCN modeled with transcriptomic data from two of the earliest *H. brasiliensis* clones to identify novel patterns between the genes involved in *H. brasiliensis* response to cold stress, aiming to supply a deeper insight on how young rubber tree plants cope with low temperatures. The use of the transcriptome from a cold experiment with two genotypes, one resistant and one susceptible, allowed the identification of central genes in the created networks underlying cold stress in rubber trees. Thus, this study is one of the pioneers in

the association of molecular mechanisms of Hevea through GCNs, being the first one to unveil rubber tree cold stress responses in important commercial varieties.

2 Material and Methods

2.1 RNA-Seq Experiment

In this work, two genotypes of *H. brasiliensis* recommended for escape areas (Mantello et al., 2019), GT1 and RRIM600, were evaluated. Under low temperatures, RRIM 600 presents an avoidance physiological strategy while GT1 balances between photosynthetic/growth activities and stress response (Mai et al., 2010; Mantello et al., 2019). The cold experiment consisted of exposing three six-months-old seedlings of each genotype to a temperature of 10ºC for 24 hours (12h light/12h dark). During this period, leaf material was collected at a time series of 0 hours (0h - control), 90 minutes (90m), 12 hours (12h), and 24 hours (24h). The RNA extracted from these samples was employed to construct cDNA libraries, and it was subsequently sequenced using an Illumina Genome Analyzer IIx with the TruSeq SBS 36-Cycle Kit (Illumina, San Diego, CA, USA) for 72 bp PE reads. The transcriptome assembled by Campos Mantello et al. (2019) was used for the analyzes of the present work.

2.2 Bioinformatics and Differential Gene Expression Analysis

The reads of each genotype evaluated with their respective times and replicates of treatment were mapped with the Bowtie2 aligner (Langmead & Salzberg, 2012) in the reference genome (Tang et al., 2016). The estimated abundance of reads for each sample was calculated by RSEM (Li & Dewey, 2011) and, subsequently, a matrix was constructed with expression values and abundance estimates between samples and replicates. Genes with a minimum of 10 counts per million (CPM) in at least three samples were maintained for further analyses. These samples were normalized by the quantile method and transformed into log2 counts per million (log2cpm) by the edgeR package (Robinson et al., 2010).

From the normalized data, a principal component analysis (PCA) was performed using the R software, to explore the data and verify the behavior of the samples. For DEG analysis, the datasets from GT1 and RRIM600 were combined in an attempt to create an unique *H. brasiliensis* expression dataset. An empirical Bayes smoothing method implemented in the edgeR package

(Robinson et al., 2010) was used to identify DEGs between cold exposure sampling times, in which the following comparisons were performed: (i) 0h vs 90m; (ii) 0h vs 12h; (iii) 0h vs 24h; (iv) 90m vs 12h; (v) 90m vs 24h; and (vi) 12h vs 24h. The p-values were adjusted using a Bonferroni correction and genes with p-value ≤ 0.05 were considered DEGs.

Rubber tree DEGs were compared between the time-points evaluated with Venn plots constructed with the venn R package (Dusa, 2017). For all genes, we selected the respective annotation obtained with the Trinotate v2.0.1 pipeline (Bryant et al., 2017; https://trinotate.github.io/). Untranslated transcripts were searched against the SwissProt/UniProt database using BLASTX and filtered with an e-value of 1e-5 and placed into a tab-delimited file. Transcripts were also associated with Gene Ontology (GO) (Harris et al., 2004).

2.3 Gene Coexpression Network (GCN) Analysis

The GCN was inferred through R Pearson correlation coefficients with a cut-off of 0.8 (Van Noort et al., 2004). The R values of the GCN were normalized using the highest reciprocal rank (HRR) approach limited to the top 10 connections, and the heuristic cluster chiseling algorithm (HCCA) was used to organize the network into communities (Mutwil et al., 2010). Only the top 10 Pearson R correlation coefficients considering a 0.8 cut-off were retained for each gene, being disconsidered the genes disconnected.The HRR networks and the HCCA results were visualized with Cytoscape software v.3.8.0 (Shannon et al., 2003).

2.4 Cold Stress Association

The number of DEGs in the clusters was used to pinpoint the most probable groups involved in *Hevea* cold stress response. Hub genes in the selected clusters were analyzed using the node degree distribution (DD), betweenness centrality (BC) and stress centrality distribution (SC) parameters of the networks that were obtained using the Cytoscape software v.3.8.0 (Shannon et al., 2003). Genes with high DD that presented high values of BC and SC were considered highly influential in the selected clusters' co-expression dynamics. If a highly connected node has low BC and SC values it was not selected as a hub. For each network cluster, at least one hub gene was identified, and they were evaluated by their functional annotation and/or the genes they interacted with.

For identifying molecular associations with cold stress response, we selected the clusters that, together, account for 70.2% of the DEGs identified. We considered as associated clusters those that contain at least 50% of their genes as DEGs, expanding the module across these clusters' neighbors considering a minimum required frequency of 20% of DEGs inside a cluster (Supplementary Table 6 - Access in https://docs.google.com/spreadsheets/d/1UynT3MQvcdZVKOEBUKuAwpMaYVZ17Ds3v_BdCU9hAWE/edit?usp=sharing).

2.5 Functional Enrichment

As functional coherence between co-expressed genes belonging to the same cluster is expected, the analysis of enrichment of GO terms was performed in each cluster identified by the HCCA. Overrepresented GO terms in the clusters were determined with BiNGO plugin v.3.0.4 (Maere et al., 2005) using a customized reference set created from the transcriptome annotation data. A hypergeometric distribution and false discovery rate (FDR) < 0.05 were used in the analyses. GO enrichment analyses were performed with EnrichmentMap plugin v.3.3.1 (Merico et al., 2010) with FDR Q-values < 0.05 and the BiNGO output files. Both plugins were used in the Cytoscape software v.3.8.0 (Shannon et al., 2003).

3 Results

3.1 Bioinformatics and Differential Expression Analysis

Approximately 530 million paired-end (PE) reads were obtained for the RRIM600 genotype and 633 million for the GT1 genotype. After quality filtering, around 933 million PE reads were retained and used to assemble the transcriptome in the Trinity software. A total of 104,738 isoforms (51,697 genes) were identified with sizes between 500 bp and 22,333 bp, with an average of 1,874 bp and N50 of 2,369. From these genes, we could identify a total of 74,398 unique proteins related to 9,417 different GOs (Supplementary Table 1 - Access in https://docs.google.com/spreadsheets/d/1AkQICDVW60e6Ftrdfr8QjHVx814Ve22qGNvUKkjkBqI/edit?usp=sharing).The DEG analyses, resulted in a final amount of 30,407 genes that, analyzed through a PCA,revealed a distinct profile of samples belonging to each different genotype (Figure 1A).

The amount of DEGs was different in each period comparison (Table 1), as there were different profiles of genes associated with each period analyzed (Supplementary Table 2 - Access in https://docs.google.com/spreadsheets/d/1oGunmT7okMwkCnXkxvV1XkaTmVSoxjRc4z04hi4OLvI/edit?usp=sharing). Although there were evident intersections among the tests established (Figure 1B), it is clearly observed a joint mechanism of several genes for cold response. About 93% of the down-regulated genes and 96% of the up-regulated genes could be annotated. Regarding the GO enrichment analysis, the full DEG set was enriched for response to stress and response to chitin terms. The down-regulated group of genes did not present any overrepresented category. In contrast, the up-regulated genes' group was enriched for the following terms: response to chitin, defense response, response to wounding, cell death, photoprotection, calcium ion transmembrane transporter activity and integral to plasma membrane.

Table 1. Number of *Hevea brasiliensis* differentially expressed genes (DEGs) that were modulated by cold stress.

Time period comparison	**DEGs**	**Up-regulated DEGs**	**Down-regulated DEGs**
0h vs 90m	32	32	0
0h vs 12h	673	558	115
0h vs 24h	1,208	852	356
90m vs 12h	526	455	71
90m vs 24h	917	700	217
12h vs 24h	18	15	3
(0h + 90min) vs (12h + 24h)	2,436	1,575	861

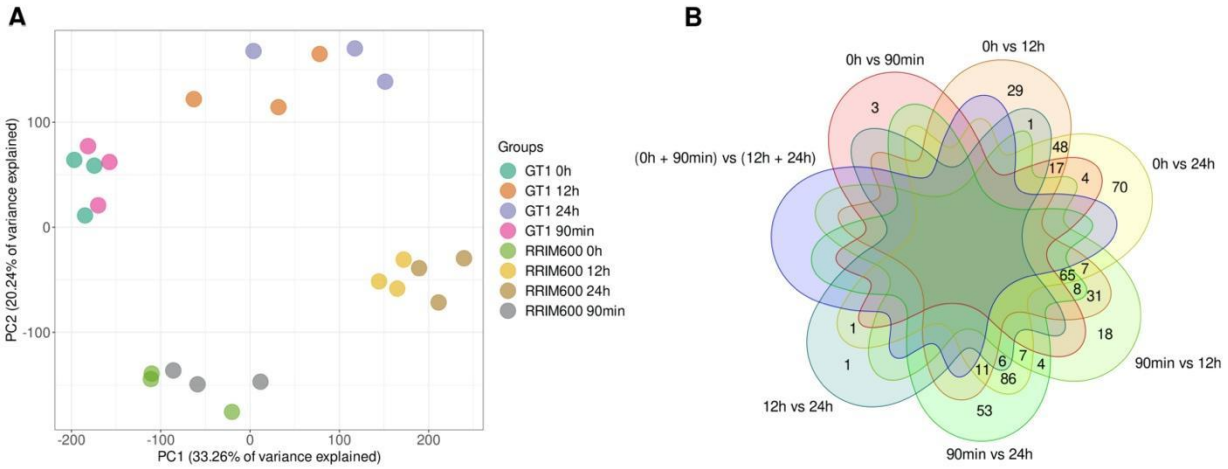

Figure 1. (A) Principal component analysis (PCA) scatter plot performed on the first two principal components (PCs) of rubber tree transcriptome data (GT1 and RRIM600 genotypes). Each point represents a different sample, colored according to the respective genotype. (B) Venn diagrams for differentially expressed genes (DEGs) found on the conditions established.

After 90 minutes of cold treatment, only thirty-two transcripts were characterized as DEGs, all of them were up-regulated during the plantlets' early response to cold stress. Of these, 26 genes were successfully annotated (Supplementary Table 1 - Access in https://docs.google.com/spreadsheets/d/1AkQICDVW60e6Ftrdfr8QjHVx814Ve22qGNvUKkjkBqI/edit?usp=sharing). The annotated DEGs are known to be induced by cold as well as other abiotic stressors, such as drought and salt stresses. They are involved with cell wall tightening processes, growth inhibition, oxidative-stress protection, calcium (Ca2+) signaling and cold-induced RNA processing. The late response, considered as the interval between twelve hours and 24 hours of cold treatment, contained 18 DEGs, including genes that stimulate an increase in the membrane diffusion barriers and cell wall modifications.

One hundred and thirty eight DEGs were annotated with the GO term DNA-binding transcription factor activity, being 103 up-regulated and 35 down-regulated. All of them, but one, were grouped in the cold stress response gene clusters (see below). An analysis of overrepresented GO terms in the 138 DEGs showed that they are involved with the regulation of cellular metabolic processes: negative and positive regulations are modulated by the up- and down-regulated groups respectively. While the up-regulated genes are responsive to stress and other stimuli, the down-

regulated genes promote plant development and growth. All transcripts annotated as members of the Apetala2/Ethylene-responsive transcription factors (AP2/ERF) family and that were identified as DEGs (38) were up-regulated. DEGs annotated as transcriptional repressors of Jasmonic Acid (JA)-mediated responses (i.e.: TIFY/JAZ proteins) were also up-regulated.

Non-specific serine/threonine Protein kinases (EC: 2.7.11.1) were the most represented enzymes among the annotated DEGs: 165 were up-regulated while 57 were down-regulated. The up-regulated kinases have an overrepresentation of GO terms related to reproduction processes, response to Abscisic acid (ABA) stimulus, calmodulin binding and signal transmission, while the down-regulated kinases were enriched for post-translational protein modification. The second most differentially expressed enzyme-types were RING-type E3 Ubiquitin Transferases (EC: 2.3.2.27) with 42 and 11 genes up- and down-regulated respectively. The RING E3 ubiquitin proteins are essential for the Ubiquitin Proteasome System (UPS) due to their roles in different developmental processes and stress responses, as these proteins recruit the substrate to be degraded. Cho et al., 2017). In addition, genes similar to enzymes from the Gibberellin (GA) biosynthetic pathway were down-regulated, while enzymes that attenuate JA-mediated stress response were up-regulated. Interestingly, enzymes that synthetize JA precursor molecules (Oxylipins) were up-regulated as well.

3.2 Rubber Tree GCN Analysis

From the transcriptome data of GT1 and RRIM600 genotypes, an HRR network was modeled, composed of 27,220 nodes across 50,650 connections (edges), in order to represent the cold response of the species (Figure 2A). The major connected component within the network is composed of 25,608 nodes (94.1%). There were also other 626 components for the remaining 1,592 genes, with a gene quantity inside each component ranging from 2 to 11 nodes. In order to identify putative gene associations within network structure, we analyzed the network with the HCCA, identifying 832 clusters (Figure 2B). From these sets of genes, 626 corresponded to the disconnected components (mean cluster size of ~3), and 206 to the core GCN structure (mean cluster size of ~124), comprising clusters with sizes ranging from 45 to 280 genes (Supplementary Table 3).

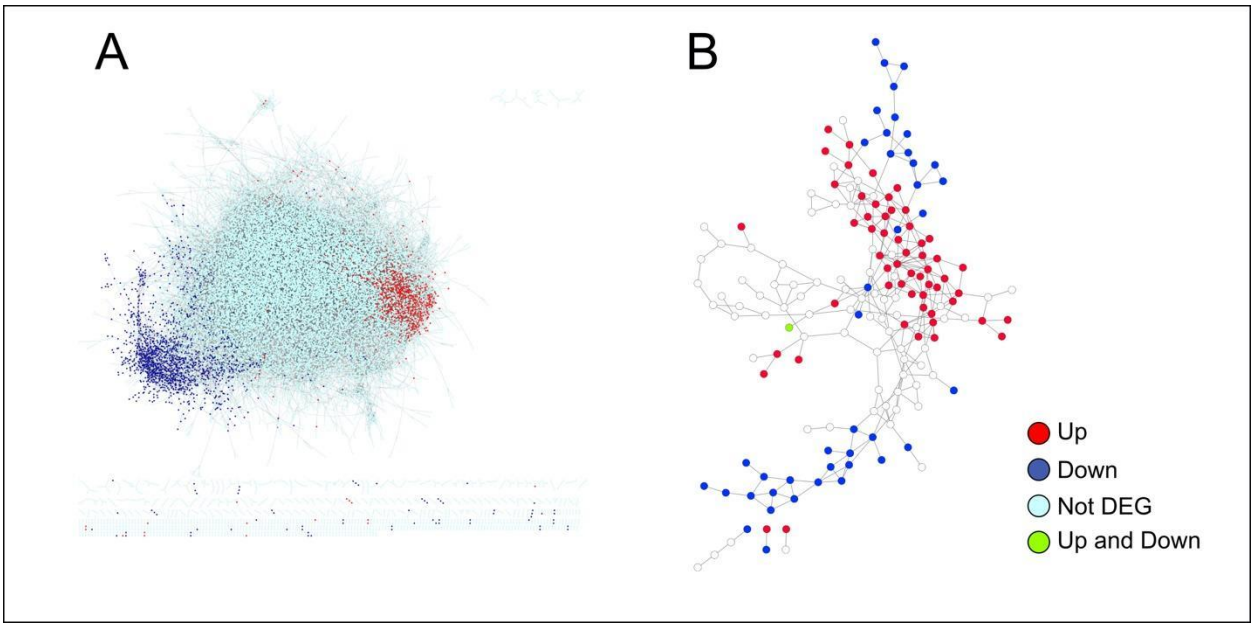

Figure 2. (A) Gene coexpression network (CGN) modeled; each node represents a gene colored according to the differential expression analysis, and each connection a minimum R Pearson correlation coefficient of 0.8 (corrected with the highest reciprocal rank approach). (B) GCN network contracted according to the groups identified by the heuristic cluster chiseling algorithm (HCCA); each node represents a cluster colored according to the presence of differentially expressed genes.

From the 206 main clusters, 39 (14%) had GO terms that were significantly enriched, as highlighted in Figure 3 for biological process GO terms. Response to stress was the most overrepresented term among clusters, encompassing 1,039 genes across 9 groups, which was expected considering the cold experimental design. Two of these clusters (c14 and c28) were enriched for developmental processes as well, indicating the tight correlation between environmental stimulus and growth. Interestingly, we also observed seven clusters enriched for virus replication and transposition.

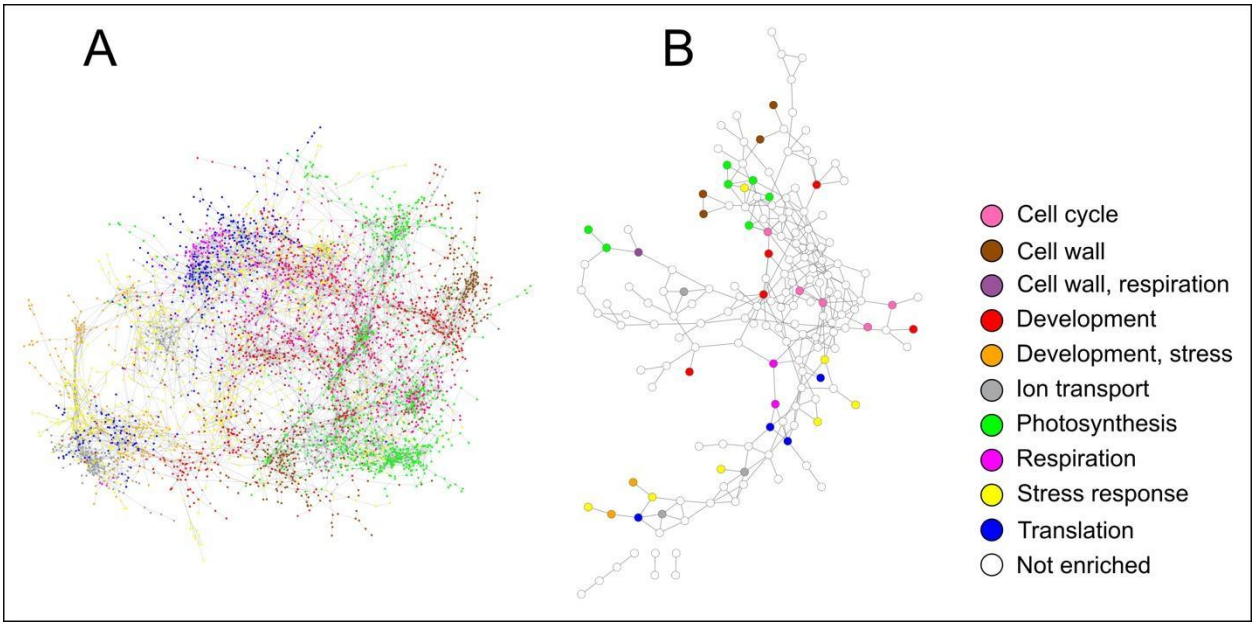

Figure 3. (A) Gene coexpression network (CGN) modeled; each node represents a gene colored according to the enriched gene ontology (GO) term found in the related cluster, and each connection a minimum R Pearson correlation coefficient of 0.8 (corrected with the highest reciprocal rank approach). (B) GCN network contracted according to the groups identified by the heuristic cluster chiseling algorithm (HCCA); each node represents a cluster colored according to the GO term enriched.

The gene centrality measures (Supplementary Table 3 - Access in https://docs.google.com/spreadsheets/d/1lg0DBV6vXIO7zTrgqNdqpBoJJYSDlpurZ_3mUc5Kmr4/edit?usp=sharing) showed that, of the total of 27,220 genes inserted into the network, there were only 507 genes with the maximum permitted connections (ten). One of them, annotated as "probable cytochrome c biosynthesis protein" (CCBS), also presents the highest values of SC and BC centralities, indicating that this gene exerts great influence in the network. The gene belongs to cluster c8, which was enriched for photosynthesis, but also for cellular respiration and transposition. By evaluating the inter cluster connections in the HCCA-based contracted network (Figure 3B and Supplementary Table 4 - Access in https://docs.google.com/spreadsheets/d/1-peHUKs684MmvI74qUyinc7m9MF9QiyIqX_xHgmIK7U/edit?usp=sharing), we found that the cluster with the highest number of connections is c117 (nine edges), being enriched for developmental processes.

3.3 Gene Clusters Mostly Related to Cold Stress Response

Even though the DEGs identified were dispersed among the GCN (Figure 2A), specific patterns could be observed in several clusters. It was possible to identify 1,751 DEGs involved in clusters associated with cold stress response (Supplementary Table 5 - Access in https://docs.google.com/spreadsheets/d/1LU2geL64NmrEPg88SwoTZ5JYPhevc72y32pD5UvVzCA/edit?usp=sharing) (Figure 5 A). Expanding the module across these clusters´ neighbors, we could establish three GCN modules for the 31 clusters selected: (i) a down-regulated group with 12 clusters, (ii) an up-regulated group with 11 clusters; and (iii) an up-regulated group with 3 clusters (Figure 4 B). Even though there were additional clusters containing DEGs, only the most pronounced ones were used for novel inferences. Among the DEGs in the selected clusters, 480 genes were annotated with enzyme code (EC) numbers, being 339 up-regulated and 141 down-regulated transcripts, and 180 DEGs were assigned to KEGG pathway maps.

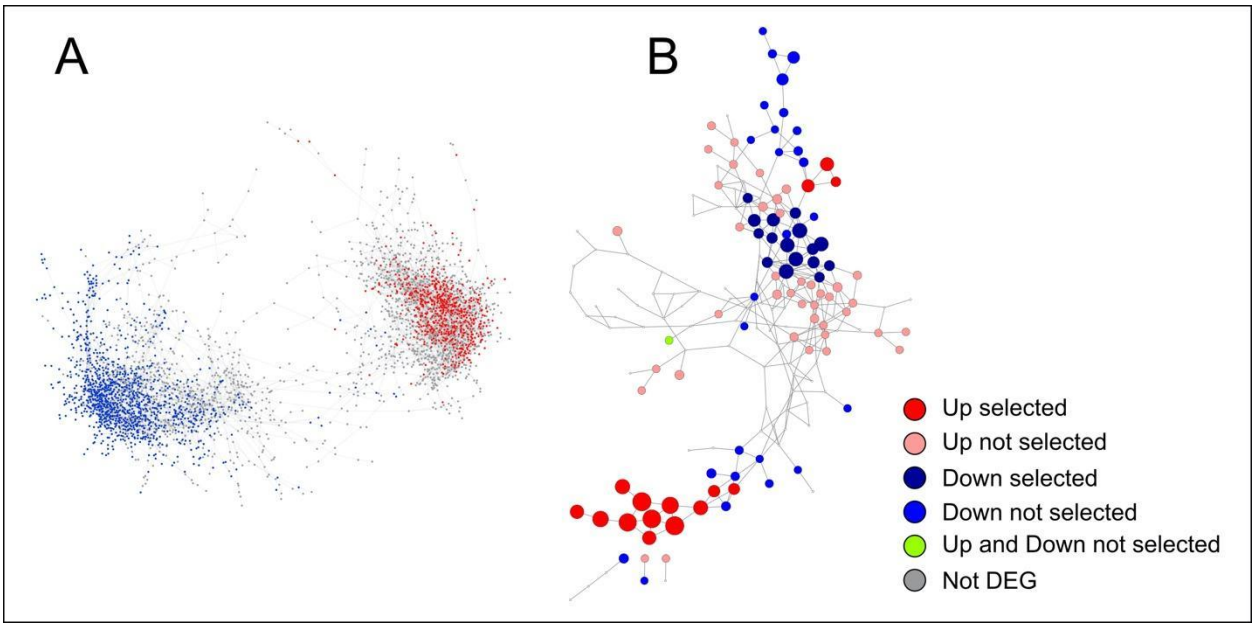

Figure 4. (A) Gene coexpression network (GCN) colored according to the modules considered as associated with cold response; each node represents a gene, and each connection a minimum R Pearson correlation coefficient of 0.8 (corrected with the highest reciprocal rank approach). (B) GCN network contracted according to the groups identified by the heuristic cluster chiseling algorithm (HCCA); each node represents a cluster colored according to modules selected and sized according to the proportion of DEGs (Supplementary Table 5 - Access in https://docs.google.com/spreadsheets/d/1LU2geL64NmrEPg88SwoTZ5JYPhevc72y32pD5UvVzCA/edit?usp=sharing).

The down-regulated module (i), as a whole, did not present any overrepresented GO category; however the cluster c68, by itself, was enriched for cell cycle. In this module, we could identify nineteen hub genes. Almost all hubs were late-responsive genes. Among the hubs, there are genes similar to proteins that inhibit the flowering process and the ABA signaling pathway (Supplementary Table 6).

The GO enrichment of the module (ii) showed that signaling processes were amplified since MAP kinase kinase kinase and calcium ion (Ca^{2+}) transmembrane transporter activities were overrepresented. The group was also enriched for response to stress, photoprotection and plant-type hypersensitive response. Additionally, we also observed in (ii) clusters enriched for specific GO terms. Twenty-five early response genes were grouped into cluster c14, which was the cluster with more overrepresented GO categories among the groups of the modules selected, being enriched for response to high light intensity, response to reactive oxygen species and transcription regulation activity. Other clusters from this module were enriched for stress response (c28, c126 and c159), ion transport (c34), development (c28) and translation (c143). Eighteen hubs were identified in module (ii), three of them being early-responsive genes. Intriguingly, the second transcript with the highest BC and SC values in the whole network is in cluster c28 and does not have a functional annotation. It has seven connections, four of which with genes from different clusters. Its sequence is conserved among plant species and it is described as an uncharacterized protein.

The genes clustered in the second up-regulated module (iii), according to the enrichment analysis, are mostly involved with carbohydrate metabolic processes, lipid phosphorylation and phosphatidylinositol phosphate biosynthetic processes. By itself, cluster c106 was enriched for developmental growth and water homeostasis. Three late-responsive and two non-DEG genes were identified as hubs in module (iii).

The GO terms identified in the modules selected were summarized into several biological processes, separated into up (Figure 5A) and down-regulated (Figure 5B) associated categories. Although up and down-regulated DEGs from these modules present evident similarities into the GO visualization, it is clearly observed that the same biological processes are affected in specific ways in Up modules (Figura 5 A) and Down module (Figure 5 B).

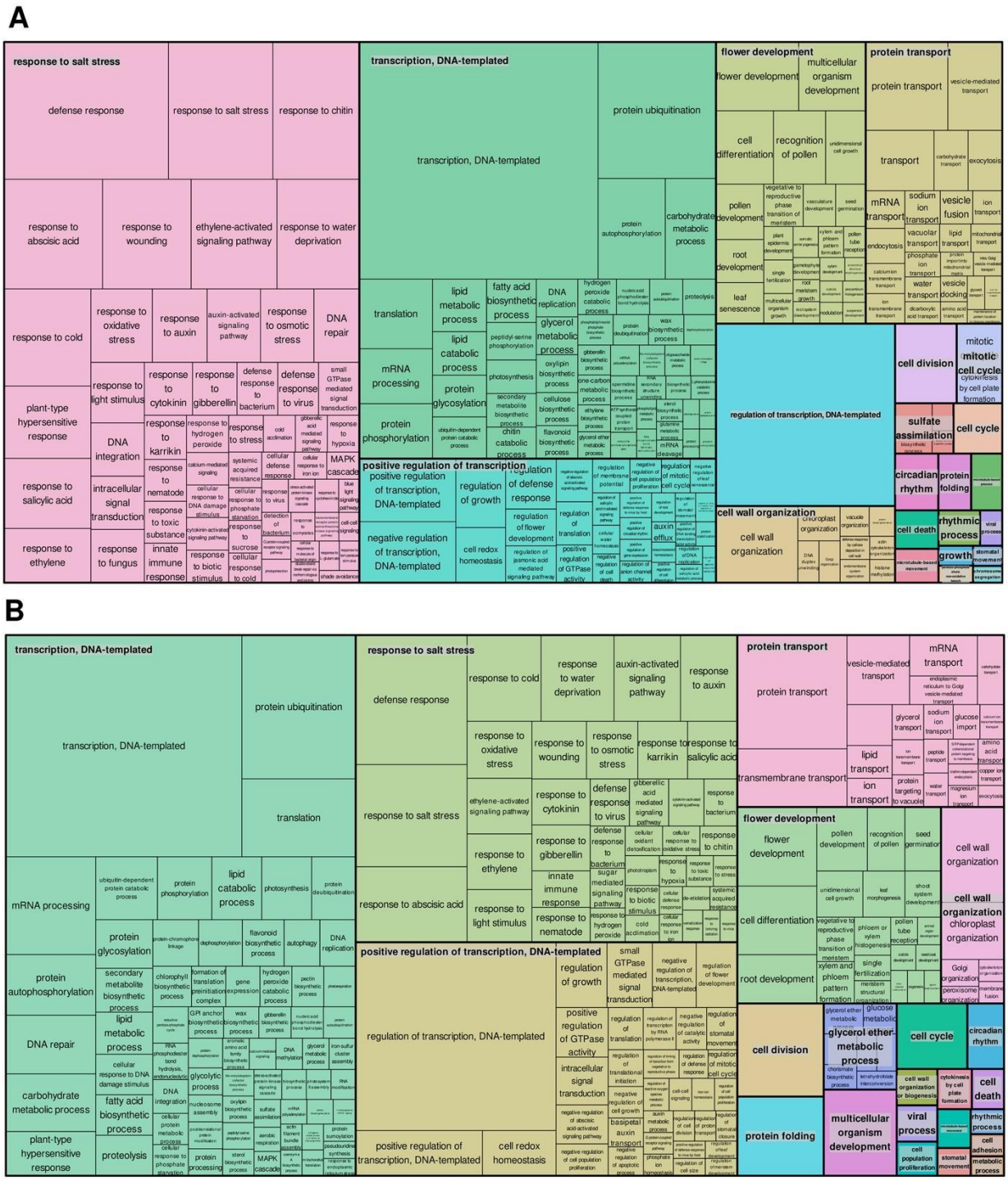

Figure 5. Gene ontology (GO) terms for the cold response modules selected: (A) Up modules; (B) Down module.

4 Discussion

The optimal conditions for *H. brasiliensis* development are temperatures between 22-30 ºC, relative humidity of 70% or higher, and annual rainfall between 1,500 and 3,000 mm. Currently, rubber tree plantations are mostly located in Southeast Asia, which has the ideal edaphoclimatic conditions for the crop development. Interestingly, such varieties are genetically closer to genotypes derived from the southern part of the Amazon basin (Souza et al., 2015), also presenting an adaptation capacity to the Amazon Rainforest dynamics. However, in Brazil, the champion region in natural rubber productivity, the state of São Paulo, is located in suboptimal conditions. Cold weather and fluctuations in rainfall levels directly affect plant development, impacting rubber production (Rao & Kole, 2016). In this context, Brazilian rubber tree breeding programs have been focused on the development of genotypes with high productivity and tolerance to the stress imposed by these areas of higher productivity, especially cold, which inhibits biochemical reactions, reduces photosynthetic capacity and alters the permeability of the plant membrane (Sage & Kubien, 2007; Sevillano et al., 2009; Mai et al., 2010).

The adjustment and adaptation of the rubber tree to the climate of its current places of production depends on its acclimatization capacity. This process is essential for the development of plants that show tolerance to freezing when exposed to low temperatures (Tomashow, 1999). The expression of specific genes is altered at the transcriptional level during the cold acclimation process, forming several cooling-responsive pathways from a signaling network (Tomashow, 1999; Carvallo et al., 2011; Jeon & Kim, 2013), which we were able to clearly visualize in the combined GCN of two of the earliest rubber tree clones, GT1 and RRIM600, being RRIM600 the most planted clone in Brazil. GT1 is a primary clone obtained through open pollination of the wild seedlings introduced in South East Asia and their unselected progeny. RRIM600 was selected in the first rubber tree breeding cycle, when breeders first used controlled pollinations. Both genotypes are prime progenitors of modern rubber tree clones (Priyadarshan et al., 2009). Considering rubber tree long life cycle and the few generations that occurred these clones are substantially close to its wild ancestors, therefore they may be considered as genotypes bearing the wild *Hevea* natural genetic variation. Thus, the work carried out in this paper, which starts from transcripts of genotypes that represent a good part of the genomic characterization of rubber trees, opened paths for understanding the response to cold in the species and which biological pathways to focus on the next steps of rubber tree breeding.

The understanding of the global cold response mechanism of the species becomes attractive so that we can advance in the improvement of rubber production. Our results showed that there are differentially expressed genes that act in a convergent strategy to cold stimulation, considering all transcriptional triggering of GT1 and RRIM600 when exposed to cold.

The identification of genes modulated in response to cold showed that there are contrasting amounts of DEGs according to the time of exposure to cold. The comparison between 0h and 24h stages was the most expressive in number of DEGs, while 0h and 90min was the interval that presented the lowest number of DEGs, suggesting that the seedlings are able to withstand a short period of cold exposure without massive activation or repression of genes. After twelve hours of exposure to the cold, we can observe that both genotypes underwent a reprogramming in their expression profiles (DEGs identified in 0h vs 12h), with the new changes being practically maintained over the next twelve hours (DEGs identified in 12h vs 24h).

The most prominent transcription factors among the DEGs were members of the *ERF* family. *ERFs* are activated by the ethylene signaling pathway and regulate the transcription of ethylene-responsive genes, which negatively impact plant growth (Müller and Munné-Bosch, 2015; Van den Broeck et al., 2017). The *ERF* family in rubber tree is comprised of 115 members (Duan et al., 2013) and the overexpression of an arabidopsis *ERF1* orthologue in rubber tree plants enhanced their tolerance to abiotic stresses and augmented laticifer cell differentiation (Lestari et al., 2018). In our analysis, all degs annotated as ERFs were up-regulated, suggesting that *H. brasiliensis* plantlets maintained the ethylene-mediated stress response active during the whole experiment. Additionally, Ethylene biosynthesis enzymes were up-regulated, which indicates that ethylene plays a constitutive role in rubber tree cold stress response.

JA biosynthesis and signaling pathways seem to be toned down in the seedlings during the 24h of cold treatment. *TIFY*/*JAZ* TFs, which were up-regulated in our experiment, are specific to plants and are negative regulators of JA-induced responses. Nevertheless, their expression is induced by JA, which creates a regulatory feedback loop for fine-tuning of JA-signaling (Chini et al., 2007). Different types of stresses induce the expression of these genes and they are essential for plant growth-defense balance (Zhu et al., 2014; Major et al., 2017; Guo et al., 2018; He et al., 2020). Accordingly, enzymes that attenuate JA-mediated responses, such as IAA-alanine resistant 3 (IAR3) and Cytochrome P450 94C1 (CYP94C1) [that catalyze the turn over of JA (Hertz et al.

2012, Widemann et al. 2013)], were up-regulated as well. Despite the attenuation of JA responses, the biosynthesis of oxylipins seem to have been promoted since the expression of their biosynthetic enzymes were up-regulated.

In addition to the findings with DEG analyses, we expanded the inferences on cold response using GCNs. Considering that the cold tolerance is the result of a network of complex biochemical pathways, an appropriate way to understand such a regulation is not evaluating a single gene, but a set of connected genes corresponding to a broad molecular mechanism. The correlation of how the coordinated expression of genes involved in multiple metabolic pathways act in response regulation can be elucidated through the definition of clusters within the GCN (Umer et al, 2020; Sun et al., 2021). In this way, GCN is a promising approach to better elucidate the participation of genes linked to cooling stress.

Recently, Ding et al. (2020) modeled a GCN using several public rubber tree transcriptomes, providing insights into rubber biosynthesis. Even with a high potential, such approaches are incipient in rubber tree research and this study is the first initiative on evaluating *Hevea* cold response in a more holistic perspective.

4.1 Selection of cold response associated clusters

The selection of GCN clusters associated with the cold response allowed us to supply a range of molecular mechanisms triggered by such a stress. The starting point of rubber tree cold stress response mainly resides in cluster c14

Surprisingly, only six enzymes were early responsive to the cold treatment and all were placed in this cluster as well and four of them are RING-type E3 ubiquitin transferases. Two were annotated as RING-H2 finger proteins ATL2 (PASA_cluster_59724 and PASA_cluster_65327). RING-H2 finger proteins, in special the *ATL* genes, are involved in the regulation of plant response to different type of stresses (Bopopi et al., 2010; Serrano et al., 2006; Song et al., 2016; Kim et al., 2020), being *ATL2* an early responsive gene in Arabidopsis (Salinas-Mondragon et al., 1999). The other two were annotated as E3 ubiquitin-protein ligase RING1 (PASA_cluster_150375 and PASA_cluster_47183), which are involved in the regulation of programmed cell death during plant hypersensitive response (Lin et al., 2008; Lee et al., 2011).

Considering the subnetwork formed with the first up-regulated module, c14 is solely connected to cluster c159, indicating a direct mechanism associated. In addition to being enriched for response to stress and protein ubiquitination, the cluster c159 has transcripts annotated as ERFs, calmodulin binding proteins, serine/threonine protein kinases and disease-responsive genes. Three early-responsive transcripts are present in this cluster, but only two were annotated: Nematode resistance protein-like HSPRO2, a hub gene of this cluster (see below), and BON1-associated protein 2 (BAP2), a gene that is connected to two c14 genes. HSPRO-like proteins are associated with disease resistance, being up-regulated after pathogen infection (Murray et al., 2007; Nemchinov et al., 2017). BAP proteins are negative regulators of cell death in plants, which is essential for plant development and hypersensitive response, and their expression is modulated by temperature (Yang et al., 2007; Cao et al., 2019; Hou et al., 2018). c159 is connected to two other clusters: c53, which did not present any overrepresented GO category but has transcripts annotated as proteins involved with protein phosphorylation and serine/threonine kinase activity, and cluster c143.

It was possible to observe an over representation of the transmembrane transporter activities of MAP kinase kinase and calcium ion (Ca2+) transmembrane, which is already a recognized mechanism of action to cold stress, where several metabolites act as low temperature sensors, after which cells release calcium through phosphatidylinositol signaling and inositol phosphate metabolism through the MAPK signaling pathway . This MAPK signaling pathway triggers signal transduction via TFs such as ERF, bHLH and MYB TFs (Sun, 2021). The c34 cluster, the most connected cluster in the first up-regulated group, was enriched for transmembrane transport of Ca2+, widely known as a second messenger in plants, being (Zhu, 2016; Yuan et al., 2018; Michailidis et al., 2020). Low temperature stimulus is well established as a main trigger of cell Ca2+ influx by the activation of Ca2+ channels, which leads to the Ca2+-signaling cascade of plant cold stress response (Mori et al., 2018; Liu et al., 2021; Mao et al., 2021). Considering the overrepresentation of proteins with Ca2+ transporter activity in cluster c34, it could be seen as the cluster controlling Ca2+-influx, therefore regulating Ca2+-signaling, which agrees with the cluster central position in the up-regulated module (ii).

The remaining five clusters in module (ii) did not present any overrepresentation of GO terms in the enrichment analysis, nevertheless the genes present in these clusters are involved with

signaling processes, response to stress and transcription regulation. Cluster c53 houses members of *ERF* and *TIFY*/*JAZ* transcription factor families.

"Positive regulation of protein kinase activity" and "DNA-binding transcription factor activity" were also over-represented GO terms in the c28 cluster, as well as enhanced stress response mechanisms and developmental processes. Among the 108 transcripts that have a functional annotation in this cluster, 25 (23%) are TFs involved in the regulation of plant defense response. The most represented TF family was WRKY (eight members), which is one of the largest plant-specific TF families and they participate in the regulation of plant growth and development as well as promoters of plant stress response mechanisms by activation and inhibition of target-genes transcription and modulation of signaling cascades (Jiang et al., 2017). This cluster also harbors transcripts similar to enzymes from the ethylene biosynthesis pathway, calmodulin-binding proteins and small auxin up-regulated RNA (SAUR) proteins. These SAUR genes are essential growth factors for regular plant development and adaptive growth under stress conditions. They are responsive to different environmental factors and to other phytohormones besides auxin (Stortenbeker and Bemer, 2019; He et al., 2021). In addition, this cluster has a strong candidate for further analysis into rubber tree cold stress response: PASA_cluster_65283, the uncharacterised protein with the second highest BC and SC values in the whole GCN. Five of its connected transcripts were annotated and the proteins are involved with plant meiotic recombination (Homologous-pairing protein 2 homolog, c143) (Uanschou et al., 2013; Shi et al., 2019), zeaxanthin synthesis (Beta-carotene 3-hydroxylase 1, chloroplastic, c106) (Fiore et al., 2006), JA-dependent defense response (Suppressor of NPR1-1 Constitutive 4, c28) (Bi et al., 2010), stress-induced osmotic solutes accumulation (Probable polyol transporter 4, c28) (Noiraud et al., 2001) and Ca^{2+}-signaling (Probable calcium-binding protein CML36, c28) (Astegno et al., 2017).

Also linked to cluster c28, presenting only this connection in the HCCA network, cluster 126 showed enrichment for stress response. There was an overrepresentation of GO terms for phenylalanine biosynthesis and sphingolipid biosynthesis processes. Phenylalanine, apart from its role in protein biosynthesis, is the precursor of several plant phenolic compounds, such as lignin, flavonoids and anthocyanins (Maeda and Dudareva, 2012), and salicylic acid (SA) (Lefevere et al., 2020). Sphingolipids constitute a significant part of plant plasma membrane lipids and are also

involved in the control of PCD and mediating SA-signaling in plant stress response (Pata et al., 2010; Alden et al., 2011; Rivas-San Vicente et al., 2013).

The cold exposure also triggered the up-regulation of enzymes involved in the synthesis of protective photosynthetic pigments, such as zeaxanthin and lutein (c106) (Dall'Osto et al., 2006), and lipoic acid (lipoamide) (c194), which protects against oxidative stress (Navari-Izzo et al., 2002). Several enzymes from the nucleotide sugars' metabolic pathway were up-regulated (c101, c106, c121, c130, c143, c16, c161, c34, c53), showing the importance of cell wall biosynthesis activation and also being able to function as signaling molecules, being sugar donors for the targeted-glycosylation of different compounds (Figueroa et al., 2021). Interestingly, enzymes from the methionine metabolism pathway that produce ethylene as their final product were also up-regulated (c121, c28, c34), as well as, α-linolenic acid metabolism enzymes that synthetize oxylipins, which are jasmonic acid (JA) precursor molecules that also have signaling functions in cold stress (c126, c159, c34, c53) (Du et al., 2013; Maynard et al., 2018; Wang et al., 2020).

The translation process, more specifically for negative regulation of translation, was enriched in o ther up-regulated cluster, the luster c143, which in the HCCA network is connected to clusters c34 and c28. . This group has three members of the C Carbonatabolite-Repressor 4 (CCR4)–associated factor 1 (CAF1) gene family that were up-regulated in response to the cold treatment. These deadenylase proteins are part of the CCR4- negative on Tata (CCR4-NOT) complex that promotes the deadenylation of mRNAs, which leads to the suppression of translation and mRNA degradation (Collart 2016, Fang et al., 2020). In plants, CAF1 proteins were shown to be necessary for proper development and stress response processes (Sarowar et al., 2007; Liang et al., 2009; Walley et al., 2010; Shimo et al., 2019; Fang et al., 2020, 2021; Wang et al., 2021). In addition, stress-responsive TFs, nucleotide sugar biosynthesis' enzymes and cell wall biogenesis' proteins were assigned to c143 as well.

Cold acclimatization and response to low temperatures was also associated with cluster 101, where there is positive expression of cognate 70 kilodalton heat shock proteins (Neven et al., 1992) in the period between 90 min and 24 hours of stress by cold in rubber tree. In addition, cluster c101 has the action of BAM1, a receptor protein kinase involved in plant development (DeYoung et al., 2006). This protein also has a role in drought stress response, being required for

long distance signaling for stomatal closure (Takahashi et al., 2018). c101 also houses a transcript similar to DELLA protein RGL1, which is a negative regulator of growth promoting phytohormone gibberellin (GA) signaling pathway (Wen and Chan, 2002). Poplar plants overexpressing this gene showed severe dwarfed phenotypes (Busov et al., 2006). This cluster has a regulator of PCD as well. Myb108 is a negative regulator of ABA biosynthesis and ABA-induced cell death (Cui et al., 2013) and is highly induced in rose plants under chilling (4°C) and freezing (-20°C) treatments. Arabidopsis plants overexpressing the rose ortholog showed improved cold tolerance and better performance under other stresses as well as a shorter growth cycle than the wild type (Dong et al., 2021).

The cluster with the most enzymes mapped to the KEGG pathways was c121, with forty-four transcripts assigned to EC numbers. Enzymes that synthesize scopolin and lignins in the phenylpropanoid biosynthesis pathway were placed in this cluster. The coumarin scopolin and its precursor, scopoletin, accumulate in plant tissues in response to stress conditions (Kai et al., 2006; Zandalinas et al., 2017) and are believed to be involved in ROS scavenging (Chong et al., 2002; Lee et al., 2013). Members of the plant ACT domain-containing protein family (ACR4 and ACR8) are present in cluster c161, and their expression is induced by cold treatment in Arabidopsis (Hsieh and Goodman, 2002). Regarding programmed cell death PCD (Programmed Cell Death), c161 bears both proteins that positively (E3 ubiquitin-protein ligase RING1) or negatively (Accelerated cell-death ACD11) regulate cell death. Expression of RING1 is induced by biotic stress and the protein is necessary for the activation of the PCD process (Lin et al., 2008; Lee et al., 2011). ACD11 is a sphingolipid transfer protein that controls cell death through the regulation of sphingolipid levels (Brodersen et al., 2002; Simanchu et al., 2014). It is down-regulated by the UPS machinery after pathogen infection (Liu et al., 2017), however ACD11 transiently accumulates under low concentrations of ABA and under salt and drought stress and its overexpression confers abiotic stress tolerance (Li et al., 2020).

Cluster c194 has proteins involved with negative regulation of SA-mediated PCD (MACPF domain-containing proteins CAD1 and NSL1) (Tsutsui et al., 2006, Noutoshi et al., 2006), regulation of photoperiod gene expression and circadian clock (WD repeat-containing protein LWD1) (Wu et al., 2008; Wang et al., 2011), sugar transporters involved with cell wall biogenesis (UDP-galactose transporter 2) (Norambuena et al., 2005), and plant growth and stress response

(Sugar transport protein 13) (Schofield et al., 2009; Lee and Seo 2021), besides having lignin synthesizing proteins as well.

4.2 Identification of key elements within cold response associated modules

In addition to evaluating the functional profile of all the associated genes within the modules selected, we performed inferences into the importance of the genes with high centrality within a GCN - hubs, which may be described as core regulators (Amrine et al., 2015) . Through the criteria defined for selecting hubs, we could identify the hub for each cluster of the cold stress gene modules , being considered key elements into the cold resistance definition (Carlson et al., 2006; Koido et al., 2018).

Among the nineteen hub genes of the cold response cluster, two had functional annotation indicating a down-regulation of photosynthetic pathway genes (clusters c42 and c166). The c42 hub gene was annotated as lycopene beta cyclase, chloroplastic/chromoplastic (LCYB), an enzyme of the carotenoid biosynthesis process. This hub highlights the importance of the formation of pigment-protein complexes in the photosystems protecting the machinery from reactive oxygen species (ROS) created by abiotic stresses (Dall'Osto et al., 2014; Shi et al., 2015; Kang et al., 2018). The hub from cluster c166 was annotated as OTP51, a pentatricopeptide repeat-containing protein, that is required for the proper assembly of photosystems I and II in Arabidopsis and rice (de Longevialle et al., 2008; Ye et al., 2012). Combined with low temperature, light promotes an enhancement in transcription of cold-responsive and photosynthesis-related genes (Soitamo et al., 2008). The down-regulation of both genes in rubber trees might be linked to the reduction of photosynthetic activity due to cold stress; nevertheless this modulation was detected in the dark period of the experiment.

In addition to the regulation of photosynthetic activity from the cold down-regulation genes, it was also possible to identify two hubs representing proteins involved in abscisic acid (ABA) signaling, which is essential for development and responses to abiotic stress. A transcript similar to a protein phosphatase 2C (PP2C) is one of the hubs in cluster c171. Chao et al. (2020), in a genome-wide identification and expression analysis of the phosphatase 2A family in rubber trees, identified cis-acting elements related to the low temperature responsiveness (LTR) category. Under non-stress conditions, PP2C proteins negatively regulate the ABA-mediated signaling

pathway by inactivating ABA-responsive genes. Once under stress, ABA-receptors bind PP2Cs and inhibit their activity, consequently activating ABA signals (Cai et al., 2017). The other hub gene belongs to cluster c134 and was annotated as zinc finger CONSTANS-like 4 protein (COL4), a transcription factor. COL4 is a flowering inhibitor by repressing the flowering locus T (FT) gene expression (Steinbach, 2019) and, strikingly, it is also involved in abiotic stress responses through the ABA-dependent signaling pathway in Arabidopsis (Min et al., 2015). Considering that ABA concentration might have increased in the rubber trees during the cold exposure, which is enforced by the down-regulation of a transcript similar to a PP2C protein, one could expect that this gene would be up-regulated instead. ABA is known for delaying flowering, nevertheless, under severe drought conditions, ABA up-regulates florigen genes, which is part of the drought escape (DE) strategy. DE prompts plants to accelerate their vegetative growth and reproduction stages during the high water availability period. In the drought season, the plants enter a dormant stage (Verslues and Juenger 2011; Yildirim and Kaya 2017).

The down-regulation of three other strongly co-expressed hub genes, directly connecting three different clusters (c184, c171 and c153) , detected in the last phase of the cold experiment, also suggests an increase in ABA synthesis by the *H. brasiliensis* plantlets connected to an inhibition of growth and the photoperiod. .The hub identified for cluster c184 was annotated as homeobox-leucine zipper (HD-Zip) protein HAT5 (AtHB1) and it was detected as a down-regulated gene after 12 hours of rubber tree cold exposure. HD-Zip transcription factors seem to be a plant-specific TF family and its members take part in plant development and stress response (Ariel et al., 2007), each member having its own expression pattern under different environmental conditions (Li et al., 2019; Li et al., 2020). This hub was connected to not only c171 hub, but also to c153 hub, which was annotated as leaf rust receptor-like kinase 10-like (LRK10L) protein, that have an Arabidopsis' homolog gene involved in ABA-signaling

The hub from the cluster c156 was annotated as histone acetyltransferase of the MYST family (HAM) 1/2 proteins, which are involved in UV-B DNA damage repair (Campi et al., 2012). Interestingly, these proteins positively regulate the expression of FLOWERING LOCUS C (FLC) gene, therefore being a flowering repressor as well (Xiao et al., 2013). The down-regulation of this transcript in *H. brasiliensis* plantlets might be due to a decrease in damaged DNA by UV-B because of the absence of light. In addition, the down-regulation of c196 hub annotated with Down

Homeobox protein BEL1 homolog from different metabolic processes that are flowering repressors (Bhatt et al., 2003) suggests that rubber trees may have a DE-type strategy to deal with abiotic stresses.

Considering all these key elements of transcriptional regulation of rubber trees in response to cold, we were able to reveal by GCN the general genetic responses to cold stress of two important clones of H. *brasiliensis*, which serve as a basis for the rubber breeding. As a tropical tree species, rubber tree most probably did not have to deal with prolonged periods of low temperatures during its evolution; nevertheless the few wild genotypes that were successfully introduced in Southeast Asia carried so much genetic diversity that breeding for varieties that are cold tolerant is still possible. As a result of the GCN strategy applied in this study, we could visualize *Hevea*'s primary reprogramming of gene expression and the relationship among the genes involved in the cold stress response. In the short period of cold exposure used in this work, the plantlets activated the ethylene-mediated signaling pathway since the beginning of the stress treatment and kept ethylene signaling active. The programmed cell death plays a major role in the rubber tree cold response process, being tightly regulated by the signaling cascades. Growth inhibition and cell wall thickening are implemented by the plantlets, which can be correlated to a possible drought escape strategy triggered by the cold stress. In view of the genotypes analyzed, our results may represent the species' genetic stress responses developed during its evolution. The understanding of how *H. brasiliensis* copes with low temperature stress can improve greatly the breeding strategies for this crop as well as emphasize the importance of rubber tree genetic diversity preservation, since it has such a narrow genetic base, is being impacted by climate change and is the only source for large scale rubber production.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

AS, CS, CM and RV conceived the project; CS, SB, AA, FF, RB and RV performed the analyses and wrote the manuscript. All authors reviewed, read and approved the manuscript.

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