Novel insights into the cold resistance of *Hevea brasiliensis* through coexpression networks

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Abstract

*Hevea brasiliensis*, a tropical tree species from the Amazon rainforest, is the main source of natural rubber worldwide. Due to the high pressure of fungal diseases in hot, humid regions, rubber plantations have been moved to “escape areas”, which are dryer and have lower temperatures during the winter. Here, we combined gene expression data of a primary (GT1) and a secondary (RRIM600) young rubber tree clones, which present different cold tolerance strategies, to analyze rubber tree gene expression regulation during 24 h of cold exposure (10°C). Together with traditional differential expression approaches, a RNA sequencing (RNA-seq) gene coexpression network (GCN) comprising 27,220 genes was established in which the genes were grouped into 832 clusters. In the GCN, most of the rubber tree molecular responses to cold stress were grouped in 26 clusters, which were divided into three GCN modules: a downregulated group comprising 12 clusters and two upregulated groups comprising eleven and three clusters. Considering the three modules identified, the general *Hevea* response to short-term cold exposure involved downregulation of gibberellin (GA) signaling, complex regulation of jasmonic acid (JA) stress responses and programmed cell death (PCD) and upregulation of ethylene responsive genes. The hub genes of the cold-responsive modules were subsequently identified and analyzed. As a result of the GCN strategy applied in this study, we could not only access individual DEGs related to the *Hevea* cold response, 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 genetic stress responses of the species, developed during its evolution, since the varieties chosen for this work are genotypes that were 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, which has a narrow genetic base, is 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 to the Amazon rainforest and belonging to the Euphorbiaceae family (Priyandarshan and Clément-Demange, 2004). Although rubber trees are the main source of natural rubber worldwide (Pootakham et al., 2017; Gonçalves and Fontes, 2009; De Faÿ and Jacob, 1989), this tree species is a recent crop and is still in domestication (Priyadarsan and Clément-Demange, 2004). The dispersal and domestication of rubber trees worldwide is based on only approximately 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. Thus, until the present day, almost all commercial varieties of *H. brasiliensis* are derived from these seedlings; therefore, their genetic variability is quite narrow (Gonçalves and Fontes, 2009; Souza et al., 2015).

Although the Amazon Basin provides ideal conditions for rubber tree cultivation, the occurrence of 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 suboptimal areas (or “escape areas”), such as the Brazilian central and southeastern regions, which are dryer and present lower temperatures during the winter (Priyadarshan et al., 2009). Southeast Asian countries, whose climate conditions are similar to those of the Amazon 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) and have expanded to suboptimal areas as well, such as southern China and northeastern 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 escape areas, low temperatures heavily affect *H. brasiliensis* development, causing damage to leaves and latex production (Priyadarshan et al., 2005). For rubber tree plantations, prolonged low-temperature periods or frost occurrence can result in plant death (Priyadarshan et al., 2009). Cold stress triggers a massive reprogramming of gene expression in plants such that their metabolic processes are altered to cope with the cold environment (Theocharis et al., 2012). To promote breeding of cold tolerance in *H. brasiliensis*, a more in-depth understanding of the cold response mechanisms of this species is necessary.

In recent years, due to the impact of low temperatures in rubber tree plantations and rubber production, there has been an increase in the number of studies about *H. brasiliensis* molecular cold stress responses. The CBF-regulon genes *HbCBF1*, *HbICE1* and *HbICE2* have been identified and characterized, and their overexpression enhances cold tolerance in Arabidopsis (Cheng et al. 2015, Yuan et al. 2017, Chen et al. 2019). Long periods of low temperatures induce the demethylation of rubber tree cold-responsive gene promoters, while hypermethylation is observed during warmer periods (Tang et al. 2018). Jasmonic acid (JA) has been shown to modulate and enhance *Hevea* cold tolerance (Deng et al. 2018a, Chen et al. 2019), and cold-tolerant clones exhibit the upregulation of
calcium (Ca2+) signaling-related genes and reactive oxygen species (ROS)-scavenging enzymes. Through comparative genomics involving cold-resistant and cold-sensitive clones, thousands of differentially expressed genes (DEGs) have been identified in response to cold stress (Cheng et al. 2018, Deng et al., 2018, Mantello et al., 2019). Although such works have brought a wealth of information regarding the rubber tree molecular cold stress response, how these mechanisms come together and orchestrate this response needs to be better understood. Recently, Ding et al. (2020) modeled a gene coexpression network (GCN) and associated a network module in response to cold treatment. Although the cold response mechanisms were not assessed, the created GCN represents a rich source of data for investigating cold responses.

Here, we carried out a study based on a GCN modeled with combined transcriptomic data from two of the earliest H. brasiliensis clones to identify novel patterns between the genes involved in the species response to cold stress. The GCN use of the transcriptome data from a cold stress experiment with these genotypes, one resistant (RRIM600) and one tolerant (GT1), allowed the analysis of highly associated genes and their classification into clusters, some of which were enriched for different biological processes. Three main cluster modules for rubber tree cold stress response were delimited, which enabled the identification of central genes in the created networks underlying cold stress in rubber tree. This study aimed to provide insights on how this species copes with low temperatures by the use of clones with distinct cold response strategies and that are part of the basis of rubber tree domestication and breeding processes. This work is one of the pioneering works in determining the association of the molecular mechanisms of Hevea through GCNs and is the first to present rubber tree cold stress response mechanisms as an integrated network.

2 Material and Methods

2.1 RNA Sequencing (RNA-Seq) Experiments

The RNA-seq experiment was based on two clones of H. brasiliensis recommended for escape areas (Mantello et al., 2019): RRIM600 and GT1. Under low temperatures, RRIM600 presents an avoidance physiological strategy, while GT1 employs a balance between photosynthetic/growth activities and stress responses (Mai et al., 2010; Mantello et al., 2019). Raw RNA-seq data generated by Campos Mantello et al. (2019) (BioProject, PRJNA483203; Sequence Read Archive (SRA), SRX4491391-SRX4491414) were used for the analyses of the present work. The cold experiment consisted of exposing three six-month-old plantlets of each clone, which were provided by the Seringueira Center, Votuporanga, São Paulo, Brazil, to a temperature of 10°C for 24 h (12 h light/12 h darkness). During this period, sampling was carried out for each biological replicate by random collections of one green leaf at the mature stage, at 0 h (0h, control), at 90 mins (90m), at 12 h (12h), and at 24 h (24h) post-treatment. The RNA extracted from these samples was used to construct 24 cDNA libraries (three replicates of each clone for each time points). The 24 samples were randomly pooled (four samples per pool) and clustered using the TruSeq PE Cluster Kit on the cBot platform (Illumina, Inc., San Diego, CA, USA). The samples were sequenced using an Illumina Genome Analyzer IIx with the TruSeq SBS 36-Cycle Kit (Illumina, Inc., San Diego, CA, USA), for 72 bp paired-end (PE) reads.

2.2 Bioinformatics and Differential Gene Expression Analysis

The reads of each library considering their time points and genotypes' biological replicates were mapped with Bowtie2 v.2.2.6 (Langmead & Salzberg, 2012) to the reference transcriptome. The estimated abundance of reads for each sample was calculated by RSEM v.1.2.28 (Li & Dewey, 2011) using a Trinity accessory script (align_and_estimate_abundance.pl). 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 kept for further analyses. These samples were normalized by the quantile method and transformed into log2cpm by the edgeR v.3.34.1 package (Robinson et al., 2010)

To explore the data and verify the behavior of the samples a principal component analysis (PCA) was performed in R (R Core Team 2020) using a singular value decomposition from the standardized log2cpm data (Z-score normalization). For DEG analysis, the datasets from GT1 and RRIM600 were combined in an attempt to create a unique \textit{H. brasiliensis} expression dataset. An empirical Bayes smoothing method implemented in the edgeR v.3.34.1 package (Robinson et al., 2010) was used to identify differentially expressed genes (DEGs) between the time points sampled. The eBayes function with a minimum ratio of 0.01 was used to determine whether a gene is differentially expressed. 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 for which the p-value was ≤ 0.05 were considered DEGs.

The identified DEGs were compared between the time points with Venn plots, which were 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; \url{https://github.com/Trinotate/Trinotate.github.io}). Transcripts were queried against the SwissProt/UniProt database using BLASTX (e-value of 1e-5), and placed into a tab-delimited file. Transcripts were also associated with Gene Ontology (GO) terms (Harris et al., 2004).

2.3 GCN Analysis

The GCN was inferred through R Pearson correlation coefficients with a cutoff 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). The HRR networks and the HCCA results were subsequently 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 likely groups involved in the \textit{Hevea} cold stress response. Hub genes in the selected clusters were analyzed using the node degree distribution (DD), betweenness centrality (BC) and stress centrality (SC) distribution parameters of the networks obtained using Cytoscape software v.3.8.0 (Shannon et al., 2003). Genes that had a high DD and that presented high values of BC and SC were considered highly influential in the selected clusters’ coexpression dynamics. For each selected network cluster, at least one hub gene was identified, and they were evaluated according to their functional annotation and/or according to the genes with which they interacted.

2.5 Functional Enrichment

Overrepresented GO terms in the clusters were determined with the 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 for 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 Cytoscape software v.3.8.0 (Shannon et al., 2003).

3  Results

3.1  Bioinformatics and Differential Expression Analysis

Approximately 530 million PE reads were obtained for the RRIM600 genotype, and 633 million were obtained for the GT1 genotype. After quality filtering, approximately 933 million PE reads were retained and used to assemble the transcriptome via 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 identified a total of 74,398 unique proteins related to 9,417 different GO terms (Supplementary Table 1).

The transcriptome data used were validated by Mantello et al. (2019). In addition to the validation of this RNA-seq data, genes that were analyzed by Silva et al. (2014) are also present in the network. Since qPCR experiments have already been performed by those who produced the data to validate the quantitative transcription data, we consider the data to be reliable for our analyses.

For DEG analyses, we employed a filtering criteria based on genes that presented at least 10 counts per million (CPM) in at least three samples, which resulted in a final number of 30,407 genes. From these genes, the PCA revealed a distinct profile of samples belonging to each different genotype (Figure 1A).

The number of DEGs per time period comparison showed different responses regarding not only the number of DEGs (Table 1) but also the associated genes (Supplementary Table 2). Although there was evident overlapping among the results (Figure 1B), a joint mechanism involving several genes for the cold response was clearly observed. We detected contrasting numbers of DEGs for the conditions established, with the highest number of DEGs identified at 0h and 24h and the smallest at 12h and 24h. Approximately 93% of the downregulated genes and 96% of the upregulated genes were annotated. Regarding the GO enrichment analysis, the full DEG set was enriched for response to stress and response to chitin terms. The downregulated group of genes did not present any overrepresented category. In contrast, the upregulated gene 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.

After 90 minutes of cold treatment, only thirty-two transcripts were characterized as DEGs, all of which were upregulated during the plantlets’ early response to cold stress. Of these, 26 genes were successfully annotated (Supplementary Table 1). The annotated DEGs are known to be induced by cold as well as other abiotic stressors, such as drought and salt stresses. These DEGs 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, 103 of which were upregulated and 35 were downregulated. All of them except one were grouped in the cold stress response gene clusters (see below). An analysis of overrepresented GO terms for 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 downregulated groups, respectively. While the upregulated genes are responsive to stress and other stimuli, the downregulated genes promote plant development and growth. All transcripts annotated as members of the Apetala2/Ethylene-responsive factors (AP2/ERF) family and that were identified as DEGs (38) were upregulated. DEGs annotated as transcriptional repressors of JA-mediated responses (i.e., TIFY/JAZ proteins) were also upregulated.

Nonspecific 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 DEGs have an overrepresentation of GO terms related to reproduction processes, response to abscisic acid (ABA) stimulus, calmodulin binding and signal transmission, while the downregulated DEGs were enriched for posttranslational 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 downregulated, respectively.

Among the annotated DEGs, four transcripts similar to Gibberellin 20 oxidase (GA20ox) protein, which belongs to the GA biosynthesis pathway, were downregulated. Transcripts similar to Allene oxide cyclases (AOC) and synthases (AOS) and Linoleate 13S-lipoxygenase (LOX), which synthesize oxylipins (i.e., 12-oxo phytodienoic acid, 12-OPDA) that are precursors of JA, were upregulated. Nevertheless, DEGs annotated as enzymes that attenuate JA-mediated stress responses were also upregulated; these include IAA-alanine resistant 3 (IAR3) and Cytochrome P450 94C1 (CYP94C1), which catalyze the turnover of JA (Heitz et al. 2012, Widemann et al. 2013).

3.2 Rubber Tree GCN Analysis

From the transcriptome data of both genotypes, we modeled an HRR network in order to represent the cold response of rubber tree (Figure 2A). This network was composed of 27,200 nodes (genes) across 50,650 connections (edges) that were grouped into 832 gene clusters. Only the top 10 Pearson R correlation coefficients (considering a 0.8 cutoff) were retained for each gene, and disconnected genes were disregarded. The major connected component within the network is composed of 25,608 nodes (94.1%). There were also 626 components for the remaining 1,592 genes, with the number of nodes in each component ranging from two to eleven. To identify putative gene associations within the network structure, we analyzed the network with HCCA, which revealed 832 clusters (Figure 2B). From these sets of genes, 626 corresponded to the disconnected components (mean cluster size of ~3), and 206 corresponded 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 shows that the complete network of transcripts from the cold experiment has a large amount of highly correlated genes, confirming the efficiency of the transcription analysis method of GCNs. The central portion of the network has the major part of genes, whose expression was not significantly different, while right and left ends concentrate the majority of DEGs. However, part B of Figure 2 highlights a region of the network where most groups contain DEGs, in which up- and downregulated transcripts responsible for a significant part of the results are shared; this is also the region where we made most of the inferences. It is possible to see in this figure that one group of DEGs can reach another with few connections. Thus, although we have a clear picture of the DEGs separated in part A of the figure, it is possible to find a region of the network in which the correlation of up- and downregulated DEGs functioning in common processes that interfere with each other can be analyzed.
Supplementary Figure 1 (part B) also shows the topological structure that seems to be the core of the main architecture of the network. This is the structure we used to analyze the DEGS and biological functions among the correlated genes. In part A of the figure, only the condensed groupings of up- and down-regulated genes are shown, with almost no connection between them, and part B shows the subnetwork that we used for most of the inferences in this work, given the concurrent connectivity between up- and downregulated genes active in response to cold stress.

Functional coherence is expected across coexpressed genes belonging to the same cluster. As such, an enrichment analysis of GO terms was performed for each cluster of genes identified by the HCCA. 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 nine groups, which was expected considering the cold experimental design. Two of these clusters (c14 and c28) were also enriched for developmental processes, indicating the tight correlation between environmental stimuli and growth. Interestingly, we also observed seven clusters enriched for virus replication and transposition processes.

In addition to the clustering structure evaluated on the GCN topology, we also assessed the gene centrality measures (Supplementary Table 3). From the 27,220 genes in the network, only 507 had 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 a great influence on the network. This gene belongs to cluster c8, which was enriched for photosynthesis, but also for cellular respiration and transposition processes. By evaluating the intercluster connections in the HCCA-based contracted network (Figure 3B and Supplementary Table 4), we found that the cluster with the highest number of connections is c117 (nine edges), which was enriched for developmental processes.

Figure 3 presents an important aspect of the network architecture, which returns similarity with the biological functions. According to the distribution of GO terms in the network architecture, it is possible to see enrichment of correlated pathways distributed in close groups in the GCN. This coherence between enrichment of correlated GO terms corroborates the application of this method as a valuable source of inferences.

3.3 Gene Clusters Mostly Related to the Cold Stress Response

Even though the DEGs identified were dispersed throughout the GCN (Figure 2A), specific patterns could be observed in several clusters. To identify molecular associations with the cold stress response, we selected the clusters that together accounted for 70.2% (1,751) of the DEGs identified. We considered as associated clusters those that contain at least 50% of their genes as DEGs (Supplementary Table 5), expanding the module across these clusters’ neighbors considering a minimum required frequency of 20% of DEGs inside a cluster (Figure 3 and Supplementary Figure 1). Interestingly, we established three GCN modules for the 31 clusters selected: (i) a downregulated group with 12 clusters, (ii) an upregulated group with 11 clusters, and (iii) an upregulated group with three clusters. Even though there were additional clusters with DEGs, only the most pronounced clusters were used for novel inferences.
Among the DEGs in the selected clusters, 480 were annotated with enzyme code (EC) numbers, being 339 up-regulated and 141 down-regulated transcripts. Moreover, 180 DEGs were assigned to KEGG pathways. Cold exposure triggered the upregulation of transcripts similar to enzymes involved in the synthesis of zeaxanthin and lutein (c106) and lipoic acid (lipoamide) (c194). Several enzymes from the nucleotide sugar metabolic pathway were upregulated (c101, c106, c121, c130, c143, c16, c161, c34, c53). Enzymes in the methionine metabolism pathway that produce ethylene as their final product were also upregulated (c121, c28, c34), as well as α-linolenic acid metabolism enzymes that synthesize oxylipins (c126, c159, c34, c53) and enzymes that promote the turnover of JA (c194, c53).

The module of downregulated genes (i), as a whole, did not present any overrepresented GO term category. This module bears transcripts similar to Gibberellin receptor GID1C protein (clusters c153 and c196), which is a positive regulator of GA-signaling (Griffiths et al. 2006). Together with the downregulation of putative GA20ox transcripts in non-selected clusters, these results indicate that rubber tree decreases GA-mediated processes during cold stress. Two transcripts annotated as Coronatine-insensitive protein 1 (COI1), an F-box protein that promotes the degradation of JAZ proteins (Thines et al. 2007), were also assigned to clusters in this module (c184 and c42).

Interestingly, members of the florigen CONSTANS gene family were downregulated and the DEGs were associated to clusters c134, c184 and c48.

GO enrichment of module (ii) showed that signaling processes were amplified, since MAP kinase kinase kinase (MAPKKK) 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 and three early-response genes were grouped into clusters c14 and c159, respectively. c14 was the cluster with the most overrepresented GO categories among the groups of the modules selected, being enriched for response to high-light intensity, response to ROS and transcriptional regulation activity. Other clusters from this module were enriched for stress response (c28, c126 and c159), ion transport (c34), development (c28) and translation (c143). Twenty-six of the 38 DEGs annotated as AP2/ERF proteins and all DEGs annotated as JAZ proteins (8) were assigned to the clusters of module (ii). In addition, five putative RING1 E3 ubiquitin-protein ligases, which are involved in the regulation of programmed cell death (PCD) during the plant hypersensitive response (Lin et al., 2008; Lee et al., 2011), were placed in clusters c14, c161 and c28. The two transcripts from c14 are early-responsive genes, being upregulated after 90m of cold exposure. Intriguingly, the second transcript with the highest BC and SC values in the whole network was in cluster c28 and was not functionally annotated; this transcript has seven connections, four of which with genes from different clusters. Its sequence is conserved among plant species and is described as an uncharacterized protein.

The genes clustered in the second module of upregulated genes (iii), according to the enrichment analysis, are mostly involved in carbohydrate metabolic processes, lipid phosphorylation and phosphatidylinositol phosphate biosynthetic processes. By itself, cluster c106 was enriched for developmental growth and water homeostasis.

The GO terms identified in the modules selected were summarized into several biological processes and then separated into up- (Figure 4A) and downregulated (Figure 4B) categories. Although up- and downregulated DEGs from these modules present evident similarities into terms of GO annotations, it is clearly observed that the same biological processes are affected in specific ways.
To evaluate the importance of genes inside these modules, we evaluated the presence of central genes inside each cluster: the hub genes. The network structure allows the insertion of ten edges for each node while respecting the minimum Pearson correlation of 0.8; however, if a highly connected node has low BC and SC values, it was not selected as a hub. As such, this maximum number of connections was not observed in all the selected hubs. In total, 39 hub genes were identified in the selected clusters: 16 for module (i), 18 for module (ii) and five for module (iii). Among the hubs, there are transcripts similar to proteins involved in developmental processes, response to stress, uncharacterized proteins and three transcripts whose sequences were not similar to any sequence in the BLASTN Nt database. (Supplementary Table 6). A BLASTN search against other Euphorbiaceae species genomes returned no results. Thus, possibly, these sequences are specific at least to the genus. Interestingly, two hub genes from module (iii) were not among the set of DEGs.

4 Discussion

The optimal conditions for *H. brasiliensis* development are temperatures ranging from 22-30°C, relative humidity of 70% or higher, and annual rainfall between 1,500 and 3,000 mm. Considering the damage of low temperatures to rubber production, rubber tree breeding programs worldwide have been focusing on the development of genotypes with both high productivity and tolerance to cold stress present in escape areas. Thus, the work carried out in this paper, which starts from transcripts of genotypes that represent a good portion of the genomic makeup of commercial rubber tree clones, opened avenues for understanding the response to cold stress in the species.

GT1 is a primary clone obtained through open pollination of the wild seedlings introduced in Southeast Asia and their unselected progeny. RRIM600 was selected in the first rubber tree breeding cycle, when breeders first used controlled pollination. Both genotypes are prime progenitors of modern rubber tree clones (Priyadarshan et al., 2009). Considering the long life cycle of rubber trees and the low number of generations that have occurred, these clones are substantially close to their wild ancestors; therefore, they may be considered as genotypes bearing the wild *Hevea* natural genetic variation. For this reason, we used these genotypes to make inferences concerning *H. brasiliensis* cold resistance.

We started our analyses by identifying genes modulated at different time points of cold exposure. The small number of responsive genes may indicate that the plants are able to withstand a few hours of cold exposure without massive activation or repression of genes. After twelve hours of exposure to the cold, we observed that the plantlets underwent a reprogramming in terms of their expression profiles (DEGs identified at 0h vs. 12h), with the new programming essentially being maintained for the next twelve hours (DEGs identified at 12h vs. 24h). Similar results were observed in rubber tree comparative transcriptome works as well (Cheng et al. 2018, Deng et al. 2018a, Mantello et al. 2019) and a higher number of DEGs was identified in the cold-tolerant genotypes (Cheng et al. 2018, Mantello et al. 2019). Our results indicate that the species, and not only selected clones, has a delayed response to low temperatures in terms of gene expression modulation.

In addition to DEG analyses, we constructed GCNs to expand our inferences concerning genes involved in the cold response. Analyses based on such a structure are a promising approach to better elucidate the participation of genes associated with cold stress. Considering that such tolerance is the result of a network of complex biochemical pathways, an appropriate way to understand such regulation is evaluating not a single gene but rather a set of associated genes corresponding to a broad molecular mechanism. As such, we did not search for specific genes inside the GCN; instead, the structure and connections of the GCN helped guide us to identify potential candidate genes and interactions that might play essential roles in the rubber tree cold stress response. The correlations of
how the coordinated expression of genes involved in multiple metabolic pathways act in unison can be elucidated through the definition of clusters within the GCN (Umer et al., 2020; Sun et al., 2021). Recently, using several public rubber tree transcriptomes, Ding et al. (2020) modeled a GCN, providing insights into rubber biosynthesis. Even with high potential, such approaches are still relatively new in rubber tree research, and this study is the first initiative on evaluating *Hevea* cold response from a more holistic perspective.

### 4.1 Selection of Cold Response-Associated Clusters

From the GCN clusters selected as being associated with the rubber tree cold stress response, analysis of enzymes and correspondent metabolic pathways led to the identification of potential cold-related metabolic changes. Enzymes involved in the synthesis of protective photosynthetic pigments (i.e., zeaxanthin and lutein) (Dall'Osto et al., 2006) and lipoic acid (lipoamide), which protects against oxidative stress (Navari-Izzo et al., 2002), were upregulated. Nucleotide sugars are essential for cell wall biosynthesis and can function as signaling molecules, being sugar donors for the targeted-glycosylation of different compounds (Figueroa et al., 2021). The GCN indicates that ethylene biosynthesis is correlated with nucleotide sugar pathway enzyme expression (c121, c34) as well as the synthesis of oxylipins (c34). Oxylipins are synthetized in the plastids and then are transported to the peroxisomes for the synthesis of JA. These molecules have signaling functions, working independently from JA in response to stresses (Du et al., 2013; Maynard et al., 2018; Wang et al., 2020). JA mediates rubber tree response to cold stress, nonetheless cold-tolerant clones promote the downregulation of *COII* while *JAZ* genes are upregulated (Deng et al. 2018a). Taken together, our results suggests that the primary rubber tree strategy might be employing OPDAs as signals to trigger short-term cold responses rather than JA, which might be transiently used in specific periods.

MAPK and Ca\(^{2+}\)-signaling processes increased during cold exposure. This mechanism of action in response to cold stress has been reported in other studies, where several metabolites were found to act as low-temperature sensors; in response cells release calcium through phosphatidylinositol signaling and inositol phosphate metabolism through the MAPK signaling pathway. This MAPK signaling pathway in turn triggers signal transduction via transcription factors (TFs) such as ERFs, bHLHs and MYBs (Sun, 2021). Mantello et al. (2019) detected the upregulation of transcripts similar to Calmodulin-like proteins (CML) in rubber tree during cold stress in both RRIM600 and GT1 genotypes. Here we also identified several upregulated transcripts annotated as CMLs and Calcium-dependent protein kinases (CDPKs), and the central cluster of module (ii) (c34) was enriched mainly for cellular Ca\(^{2+}\) flux processes, indicating that Ca\(^{2+}\)-signaling has a major role in rubber tree cold response. Cheng et al. (2018) observed an upregulation of *MAPK* and *MAPK3K* genes both in the cold-tolerant (CATAS93-114) and in the cold-susceptive (Reken501) clones; however, the expression of *MAPK3K* genes was higher in the tolerant genotype. Accordingly, our results demonstrate that the MAPK3K signaling cascade is enhanced in rubber trees under low temperature stress and that it seems to be a *H. brasiliensis* basal line of defense against the cold environment.

The starting point of the rubber tree cold stress response mainly lies in cluster c14, which harbors several sequences annotated as ethylene-responsive transcription factors (ERFs). 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; den Broeck et al., 2017). The *ERF* TF family in rubber tree comprises 115 members (Duan et al., 2013), and the overexpression of an Arabidopsis *ERF1* ortholog in rubber tree plants enhanced their tolerance to abiotic stresses and augmented laticifer cell differentiation (Lestari et al., 2018). The rubber tree *ERF* genes expression is modulated by low temperature, either up- or downregulated (Gong et al., 2018). Deng et al. (2018a) observed that a cold-tolerant clone downregulated genes that promoted ethylene-signaling,
suggesting that the cold-tolerant clone repressed the ethylene-mediated response during cold stress. In our analysis, all DEGs annotated as genes encoding ERFs were upregulated, suggesting that *H. brasiliensis* plantlets maintained the ethylene-mediated stress response activity during the whole experiment. The upregulation of ethylene biosynthesis-related enzymes also indicates that ethylene might have a primary role in the rubber tree cold stress response.

In addition to being enriched for response to stress and protein ubiquitination, cluster c159 has transcripts annotated as ERFs, calmodulin binding proteins, serine/threonine protein kinases and disease-responsive genes. c159 also harbors two *BON1-associated protein 2* (*BAP2*) genes, one being an early-responsive gene. BAP proteins are negative regulators of cell death in plants, which is essential for plant development and the hypersensitive response, and *BAP* expression is modulated by temperature (Yang et al., 2007; Cao et al., 2019; Hou et al., 2018).

Cluster c143 was enriched for the translation process, more specifically for negative regulation of translation. This group contains three members of the *Carbon Catabolite-Repressor 4 (CCR4)-associated factor 1* (*CAF1*) gene family that were upregulated in response to cold treatment. These deadenylase proteins promote translation suppression 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-related enzymes and cell wall biogenesis proteins, were assigned to cluster c143.

In addition to being enriched for stress responses and developmental processes, the GO terms “positive regulation of protein kinase activity” and “DNA-binding transcription factor activity” were overrepresented in cluster c28. Among the 108 transcripts that were functionally annotated, 25 (23%) are TFs involved in the regulation of the plant defense response. The most represented TF family in this cluster was the WRKY family, with eight members. WRKY TFs are one of the largest plant-specific TF families, and they participate in the regulation of plant growth and development as well as promote plant stress response by activating and inhibiting target gene transcription and modulating signaling cascades (Jiang et al., 2017). Cluster c28 also harbors transcripts similar to calmodulin-binding proteins and small auxin upregulated RNA (SAUR) proteins, which are essential growth factors for regular plant development and adaptive growth under stress conditions (Stortenbeker and Bemer, 2019; He et al., 2021). In rubber tree, the expression of a SAUR gene was higher in a cold-tolerant clone, which also downregulated auxin signaling genes (Deng et al. 2018a). In addition, this cluster has a strong candidate for further analysis of the rubber tree cold stress response: PASA_cluster_65283, the uncharacterized protein with the second highest BC and SC values in the whole GCN. Some of its connected transcripts were annotated as proteins involved in different mechanisms in plant stress responses, such as the JA-dependent defense response and Ca²⁺ signaling.

Another cluster that also showed enrichment for stress responses was cluster c126, with 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, 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 the mediation of SA signaling in the plant stress response (Pata et al., 2010; Alden et al., 2011; Rivas-San Vicente et al., 2013).

In module (ii), cluster c34 is the main player in Ca²⁺ signaling and is enriched for transmembrane transport of Ca²⁺, which controls Ca²⁺ influx. Ca²⁺ is widely known as a secondary messenger in plants (Zhu, 2016; Yuan et al., 2018; Michailidis et al., 2020). Low temperature stimuli is well
known to be a main trigger of cell Ca^{2+} influx through the activation of Ca^{2+} channels, which leads to a Ca^{2+}-signaling cascade of proteins in response to cold stress (Mori et al., 2018; Liu et al., 2021; Mao et al., 2021).

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 in signaling processes, response to stress and transcriptional regulation. *H. brasiliensis* has 18 *JAZ* genes (Chao et al., 2019), and four DEGs annotated as TIFY/JAZ TFs were assigned to cluster c53. The TIFY/JAZ TF family is specific to plants, and its members are negative regulators of JA-induced responses. Nevertheless, their expression is induced by JA, which creates a regulatory feedback loop for fine-tuning JA signaling (Chini et al., 2007). Exogenous methyl jasmonate induces latex biosynthesis (Deng et al. 2018b), and some rubber tree *JAZ* members are involved in the regulation of laticifer differentiation and rubber biosynthesis (Deng et al. 2018b, Chao et al. 2019).

Cluster c101 has a putative gene that encodes BAM1, a receptor protein kinase involved in plant development (DeYoung et al., 2006) and is required for long-distance signaling for stomatal closure (Takahashi et al., 2018). c101 also houses a transcript similar to the DELLA protein RGL1, which is a negative regulator of the growth-promoting phytohormone gibberellin (GA) signaling pathway (Wen and Chan, 2002). Poplar plants overexpressing this gene showed severe dwarfed phenotypes (Busov et al., 2002). This cluster also contains a regulator of PCD. *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. Compared with wild-type Arabidopsis plants, Arabidopsis plants overexpressing the rose *Myb108* ortholog showed improved cold tolerance and better performance under other stresses as well as a shorter growth cycle (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 (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 PCD, c161 harbors proteins that positively (the E3 ubiquitin-protein ligase RING1) or negatively (Accelerated cell death 11 (ACD11)) regulate cell death. The expression of RING1 is induced by biotic stress, and the protein is necessary for the activation of PCD (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 downregulated by the UPS machinery after pathogen infection (Liu et al., 2017); however, ACD11 transiently accumulates in response to low concentrations of ABA and under salt and drought stresses, and its overexpression confers abiotic stress tolerance to plants (Li et al., 2020).

Cluster c194 contains proteins involved in the negative regulation of SA-mediated PCD (the MACPF domain-containing proteins CAD1 and NSL1) (Tsutsui et al., 2006, Noutoshi et al., 2006), regulation of photoperiod gene expression and the circadian clock (the WD repeat-containing protein LWD1) (Wu et al., 2008; Wang et al., 2011), sugar transporters involved in cell wall biogenesis (UDP-galactose transporter 2) (Norambuena et al., 2005), and plant growth and stress responses (Sugar transport protein 13) (Schofield et al., 2009; Lee and Seo 2021), in addition to containing lignin-synthesizing proteins.

### 4.2 Identification of Key Elements within Cold Response-Associated Modules
In addition to evaluating the functional profiles of all the associated genes within the modules selected, we inferred about the importance of these genes in terms of the cold response mechanism. Genes with high centrality within a GCN may be described as core regulators (Amrine et al., 2015), which are known as hubs. Through the criteria defined for selecting hubs, we could identify hub genes for each cluster of the cold stress gene modules, which are considered key elements in the definition of cold resistance (Carlson et al., 2006; Koido et al., 2018).

Thirty-seven of the 39 identified hub genes were identified as DEGs, and 36 were successfully annotated (Supplementary Table 6). The annotated hubs indicate that downregulation of photosynthesis, ABA signaling, inhibition of growth and, interestingly, genes involved in the flowering process are central to H. brasiliensis short-term cold response. In addition, the three unannotated hubs apparently are sequences that are specific to rubber tree, which makes them interesting targets for further research into their functions in H. brasiliensis cold stress response.

The functional annotation of two of these hubs indicated a downregulation 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. Carotenoids form pigment-protein complexes in photosystems, protecting the machinery from ROS generated by abiotic stresses (Dall’Osto et al., 2014; Shi et al., 2015; Kang et al., 2018). The hub gene 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 the transcription of cold-responsive and photosynthesis-related genes (Soitamo et al., 2008). The downregulation of both hub genes in rubber trees might be linked to the reduction in photosynthetic activity due to cold stress; nevertheless, this modulation was detected in the dark period of the experiment.

Two hub genes were annotated as proteins involved in 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 tree, identified cis-acting elements related to low-temperature responsiveness (LTR). Under nonstressed conditions, PP2C proteins were shown to negatively regulate the ABA-mediated signaling pathway by inactivating ABA-responsive genes. Once plants are under stress, ABA receptors bind PP2Cs and inhibit their activity, consequently activating ABA signals (Cai et al., 2017). A cold-tolerant rubber tree genotype (CATAS93-114) also downregulated PP2C genes while under cold stress (Deng et al. 2018). The other hub gene belongs to cluster c134 and was annotated as zinc finger CONSTANS-like 4 protein (COL4), a TF. COL4 is a flowering inhibitor that represses 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., 2014). Considering that the ABA concentration might have increased in the rubber trees during cold exposure, which is enforced by the downregulation of a transcript similar to a PP2C protein, one could expect that this gene would be upregulated instead. ABA is known to delay flowering; nevertheless, under severe drought conditions, ABA upregulates 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. During the drought season, these plants enter a dormant stage (Verslues and Juenger 2011; Yildirim and Kaya 2017).

The hub gene from 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). In Arabidopsis,
HAM1 and HAM2 proteins are expressed mainly in growing tissues and double mutants are lethal (Latrasse et al., 2008). Interestingly, these proteins positively regulate the expression of FLOWERING LOCUS C (FLC) gene, therefore, they are flowering repressors (Xiao et al., 2013). The downregulation of this transcript in *H. brasiliensis* plantlets might be due to a decrease in DNA damage from UV-B because of the absence of light. One of the hub genes for cluster c196 was annotated as Homeobox protein BEL1 homolog, which is also involved with the flowering process. BEL1 controls ovule integument identity and is required for auxin and cytokinin signaling for ovule development (Bhatt et al. 2004, Bencivenga et al., 2012). The down-regulation of four genes from different metabolic processes that are involved in the flowering process suggests that rubber tree may have a DE-type strategy to deal with abiotic stresses.

The downregulation of three other strongly coexpressed hub genes from the c184, c171 and c153 clusters detected in the last timepoint of the cold experiment also suggests an increase in ABA synthesis by the *H. brasiliensis* plantlets associated with growth inhibition and photoperiod. The hub identified for cluster c184 was annotated as homeobox-leucine zipper (HD-Zip) protein HAT5 (AtHB1), and it was detected as a downregulated gene in rubber tree after 12h of cold exposure. HD-Zip TFs seem to be a plant-specific TF family and its members take part in plant development and stress responses (Ariel et al., 2007), with each member having its own expression pattern under different environmental conditions (Li et al., 2019; Li et al., 2020). In Arabidopsis, HAT5 expression is downregulated in response to salt stress and low temperature; however, HAT expression is upregulated in response to darkness (Henriksson et al., 2005), and the HAT protein acts as a positive regulator of growth by modulating the expression of genes involved in cell elongation and cell wall composition (Capella et al., 2015). This hub gene was connected to not only the c171 hub gene but also to the c153 hub gene, which was annotated as leaf rust receptor-like kinase 10-like (LRK10L) protein. LRK10 is a disease resistance gene first described in wheat (Feuillet et al., 1997), and members of this family in wheat were shown to be induced by stress and light. Interestingly, in the dark, these genes were downregulated even though the plants were under biotic stress (Zhou et al., 2007). The homologous gene in Arabidopsis encode two different proteins: one is involved in ABA signaling and is downregulated by drought stress and ABA treatment, while the other is upregulated under these conditions, which indicates that these transcripts might regulate each other (Lim et al., 2015). These three hub genes were strongly coexpressed together in rubber trees, directly connecting three different clusters.

Considering the differences in the cold stress response of two *H. brasiliensis* clones obtained in the early years of rubber tree domestication, we attempted to have a glimpse of the general genetic processes of *Hevea* in response to cold stress. As a tropical tree species, rubber tree most likely did not have to deal with prolonged periods of low temperature 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. During 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 ethylene signaling was active throughout the stress period. PCD plays a major role in the rubber tree cold response process and is tightly regulated by signaling cascades. Moreover, growth inhibition and cell wall thickening are implemented by the plantlets, which may be associated with DE strategy triggered by cold stress. In view of the genotypes analyzed, our results may represent the genetic stress responses developed during the
evolution of this species. The understanding of how *H. brasiliensis* copes with low temperature stress can greatly improve the breeding strategies for this crop species and emphasizes the importance of rubber tree genetic diversity preservation, since this species has such a narrow genetic base, it is impacted by climate change and is the only source for large-scale rubber production.

### Conflicts of Interest

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

### Author Contributions

AS, CS, CM and RV conceived the project, and 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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References


Tables

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

<table>
<thead>
<tr>
<th>Time period comparison</th>
<th>DEGs</th>
<th>Upregulated DEGs</th>
<th>Downregulated DEGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h vs. 90m</td>
<td>32</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>0h vs. vs 12h</td>
<td>673</td>
<td>558</td>
<td>115</td>
</tr>
<tr>
<td>0h vs. 24h</td>
<td>1,208</td>
<td>852</td>
<td>356</td>
</tr>
<tr>
<td>90m vs. 12h</td>
<td>526</td>
<td>455</td>
<td>71</td>
</tr>
<tr>
<td>90m vs. 24h</td>
<td>917</td>
<td>700</td>
<td>217</td>
</tr>
<tr>
<td>12h vs. 24h</td>
<td>18</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>(0h + 90min) vs. (12h + 24h)</td>
<td>2,436</td>
<td>1,575</td>
<td>861</td>
</tr>
</tbody>
</table>
**Figure Legends**

**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 of differentially expressed genes (DEGs) detected under the established conditions.

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

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

**Figure 4.** Gene Ontology (GO) terms for the cold response modules selected: (A) Upregulated modules; (B) Downregulated modules.

**Supplementary Figure 1.** (A) Gene coexpression network (GCN), colored according to the modules considered associated with the cold response; each node represents a gene, and each connection has a minimum R Pearson correlation coefficient of 0.8 (corrected with the highest reciprocal rank (HRR) approach). (B) GCN network constructed 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).