Differential expression and structural polymorphism in rubber tree genes related to South American leaf blight resistance

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A B S T R A C T

South American leaf blight (SALB) is the worst impairment for rubber tree cultivation in the Amazon. Hevea brasiliensis clones produce high latex yields but most are SALB-susceptible (SS). So, resistance has been searched in related species. We selected defense related genes among those differentially expressed while contrasting SALB-resistant (SR) and susceptible clones from three Hevea species. SS clones over-expressed pathways responsive to salicylic (SA) and also jasmonic acid, thus entering a self-harmful cycle. SR clones displayed tightly coordinated SA-responsive and stress-control pathways. Polymorphism in the selected gene sequences supported SS × SR clone divergence.

1. Introduction

The rubber tree breeding program operated by the Brazilian Agricultural Research Corporation at Embrapa Western Amazon (Manaus, Amazonas State) employs Hevea brasiliensis, H. guianensis, H. pauciflora and H. nitida, among other species, for experimentation in grafting and hybridization, research which supports the recommendation of technologies such as inter-specific hybrids displaying high latex productivity in addition to South American leaf blight (SALB) resistance or SALB resistant crowns grafted on highly adapted tapping panels. SALB, caused by the fungi Pseudocercospora ulei (synonymy for Microcyclos ulei), is recognized as the principal biological barrier to large-scale commercial rubber tree cultivation in the Amazon biome [1,2].

H. brasiliensis clones can be considered the best latex producers, but they are caducous and susceptible to SALB, which can lead to precocious leaf decay and spoiled latex yields. In contrast, H. guianensis var. marginata and H. pauciflora are ever green plants, tolerant or resistant to SALB [1–4] despite most clones do not exhibit high latex yields. Besides H. guianensis and H. pauciflora, H. nitida genotypes do not lose healthy leaves seasonally [5] and, interestingly, some of the hybrids from these species are tolerant or resistant to the leaf blight [1].

H. brasiliensis is also the species in the genus more frequently assessed at the molecular level. A H. brasiliensis genome draft was recently accomplished [5]. A study on the differential expression of H. brasiliensis genes enrolled in plant-pathogen interaction during the first hours post-inoculation with P. ulei is recent as well [6]. Nevertheless, large scale databases for other Hevea species are scarce.

The activation of salicylic acid (SA) responsive reactions, hypersensitivity (HR), and epidermal cell damage associated to cyanogenesis has been reported as rubber tree defense reactions against P. ulei [2,4,6]. SA, and jasmonic acid (JA) and ethylene (ET) trigger plant defense reactions, following the recognition of MAMP (Microbe Associated Molecular Patterns, also designated as PAMP, for Pathogen Associated Molecular Patterns). MAMP recognition is the first line of plant defense, based on perceiving the presence of chitin and other small molecules that interact with PRRs (Pathogen or Pattern Recognition Receptors). Reactive oxygen species (ROS) and calcium (Ca\textsuperscript{2+}) in the apoplast can also be part of MAMP and induce the production of SA and JA/ET, which is initiated in the chloroplasts. SA and JA subsequently interact with immediate downstream co-factors, which finally activate the transcription of cascades of defense related genes, and even the epigenetic remodeling of the chromatin, through histone modification. More commonly, SA is in charge of defense against biotrophic pathogens while JA/ET are involved in the reactions against herbivores and necrotrophic pathogens [see 7 for a recent review]. Nevertheless, despite SA and JA/ET pathways have been considered as mutual antagonists, the results of experimentation with mutants defective to one or more of these molecules (also designated as hormones) demonstrate that synergy can also be observed. The exposition of Arabidopsis to Penicillium chrysogenum dry mycelium induced SA and JA/ET responses simultaneously [7,8].

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The objectives of this work were to compare *H. brasiliensis* to *H. guianensis* + *H. pauciflora* clones in order to identify differentially expressed genes (DEGs), subsequently selecting for those related to plant-pathogen interaction. Leaves were collected in a region that is endemic for *P. ulei* and we considered that all the plants were equally challenged by the fungi. The metabolic processes affected by proteins encoded in 45 disease-related DEGs and their relationships to each other were assessed, to reveal interesting aspects of the interaction rubber trees - *P. ulei*, involving SA and JA-responsive pathways, under the additional stress represented by the extremely sunny equatorial climate. Finally, more than two thousands of polymorphic sites (SNPs, indels and repetitive element variations) were found in the sequences of those 45 disease related DEGs and paralog forms exclusive to the resistant or to the susceptible genotypes were identified.

2. Material and methods

Leaves in the B and C developmental stages, which are the stages when pathogen penetration-tissue colonization and sporulation can be observed, respectively [2], were collected at the main campus of Embrapa Western Amazon (3°06’S – 60°01’W), Manaus, Amazonas State, Brazil, within the region where *Pseudocercospora ulei* (Henn.)B.T.Hora & Mizubuti (synonymy for *Microcyclus ulei* Henn.)Arx) is endemic and there is high inoculum pressure [2]. The nine genotypes accessed through RNA-seq were *Hevea brasiliensis* (Willd. ex A.Juss.)Müll.Arg. clone RRIM 600 and clone RRIM 713, which are both susceptible to SALB [4,5] and the clone CNS AM 7905, an EMBRAPA breeding program selection that displays moderate resistance to SALB and is used as the tapping panel for grafting [1]; *H. pauciflora* Müll.Arg. clones CBA2, PX and PS8, which are resistant to SALB [1,2,4]; *H. guianensis* var. marginata Ducke clones Hmg 1 and Hmg 2, which are resistant to SALB [1], and a *H. guianensis* Aubl. x H. nitida Müll.Arg. (Hmg 17 x CPAA C 65) hybrid clone.

Total RNA was extracted using silica mini-columns, quantified in a micro-spectrophotometer, and evaluated in agarose gels. The cDNA libraries construction (paired ends) and NGS sequencing were accomplished in the LaCTAD facilities at the University of Campinas (São Paulo State, Brazil). Reads were mapped onto the *H. brasiliensis* reference genome [12]. Transcripts were assembled by Cufflinks [10], and differential expression analyses were performed following the Cuffdiff protocol [11]. The expression level of the consensus transcripts in *H. brasiliensis* was compared to the expression level in *H. pauciflora* and *H. guianensis*. Additional analyses were performed on 327 transcripts of proteins representing different metabolic routes, the identification of the main GO biological processes assessed and the statistical significance (P ≤ 0.05) of the clusters produced, by comparing the number of expected edges to the number of observed edges at the end of the clustering routine. In addition to the initial analysis and clustering of most of the 45 disease-related DEGs, two additional analyses were performed, separately uploading to the String servers the 25 proteins that were up-regulated in *H. brasiliensis* and those 20 up-regulated in *H. guianensis* + *H. pauciflora*.

Finally, for the 45 loci coding for the selected proteins, the consensus paralogs produced with reads from the nine rubber tree cDNA libraries were compared in order to identify species-specific structural polymorphisms. The polymorphisms were visualized using the IGV software (Broad Institute) and the IGV output was further examined by a human curator to find phenotype-specific (SALB resistant x susceptible) polymorphisms.

3. Results

The number of RNA-seq reads available from the nine *Hevea* transcriptomes ranged from 29,198 to 36,248 × 10^3. Bases displaying Q ≥ 30 were 95.03%, on average. Assembling produced approximately 25,000 consensus transcripts. Based on read frequencies, the number of DEGs (p value for the log2 fold-change ranging from 5 × 10^-05 to 1 × 10^-5) was found to range between 2,000 and 5,000 when *H. brasiliensis* transcriptomes were contrasted with *H. pauciflora* and with *H. guianensis* separately.

Among those thousands of DEGs, 327 locus were present in the data sets resulting from both (*H. brasiliensis* x *H. guianensis* and *H. brasiliensis* x *H. pauciflora*) comparisons, displaying one out of two opposite expression patterns: 1) up-regulation in *H. brasiliensis* and down-regulation in *H. guianensis* and *H. pauciflora* simultaneously, or 2) down-regulation in *H. brasiliensis* and up-regulation in *H. guianensis* and in *H. pauciflora* simultaneously. From those 327, the group of 45 items in Table 1 was selected as defense related DEGs, according to the *H. brasiliensis* reference genome annotation and the BioSystems outputs. The heat map in the Suppl. Mat. S1 is a graphical representation of the two patterns of differential expression for the 45 selected DEGs. There were differences between the transcriptomes of *H. guianensis* and *H. pauciflora* (compare G x P in the Suppl. Mat. S1). However, those differences did not cause the disruption of pattern number 1 (B > G and B > P in the Suppl. Mat. S1, first 25 rubber tree DEGs) or number 2 (B < G and B < P in the Suppl. Mat. 1, last 20 DEGs). So, the values for the expression level (kfp units) in Table 1 were calculated using RNA-seq reads from the set of *H. guianensis* reads + *H. pauciflora* reads (the resistant clones/genes) to contrast with the reads from the set of *H. brasiliensis* clones/genes (the susceptible clones/genes). The direction of the changes in the expression level is indicated by the signals preceding the numerical values: a minus sign (−) indicates expression pattern number 1 and is observed for the first 25 items in Table 1; in agreement with the heat map in Suppl. Mat. S1. The opposite pattern, pattern number 2, is indicated by a plus sign (+), and was observed for the last 20 items in Table 1, in agreement with the heat map in Suppl. Mat. S1 as well. Despite the same DEGs were included in Table 1 and Suppl. Mat. S1, the ordination of the DEGs is not the same in those two information sources. This is because, for Table 1, the 45 entries were organized according to the fold-change values, while, for the heat map in Suppl. Mat. S1, entries were displayed in descending order according to the expression level in the *H. brasiliensis* transcriptome. The correspondence between the two information sources is made by the use of the ordination numbers taken from Table 1 (# column) to identify the DEGs in the heat map (numbers in the left border of the heat map, Suppl. Mat. S1).

Homolog identity in the reference genome [5] and the BioSystems outputs diverged in a few cases, despite the fact that both identifications were made through the screening of *Arabidopsis* databases (see Suppl. Mat. S2 for the AOR coding sequence from the *A. italicana* Ler
ecotype and the 2-methylene-furan-3-one reductase-like sequence from the Col ecotype referring to item 30 homologs). This certainly occurred because during the six or seven years from the publication of the H. brasiliensis reference genome [5] and the database screenings made for the present work, data have been constantly uploaded, creating the opportunity to hit additional homologs to rubber tree sequences. Besides that, the screening of Arabidopsis protein databases (the BioSystems screening for this work) instead of non-redundant protein databases (the screening for the reference rubber tree annotation [5]) can result in discrepancies. Non redundant databases include Arabidopsis proteins and a huge amount of deduced peptides from other plant species. Nevertheless, the BioSystems and the String (see below) routines are available for a limited number of species and Arabidopsis was the best choice, since it is a dicotyledon and maybe the species better explored to model plant-pathogen interactions, and this grants the access to constantly updated, informative databases. Discrepancies were commented in the sub-sections of the Results, when it was relevant to comprehend the information in the text.

So, following the BioSystems identification of the best Arabidopsis orthologs for the proteins encoded in 327 DEGs, the association of the proteins with Gene Ontology (GO) terms and the selection of those 45 defense related DEGs/encoded proteins which are included in Table 1, the clustering was performed using the String routines (output is in Fig. 1 and see also Suppl. Mat. S3 for the Analysis Page). As an aid to the BioSystems analysis, String clustering was important to show how those 45 proteins interacted to accomplish different metabolic processes, justifying and supporting their association to ascertained GO terms. The 45 entries in Table 1, which are identified by the same formal symbols...
and/or loci designations (Table 1, Protein symbol) in Fig. 1, are henceforth referenced as “native” to the present study. In addition to these 45 “native nodes”, Fig. 1 included proteins automatically tracked from the A. thaliana String databases due to the existence of data attesting for their direct or indirect interactions with the native ones. The automatically tracked non-native proteins/nodes are, as well, identified by their formal symbols, which can be used to recover additional information about them (see also Suppl. Mat. S3A with the legends for the String clustering output).

Indeed, the protein sub-clusters/groups of nodes in Fig. 1 were automatically displayed with different colors to distinguish the metabolic processes they are enrolled in (see Suppl. Mat. S3 and S3A, Analysis and Legend pages, for the identification and statistical significance of the sub-clusters representing the principal physiological processes that were assessed during clustering). With the exception of items 5, 9, and 18, all of the proteins in Table 1 joined one of the 12 sub-clusters of nodes in Fig. 1 (see Table 1, S-C). To construct a wide picture of the leaf metabolic status based on the relationships of as many as possible of the native proteins to some of the sub-clusters/metabolic process. Nodes of the same color pertain to the same sub-cluster/metabolic process (see details in Supp. Mat. S3).

Fig. 1. Proteins coded by differentially expressed defense-related Hevea spp. genes clustered according to their relationships to each other and based on the information for A. thaliana orthologs available in the String databases. Each node is a protein indicated by its symbol. Native proteins are those included in Table 1. The other proteins were automatically tracked from the String databases to produce an overview of the cellular metabolism while represented through the association of as much as possible of the native proteins to some of the sub-clusters/metabolic process. Nodes of the same color pertain to the same sub-cluster/metabolic process (see details in Supp. Mat. S3).

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The major cluster in Fig. 1 (specimens 1 to 45) was subsequently divided into two representations of metabolic processes accomplished only by proteins up-regulated in the H. brasiliensis clones (specimens 1 to 25 in Table 1, Suppl. Mat. S4) or just by proteins up-regulated in the H. guianensis + H. pauciflora resistant clones together (specimens 26 to 45 in Table 1, Suppl. Mat. S5), to construct theoretical scenarios, ignoring down-
regulated proteins (specimens 26 to 45 for Suppl. Mat. S4 and 1 to 25 for Suppl. Mat S5) as if they were not expressed at all. These theoretical scenarios were useful to estimate the consequences, e.g., of silencing down-regulated proteins in each of the two sets of rubber tree clones/genotypes.

The second section of the Results is about the structural polymorphisms found in the transcripts produced with reads from the different RNA-seq libraries.

3.1. Metabolic pathways running in the rubber tree leaf cells collected for RNA-seq

3.1.1. Membrane proteins and processes occurring in the organelles

The proteins in the red sub-cluster in Fig. 1, according to the associated GO terms, are all related to processes that take place in the organelles (chloroplasts in particular) and involve trans-membrane trafficking.

The native members in the red sub-cluster (Table 1, S-C = R) up-regulated in H. brasiliensis and down-regulated in H. pauciflora + H. guianensis, and so following the expression pattern number 1 (see above), were items 7 (ortholog to AT4G24350), 21 (AT1G05675), 22 (AT3G47070), and 25 (ABC27). These four proteins lack characterization even in A. thaliana and, for this reason, the BioSystems output for three out of the four only included the homologous loci identities. In addition, proteins 21 (AT1G05675) and 25 (ABC27) did not cluster with those up-regulated in H. brasiliensis (Suppl. Mat. S4). Item 18 by its turn was up-regulated in H. brasiliensis as well but did not cluster at all (Fig. 1, down in the right corner). Absence of edges tracking these native proteins to a sub-cluster may just mean that an even higher order of interactions would be necessary to attract them. On the other hand, maybe these proteins have roles in metabolic routes not sufficiently explored. Intriguingly, when those three “outliers” up-regulated in the susceptible genotypes (items 18, 21 and 25) were uploaded to the String servers as an independent set of baits for clustering, a tiny protein cluster associated with the metabolic process designated as “ribosomal sub-unit export from nucleus” (GO:0000054) was produced, including a proteasome activating protein (PA200, Suppl. Mat. S6). Ribosomal sub-units are assembled in the nucleoplasm through the joining of rRNA and proteins imported from the cytoplasm. Assembled sub-units are then exported back into the cytoplasm through the recruitment of transporters [17], a process which occurs during apoptosis [18]. Despite ABC27 joined the cluster of proteins in Suppl. Mat. S6, involved in “ribosomal sub-unit export from nucleus”, reports on the specific participation of ATP-binding transporters in such a process could not be recovered. According to the homologous locus AT5G39040 description in Arabidopsis, the rubber tree ABC27 would more likely be involved in the transportation of Fe–S clouds in mitochondria.

Taking all this together, it can be considered that susceptible rubber tree plants were going through organelle recycling and non-programmed cell death (apoptosis).

By their turn, also in the red sub-cluster (Table 1, S-C = R), items found to be up-regulated in H. pauciflora + H. guianensis and down-regulated in H. brasiliensis, in opposition to the proteins described in the previous paragraph, were the homologs to CNGC4 (encoded in transcript 29, Table 1), AOR (encoded in transcript 30, corresponding to the locus AXX17-AT1G24910 ecotype Landsberg Erecta, bioproject PRJNA311266 in the NCBI), AT4G02530 (item 32), MSL10 (item 37), and LRR1 (item 43).

CNGC4 codes for a plasma membrane calmodulin-binding protein, which is also known as DND2 for “defense, no death” and MHL1 as well. It is associated with the “plant-type hypersensitive response” (HR) [19]. CNGC4 can homodimerize or heterodimerize with CNGC2, its closest paralog, to form cation gates that actively transport Ca2+ across the plasma membrane. Though their development of the HR response is impaired, A. thaliana cngc4 loss-of-function mutants exhibit autoimmune phenotypes, with constitutive expression of pathogenesis-related genes, elevated levels of SA, and, in some circumstances, the production of spontaneous HR-like lesions in the absence of pathogen infection or when infected by avirulent pathogens [20].

AOR is involved in the initial steps of reactive carbonyl detoxification in chloroplasts. Short-chain carboxyls are produced by stress-triggered oxidation of membrane-bound long-chain fatty acids, such as linoleic and linolenic acids, and could harm the photosynthetic apparatus [21].

MSL10 is associated with 20 GO terms, such as programmed cell death in response to ROS (GO:0097468), detection of mechanical stimulus (GO:0050982), gated channel activity (GO:0022836), and substrate-specific trans-membrane transporter activity (GO:0022891), in addition to “phyllode development” (GO:0090693, see additional information below). MSL10 could serve as an early signal of wounding [22]. Resembling CNGC4 described previously, MSL10 participates in ion-gated transport and programmed cell death induction as separate activities. The protein amino-terminal (N-terminal) domain can induce cell death in response to ROS accumulation. The protein carboxy-terminal (C-terminal) domain participates in the assembly of a mechanosensitive channel, which can respond to osmotic stresses that cause the plasma membrane to stretch [23]. The C-terminal could stabilize the N-terminal portion, avoiding spontaneous cell death in non-wounded plants [22].

LRR1 is a serine/threonine kinase that contains extracellular lectin motifs resembling legume soluble (L-type) lectins, which may serve in the recognition of small hydrophobic ligands, such as MAMP-related molecules. Among the L-type kinases, H. brasiliensis reference locus 110652570 (item 43) was identified as a LecRK-Iv.1 ortholog, which displays extremely high activity 52 h after exogenous SA application [24].

So, the red sub-cluster native proteins which were up-regulated in the resistant genotypes, indeed following the expression pattern number 2 (see above), have roles in the fast and effective detection of the pathogen employing trans-membrane MAMP-recognition, and in disciplined (non-constitutive) production and response to SA, prompting the cells for defense based in precisely programmed cell death coordinated with the restrain of the oxidative stress at defense-useful levels. The lack of these controlling mechanisms probably led susceptible plant cells to apoptosis and organelle recycling, as mentioned above.

The protein encoded in transcript 32, the last native member of the red sub-cluster, codes for a poorly characterized protein expressed in the chloroplast thylakoid lumen.

3.1.2. Ubiquitination versus hypersensitive response

The lime green sub-cluster, in the center of Fig. 1, is populated with proteins involved in ubiquitination and ligation of proteins to ubiquitin. The native proteins (Table 1, S-C = LMG) included in this sub-cluster which are expressed in higher levels in H. brasiliensis are UBQs 11 and 9 (items 19 and 20 in Table 1, respectively). The ubiquitination process involves covalent attachment of the highly conserved small protein ubiquitin to substrate proteins through a step-wise enzymatic cascade typically catalyzed by three different classes of enzymes: ubiquitin-activating enzyme (E1 or UBA), ubiquitin-conjugating enzyme (E2 or UBC), and ubiquitin ligase (E3), which transfers ubiquitin to the substrate. Conventionally, ubiquitination serves as the principal signal for protein degradation by the 26S proteasome [reviewed in 25]. Ubiquitination interacts with the autophagy of larger non-proteinaceous structures of cells, such as ribosomes [26], and this connects the red and the lime-green sub-clusters of proteins and the metabolic routes they represent in H. brasiliensis.

Meanwhile, proteins in the lime-green sub-cluster that were up-regulated in H. pauciflora + H. guianensis are involved in HR (GO:0010363) and Ca2+ transportation, just as those in the red sub-cluster (see above). According to the BioSystems output, the best hit for item 28 (Table 1) was AT1L2, which is another MAMP-recognizer that responds to chitin [27]. Nevertheless, the best hit for the conserved

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domains in the ortholog protein was to RPM1-interacting protein 2 (RIN2), which is involved in the positive regulation of the HR and also in the ubiquitin-dependent protein catabolic process (GO: 0006511), fitting better to the annotation as a defense (Table 1, _H. brasiliensis_ reference genome annotation). RIN2 from _A. thaliana_ and the rubber tree protein encoded by item 28 display the same C3HC4-RING type zinc finger and CUE ubiquitin component domains. RIN2 exerts localized E3 ubiquitin-ligase/transferase activities, while its partner, RPM1, is a CC-NB-LRR protein that recognizes pathogens by their specificities and induces rapid ion fluxes, sustained oxidative bursts, and a basal defense transcriptome, often accompanied by localized programmed cell death at the infection site [28]. The C-terminal of RIN2 (and RIN3) is involved in the interaction with RPM1, as well as in RIN2-RIN3 homo- or heterodimerization [29], which is necessary to accomplish the transfer of ubiquitin to substrate proteins. Thus, a decrease in the availability of ATL2/RIN2-like proteins could protect RPM1 from degradation, inducing the downstream defense reactions, including cyanogenesis, to be primed. Kawazaki et al. [29] reported this hypothesis but could not fully demonstrate it. Considering the reduction in gene expression by almost two folds in _H. brasiliensis_ relative to _H. guianensis_ + _H. paufficola_ (Table 1), a decrease in the availability of the ATL2/RIN2 protein and the priming of defense reactions can be anticipated in the first genotypes.

**NTR1:2** (coded in transcript number 34, Table 1) is an integral plasma membrane transporter that responds to Ca$^{2+}$ and moves nitrate and asbsic acid (ABA) molecules non-competitively [30], a process which may modulate growth rhythms according to the needs to activate or not defense related stress-control.

Resistant rubber trees would, so, have more than a set of up-regulated genes involved in pathogen recognition and ion movement across membranes, because proteins in the lime-green sub-cluster (Table 1, S-C = LMG) would join those in the red sub-cluster (Table 1, S-C = R) to trigger tightly controlled defense reactions. Meanwhile, growth, or at least photosynthesis and the primary metabolism, would not be put aside, if the influx of ABA was in tune with the efflux of Ca$^{2+}$ to the apoplast, where it can contribute to characterize MAMP (and additional physiological processes that are described below). Instead, in susceptible genotypes, the slower or mild MAMP-recognition due to the down-regulation of _RLK1_ and _ATL2/RIN2_ and _MSL10_ would aid the decreased expression of _CNGC4_, in the arise of which could be considered a delayed and disorganized excessive defense reaction.

### 3.1.3. Cell-cell junction, cell wall modification and cytoplasm detoxification are up-regulated in _H. guianensis_ + _H. paufficola_

With the exception of CBL2 that was tracked from the String databases, the small blue sub-cluster (Table 1, S-C = B) in the upper corner of Fig. 1 consists entirely of members native to the present study, which were up-regulated in the resistant _H. guianensis_ + _H. paufficola_ genotypes. The two members of the brown sub-cluster (Table 1, S-C = BR) that intersected the blue sub-cluster in Fig. 1 followed exactly the same trend. So, the blue and the brown sub-clusters are populated exclusively with proteins encoded in DEGs that followed expression pattern number 2.

Proteins HB-12, PMR6, and MEE, encoded in transcripts 31, 35, and 40 in Table 1, are involved in biological processes such as “response to biotic stimuli” (GO:0009607), “response to fungi” (GO:0009620), and “response to chitin” (GO:001200). HB-12 is a leucine zipper protein that responds to oxidative stress, water stress, and asbsic acid. The coordinated expression of HB-7 and HB-12 could modulate growth in the context of water stress [31]. In _A. thaliana_, PMR6 is involved in JA/SA-independent resistance mechanisms and exhibits pectate-lyase enzymatic activity, which is important for cell wall properties modifications [32] and drought tolerance [33]. MEE proteins have DNA binding domains and are most likely involved in DNA repair in the context of rubber tree leaves considering the description of its characteristics in _Arabidopsis_.

Still related to the cell wall, in the brown sub-cluster, item 33 encodes a xyloglucan hydrolase, and item 39 encodes an ortholog to PGIP1, a polygalacturonase inhibitor protein, which inhibits pectin-degrading enzymes produced by fungal pathogens. PGIP has been demonstrated to be up-regulated upon infection of _A. thaliana_ by _Stemphylium solani_ [34].

Back to the native proteins in the blue sub-cluster, item 27 from Table 1 code for the ortholog to _A. thaliana_ CYPK14 in rubber trees. CYPK14 takes part in the Ca$^{2+}$-dependent signal transduction mechanism, forming pairs with calcineurin B-like (CBL) proteins. CBLs could be sensors of Ca$^{2+}$ concentration and CIPks, the signal transducers. CBL/CIPK complexes, which are present in a variety of cell membranes, regulate calcium storage and release into the cytoplasm, and import and export to the apoplast, the tonoplast/vacuole, the endoplasmic reticulum, the mitochondria, and the chloroplasts. Specifically, CBL2/CIPK14 complexes were found to be located in the _A. thaliana_ tonoplast membrane [35]. It will be interesting to search for the rubber trees to CBL partner of the CIPK14, but we can already speculate that the Ca$^{2+}$-responsive pathways would include CNGC4, MSL10, and NTR1:2, which joined the red and the lime-green sub-clusters.

Item 38 by its turn code for a plasma membrane-ATPase associated with the GO terms “cell-cell junction” and “symplast”, and designated by the symbol HA2. HA2 is an integral membrane protein that may also be located in the internal membrane system, including the Golgi apparatus and the vacuolar membrane, or in plasmodesmata membranes, according to the corresponding _Arabidopsis_ locus (Table 1) description. It is important for the symport of molecules accompanied by H$^{+}$-ca
tions. HA1 and HA2 are paralogs and apparently play redundant functions in most plant organs, though HA2 expression has been primarily studied in the roots. ha2 mutants display reduced growth when subjected to high external pH or potassium concentrations and grow better than wild-type plants when exposed to toxic cations and other intoxicating molecules, such as amino-glucose antibiotics. Nevertheless, when the H$^{+}$ transportation is reduced by an increased external concentration of potassium, imports of toxic molecules are reduced in wild-type plants [36].

Is it possible that the Ca$^{2+}$ ions in the resistant genotypes play a role similar to that of potassium [37] and contribute to the prevention of cellular intoxication during cyanogenesis? If the consequences of the three-fold decrease in HA2 expression observed for _H. brasiliensis_ (Table 1) can be compared to the ha2 loss of function mutations reported for _A. thaliana_ [38], than it can be considered that these two genotypes would grow better under intoxicating conditions than their counterparts in the same genus. Meanwhile, _H. guianensis_ + _H. paufficola_ would rather be prepared to control and limit inter-cellular pectin degradation and water loss resulting from fungi exploration of the stomatal cells by moving Ca$^{2+}$ among the different cellular compart-
ments. The increase in extra-cellular Ca$^{2+}$ driven by NTR1:2 (Table 1, item 34, lime green sub-cluster) would facilitate the import of ABA from the apoplast to spread stomatal closure, balancing growth and defense-related metabolic pathways. And, maybe, Ca$^{2+}$ in the apoplast would assure that free HCN$^{-}$ an other intoxicating molecules produced out-side the cell membrane (see below) could not reach the cytoplasm for a while. As an extra advantage, Ca$^{2+}$ in the apoplast would enhance MAMP recognition. It is revealing that none of these processes would be up-regulated in the susceptible genotypes.

### 3.1.4. _H. brasiliensis_ striking defense reactions can be self-harmful

Native proteins in the dark cyan sub-cluster (Table 1, S-C = DCY) at the center of Fig. 1 and its neighbor to the right, the cyan sub-cluster (Table 1, S-C = CY), were all coded in transcripts up-regulated in _H. brasiliensis_. The same is true for the pink sub-cluster (Table 1, S-C = PK), in the bottom left corner of Fig. 1 and the sandy brown (Table 1, S-C = SBR) sub-cluster just beneath. These four sub-clusters consist almost exclusively of proteins that are responsive to JA and all of them
followed the expression pattern number 1.

Many native members of the cyan sub-cluster are TIFY/JAZ transcription factors (items 1, 6, 8, 10, 13, and 14 Table 1). TIFY/JAZ protein clusters were not observed among those produced only with proteins up-regulated in the resistant genotypes (Suppl. Mat. S4 x Suppl. Mat. S5). In addition to RLP1 (item 15), which was identified as a pattern recognition receptor (PRR) that can interact with MAMPs and LCR69 (item 23), which is homologous to PDF1.2, these proteins last mentioned are all activated in response to JA [38,39]. As the exception, the pathogenesis-related protein PR1 (item 11) that participates in more specific downstream reactions is, according to the Arabidopsis locus description, responsive to SA [see 8 too]. Interestingly, PR1 is also involved in the management of excess excitation energy, which causes plastoquinones in the chloroplasts and mitochondria to enter altered redox states, leading to cell death and tissue damage [40]. In addition, the extent of tissue damage can also be related to cyanogenesis intensity [41].

The BOL A protein (item 3, Table 1) is involved in controlling the redox status in chloroplasts or mitochondria as stated in the A. thaliana locus AT5G17560 description; the PAT1 protein (item 17, Table 1) is involved in light signal transduction mediated by Phytochrome A and the sieve element occlusion protein, SEOs (item 12, SBR Table 1), is important for the organization of phloem cells to form sieve elements. A. thaliana paralogs SEOR1 and SEOR2 can heterodimerize to each other and drive phloem constriction. In addition, SEOR2 can physically interact with RPM1-RIN4 systems and impair phytoplasma dispersion, regardless of sieve element occlusion. It has been speculated that, by reducing the availability of one of the SEOR paralogs, the other one becomes free from dimerization and available to activate defense responses instead of phloem constriction [42].

To be short, in the susceptible genotypes a second, JA-triggered, defense line against P. ulei, would be initiated, following RLP1 up-regulation, maybe because the SA-triggered defense mechanisms became mild and arrested, and insufficient to diminish the invasive capacity of the pathogen, despite possibly constitutive in the lack of sufficient CNGC4 (see the Discussion).

3.1.5. Cell wall reinforcement versus stress attenuation and renovation of the crown

The light green sub-cluster (Table 1, S-C = LGG) in the upper right corner in Fig. 1 included the native proteins LDOX (item 2) and TT4 (item 16), which are up-regulated in H. brasiliensis, following expression pattern number 1. Both proteins were found to be consistently associated with a same list of GO terms, such as cellular response to oxidative stress (GO:0009779), response to JA (GO:0009753), and response to wounding (GO:0009611). LDOX is a leucoanthocyanidin synthase that likely participates in vacuole organization as well. The synthesis of anthocyanins in the leaves aids in the protection of photosystems against intense light. TT4 is a chalcone synthase, and chalcone is a precursor for various classes of flavonoids, which are themselves important for the lignification of the cell walls and the synthesis of anthocyanins. Most of the proteins in the light-green sub-cluster are involved in phenylalanine, flavonoid, anthocyanidin, and phenylpropanoid biosynthesis and participate in the reinforcement of the cell wall by lignification, which is a common defense reaction against fungi, one of a few pathogens that can destroy lignin [43].

In the same light-green sub-cluster, the protein encoded in transcript 26, SRG1 (item 36), and PER64 (item 41, Table 1) were found to be up-regulated in H. guianensis + H. pacifica, following expression pattern number 2. PER64 is a peroxidase specifically involved in the guidance of lignin deposition, a process that is well characterized in the endodermis of roots [44]. SRG1 and MSL10 (see above) genes were both up-regulated in H. guianensis + H. pacifica and also were the only two proteins in the whole experiment found to be associated with the GO term “phyllome development” (GO:0090693), meaning “the process whose specific outcome is the progression of a phyllome over time, from its formation to the mature structure. A phyllome is a collective term for all of the different types of leaves appearing on plants”, according to the Gene Ontology database. Up-regulation in the evergreens H. guianensis and H. pacifica is certainly related to the continuous renovation of the crown, which could employ programmed cell death and controlled senescence, in contrast to H. brasiliensis plants that are caducous.

SRG1 is involved in senescence and was very recently associated with a nitric oxide-driven negative feedback that attenuates the plant’s immune response. Nitric oxide follows ROS bursts in response to pathogen attack and activates SRG1 transcription. SRG1 would hijack immune response co-repressors, such as TOPLESS, and transiently contribute to enhance defense reactions while promoting its own transcription. However, continuous accumulation of nitric oxide would eventually lead to the nitrosylation of SRG1 itself, which becomes unable to function as a transcription factor. Decreased SRG1 expression or activity results in the accumulation of PR-1 transcripts [45], and this interaction connects the light-green sub-cluster to the cyan and dark cyan sub-clusters (see above). Indeed, continuous ROS and nitric oxide accumulation could be the cause for the three-fold down-regulation of SRG1 transcription in H. brasiliensis relative to H. guianensis + H. pacifica (Table 1).

Phenylalanine-, flavonoid-, anthocyanidin-, and phenylpropanoid-related proteins were not represented in the String clusters for up-regulated H. guianensis + H. pacifica items only (Suppl. Mat. S5) as strongly as in the clusters for up-regulated transcripts in H. brasiliensis (Suppl. Mat. S4). On the contrary, susceptible genotypes did not over-express proteins dedicated to pectin modification (PMR6 in the blue sub-cluster), or cell wall extension and enhanced flexibility (XTH8 in the brown sub-cluster) or the polygalacturonase inhibitor (PGPI in the brown sub-cluster). This result is interesting because susceptible and resistant genotypes activate defense mechanisms related to the cell wall, but in the resistant genotypes cell wall properties, such as elasticity, stiffness and flexibility [32,33], were assessed while in the susceptible ones, wall thickening driven by a reduction in PMR6 expression [32] and lignification were triggered.

3.1.6. Cyanogenesis

The small purple and the yellow sub-clusters (Fig. 1) contain proteins that are committed to the synthesis of glucosinolates and cyanogenic glycosides (GO processes 0019757–0019761 and 0016137–0016139). Glucosinolates evolved from cyanogenic glycosides, and enzymes involved in glucosinolate synthesis would occur in plant species, such as A. thaliana, displaying a sort of ancestral “pre-disposition” [46]. This supports the use of A. thaliana as model for the rubber tree reactions activated in cyanogenesis.

Cyanogenic glycosides are products of the conjugation of aglycon moieties or cyano-amino acids (produced by the conjugation of HCN with amino acid residues) with glucose [4]. The most abundant cyanogenic glycoside in rubber trees is linamarin, which is produced in the leaf cytoplasm and stored in special vacuoles. Under attack, the contents of the cell vacuoles come in contact with the cytoplasm [47], and specific B-glucosidases remove the glucose moiety producing free aglycon. Then, hydroxynitrile lyases at the plasma membrane break the aglycon to produce free HCN and aldehyde or ketone [48]. This mechanism keeps cyanide away from the electron transport chains in chloroplasts and mitochondria and makes the leaf exudation produced by the lesions extremely toxic for most pathogens and herbivores, but not for P. ulei [49], as detailed in the Discussion.

The native member of the purple sub-cluster that is up-regulated in H. brasiliensis was inscribed as the valone monooxygenase 1 in H. brasiliensis reference genome (Table 1, item 4, S-C = PRP). The cyano-amino acids derived from valone through the reaction catalyzed by the valone monooxygenase are precursors of aglycons used to produce linamarin (and lotaustralin) upon glycosylation (Suppl. Mat. S7). Indeed, locus 110641550 codes for a peptide that presents better homology to
valine monooxygenases from *Manihot esculenta*, including CYP79D2 [50], and from *Ricinus communis* which are cyanogenic species, than to the CYP79B2 from *Arabidopsis*, which is a monooxygenase that uses tryptophan as substrate for the cyano-amino acid production (Suppl. Mat. S7). Nevertheless, the sequence for the *Arabidopsis* CYP79D2 is not available in the *Arabidopsis* databases. In this reason, the CYP79B2, the best homolog available for the rubber tree peptide encoded in transcript 4 was hit by the BioSystems.

The native member of the purple sub-cluster that was up-regulated in the resistant genotypes *H. guianensis* + *H. pauciflora* is an ortholog of the *A. thaliana* CYP81D1 (Table 1, item 42, S–C = PRP). The only CYP81 family members are currently included in the CYP81E subclass. Based on its homology to the *Arabidopsis* enzyme and the association with the light-green sub-cluster, CYP81D1 is a monooxygenase (cytochrome P450) that could be primarily involved in isoflavonoid biosynthesis, which is also inducible and may be defense related [51], (Suppl.Mat.S8). Nevertheless it was automatically included in the purple sub-cluster, which is populated with proteins involved in cyanogenesis. Cyanogenesis and phenylpropanoid biosynthesis and accumulation can interfere with each other [52], and this CYP81D1 monooxygenase could play a role in the modulation of defense by cyanogenesis versus growth by the production of cell wall precursors. Alternatively, the rubber tree CYP81D1 ortholog may be enrolled in cyanogenesis only, but it has not yet been sufficiently studied or characterized in rubber trees or other cyanogenic plant species.

In the yellow sub-cluster, BGLU17/24 (encoded in transcript 24, S–C = Y in Table 1) is up-regulated in *H. brasiliensis*. The peptide deduced from transcript 24 is extremely similar to linamarases from *Manihot esculenta* and *Populus tomentosa* (Fig. 2), which also produce linamarin [43]. The second protein in the yellow sub-cluster is MES5 (encoded in transcript number 44, S–C = Y in Table 1), which is up-regulated in the resistant genotypes *H. guianensis* + *H. pauciflora*. According to its homology to *Manihot esculenta* and *Populus tomentosa* proteins, MES5 is a hydroxynitrile lyase (see also Suppl. Mat. S9), acting in the last step of cyanogenesis, when the aglycon is broken and HCN⁻ is released from injured cells [4].

Indeed, taking into account CYP79D2 and BGLU17/24 up-
regulation, the increase in linamarin production and the release of aglycons from linamarin could be, respectively, 5 and 1.5 folds higher in *H. brasiliensis* (Table 1, items 4 and 24). However, these two processes would not be coordinated with the increase in HCN− release in *H. brasiliensis* P.C.S. Angelo, et al. Physiological and Molecular Plant Pathology 110 (2020) 101477

3.2. Polymorphisms identified in differentially expressed genes

Finally, the green sub-cluster of proteins, down in Fig. 1, is vastly dominated by histones and nucleosome organizers. Only one native protein encoded by a transcript included in Table 1 joined this sub-cluster: the H2B-like histone, which is ortholog to HBT4 from *A. thaliana* (item 45, G in Table 1) and is up-regulated in *H. guianensis* + *H. pauciflora*. The vast majority of proteins in this green sub-cluster were, indeed, tracked from the String databases by item 45, and are connected to proteins in the lime green, dark cyan, and red sub-clusters due to the interactions between ubiquitination, the cell cycle, and processes occurring in the chloroplasts, including chloroplast regeneration and ribosome assembly. The modification of histones can be part of defense strategies based in chromatin remodeling [7] and would be active just in resistant genotypes.

3.1.7. Histones and nucleosome organizers

In *H. brasiliensis* susceptible genotypes, the down-regulation of LRK1 and *ATL2/RIN2* and *MSL10* would delay MAMP recognition in young leaves, facilitating the occurrence of epidermal cell necrosis [2] and the evolution of the initial abaxial conidial lesions in stages B/C to the adaxial mature pycnidia in stages C/D [53]. A reduced expression of the polygalacturonase inhibitor (PGPI), which is allocated in the cell walls to decelerate pathogen invasion [34], contributed to ease the colonization process and to trigger JA responsive pathways. In these susceptible genotypes the pathosystem is supposed to have reached the epidermal cell cytoplasm and, maybe, even the vascular bundles, judging by the up-regulation of *PR1* [7] and *SEOa* [42]. And it is intriguing that in cell wall lignification and thickening was not effective (but see below). At this point, the activation of JA-triggered pathways was, probably, initiated by RLP1, which is encoded in the only MAMP-recognition gene up-regulated in susceptible genotypes, and mediated by LCR69, which is an ortholog to PDF1.2 [38,39]. The up-regulation of these two last genes in addition to four TIFY/JAZ proteins which are all responsive to JA and the simultaneous up-regulation of the *PR1* ortholog that responds to SA [7,8], indicated that SA and JA-responsive pathways were active in susceptible genotypes. SA-JA cross-talk and synergy has already been documented [8,54]. And so, despite delayed as a consequence of deficient *LRK1*, *ATL2/RIN2* and *MSL10* transcription, which was 3.9, 1.8 and 2.9 folds lower, respectively, in susceptible genotypes, SA responsive reactions would be active, enhancing cell death, due to the 1.8 folds lower expression of the *CNGC4* gene [19,20]. This “state of war” combined with cyanogenesis and direct exposure to the high light intensities of equatorial latitudes could disturb and conduct organelles to disassemble [38,40,41,55] and proteins to enter ubiquitination and recycling [25,26], thus accelerating the natural predisposition of *H. brasiliensis* toward seasonal leaf senescence.

Resistant genotypes *H. guianensis* + *H. pauciflora*, however, could display precisely controlled and rapidly triggered SA-responses following pathogen recognition, accomplished by *LRK1* and/or by *ATL2/RIN2* partners, which had the expression 3.9 and 1.8 folds increased, respectively, in resistant genotypes. The induction of HR under the control of *CHGC4* [19,20], which was also up-regulated, indicated the activation of SA-triggered responses. The deposition of delicate lignin strips associated with PER64 activity in internal leaf cellular rows [44,56], and the up-regulation of *PGPIP1*-driven fungal-polygalacturonase inhibition, both would impair the SALB spreading, even when the *PMR6* was up-regulated too. The PMR6 pectate lyase can function as a “susceptibility factor”, but up-regulation in the resistant genotypes would, simultaneously, contribute to maintain the cell wall flexible and able to extend, grant the contact of the fungi with the PGIP as soon as possible, and assure a long term up-regulation of *MSL10*, which encodes a stress-related mechano-sensitive channel able to recognize wounding [22,23], in young leaves that are expanding. Meanwhile, oxidative and osmotic stresses, in the cytoplasm and the organelles, would decrease under up-regulated Ca^{2+} trafficking and nitric oxide related mechanisms controlled by SRG1 [45,57], providing the conditions to withstand *P. ulei* and escape leaf senescence and decay. Ca^{2+} and nitric oxide could both induce JA related processes [7]. Nevertheless, JA responsiveness would be under SA control in resistant genotypes. NPR1 is necessary to stop JA-triggered responses and...
it is responsive to SA [7]. Since it was not present among the DEGs maybe NPR1 was equally expressed in resistant and susceptible genotypes at the moment of the collects. Indeed the superimposed operation of JA and SA-triggered responses in susceptible genotypes could be imputed, as mentioned above, to the delay in the initial MAMP-recognition, leading to the delay in NPR1 up-regulation in susceptible plants, while epidermal cell necrosis and tissue invasion were taking place [2,49,53].

Regarding cyanogenesis specifically, the resistant genotypes expressed the monooxygenase CYP81D1 and the MES5 lyase orthologs in higher amounts, while the susceptible genotypes over-expressed the monooxygenase CYP79B2 and the linamarase BGLU17/24 orthologs. Indeed, as already reported, susceptible genotypes can display higher cyanogenic potential (the preparation and storage of cyanogenic
precurors) and, simultaneously, lower cyanogenic capacity due to the lower or slower HCN− release in the extracellular spaces [4], which is, judging by the results presented herein, at least in part, a consequence of insufficient MES5 hydroxynitrilire lyase gene transcription. Anyway, considering the accumulation of high contents of linamarin in cells/ tissues, which can be inflated in response to JA [58], even mild MES5 lyase activity could lead to, slow but constant and cumulative production of free HCN− in susceptible genotypes.

In contrast, the SALB resistant H. guianensis and H. pauciflora would have a lower cyanogenic potential [4] due to decreased CYP79B2 monoxygenase and BGLU17/24 B-glucosidase expression, but extra-cellular HCN− could be rapidly released, as soon as the over-expressed MES5 lyase could access some aglycon. In addition, HCN− can reportedly be produced by the natural decay of the aglycon, particularly at alkaline pH [4], which could, possibly, be granted by the trafficking of Ca2+ to the apoplast, and young P. ulei mycelia have been found to present better vegetative growth in the presence of HCN− than in its absence [49]. Indeed, resistance would relay on the equilibrium among vigilance, internal stress control, maintenance of housekeeping processes that support growth and the provision of a comfortable environment for the pathogen outside the cells, which would also contribute to prevent sporulation [49] and the colonization of cellular inner spaces.

The differences between the two physiological conditions - resis- tance or susceptibility - were also supported by the identification of contrasting allele configurations that prevailed in one or the other. The H. brasiliensis clone CNS AM 7905, which has been classified as moderately resistant to P. ulei in field observations, as well as the H. guianensis x H. nitida hybrid, were distinguished by their “blended” or hybrid haplotypes. The gene sequence configurations can interfere in transcript accumulation and/or protein effectiveness, thus contributing directly to define resistant or susceptible phenotypes. The analysis of segregation and validation of the polymorphisms identified herein will make it possible for some to be used as genetic markers and to aid in the selection of resistant plants. Finally, the other 282 DEGs, some of them possibly related to defense reactions as well, must be examined (Suppl. Mat. S13).

CRediT authorship contribution statement

Paula Cristina da Silva Angelo: Conceptualization, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. Michel Eduardo Beleza Yamagishi: Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. Jeferson Chagas da Cruz: Investigation. Gilvan Ferreira da Silva: Project administration, Investigation, Writing - review & editing. Luadir Gasparotto: Investigation, Writing - review & editing.

Declaration of competing interest

Authors inform that there is no conflict of interest regarding the publication of these data.

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Appendix A. Supplementary data

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