Genome-wide analysis in *Hevea brasiliensis* laticifers revealed species-specific post-transcriptional regulations of several redox-related genes

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MicroRNA-mediated post-transcriptional regulation has been reported on ROS production and scavenging systems. Although microRNAs first appeared highly conserved among plant species, several aspects of biogenesis, function and evolution of microRNAs were shown to differ. High throughput transcriptome and degradome analyses enable to identify small RNAs and their mRNA targets. A non-photosynthetic tissue particularly prone to redox reactions, laticifers from *Hevea brasiliensis*, revealed species-specific post-transcriptional regulations. This paper sets out to identify the 407 genes of the thirty main redox-related gene families harboured by the *Hevea* genome. There are 161 redox-related genes expressed in latex. Thirteen of these redox-related genes were targeted by 11 microRNAs. To our knowledge, this is the first report on a mutation in the miR398 binding site of the cytosolic CuZnSOD. A working model was proposed for transcriptional and post-transcriptional regulation with respect to the predicted subcellular localization of deduced proteins.

Reactive oxygen species (ROS) are produced by redox reactions in plants, including aerobic respiration and photosynthesis. High levels of ROS such as \( ^1 \)O₂ (singlet oxygen), O₂°⁻ (superoxide radical), °OH (hydroxyl radical) and H₂O₂ (hydrogen peroxide) are generated during abiotic and biotic stress, as well as some plant development processes. This oxidative stress triggers disturbances in the basal redox state¹. Peroxides and free radicals damage all cellular components including proteins, lipids and nucleic acids. ROS are also described as secondary messengers that are perceivable and able to initiate adaptive mechanisms²,³. In order to detoxify the harmful ROS and maintain the redox homeostasis, plant cells contain enzymatic and non-enzymatic scavenging systems.

MicroRNA-mediated post-transcriptional regulation has been reported on ROS production and scavenging systems. This control can occur by transcript cleavage of either redox-related genes⁴,⁵, or their upstream transcription factors⁶, as well as indirectly through the repression of genes that induce hormone changes⁷ or a response to stress⁸. Although microRNAs first appeared highly conserved among plant species⁹, several aspects of biogenesis, function and evolution of microRNAs were shown to differ¹⁰. Non-conserved or species-specific microRNAs often expressed at very low levels could be detected using next-generation sequencing technology¹¹,¹². Besides microRNAs, little is known on the role of siRNAs on the expression of redox-related genes. Degradome analysis was first carried out in plant on Arabidopsis to facilitate the discovery and quantification of small RNAs cleaved targets¹³. Degradome sequencing experimentally confirmed several hundred targets in eucalyptus and populus¹⁴,¹⁵.

*Hevea brasiliensis* is the main commercial source of natural rubber, the cis-1,4-polyisoprene polymer, which is synthesized in the rubber particles of laticifers¹⁶. Latex is the cytoplasm of these articulated laticiferous vessels.
arranged in concentric rings in the phloem tissue. Latex flows out after cutting the soft bark (tapping). The application of ethephon, an ethylene releaser, to the bark stimulates latex flow and latex regeneration between two tappings\(^\text{17}\). ROS production takes place in laticifers in response to harvesting stress and consequent metabolic activity necessary for latex regeneration after tapping\(^\text{18}\). When ROS-scavenging systems cannot offset ROS accumulation, cellular dysfunctions lead to the agglutination of rubber particles\(^\text{19,20}\). This physiological syndrome, called Tapping Panel Dryness (TPD), is responsible for major losses in natural rubber production\(^\text{21}\).

Besides the evidence of ROS involvement in TPD at biochemical level\(^\text{22}\), several recent transcriptomic analyses reported that the expression of genes involved in the production and scavenging of ROS is regulated in latex. For instance, a comparison of two contrasting clones for latex yield showed that antioxidant-related genes are crucial in the regulation of latex regeneration and the duration of latex flow\(^\text{23}\). Juvenility was also found to be related to latex production. Latex from self-rooted juvenile clones created by somatic embryogenesis showed more differentially expressed genes (DEGs) related to the ROS-scavenging metabolism\(^\text{24}\). Transcriptomic analysis of a set of rubber clones showed that three and six overexpressed DEGs were involved in ROS production and ROS-scavenging, respectively\(^\text{25}\). Although all these genes were expressed in latex, several other studies did not report any significant changes in the expression of antioxidant genes in latex\(^\text{26-28}\). Post-transcriptional regulation by microRNAs was observed for some redox-related genes. Sixty-eight families of microRNAs, conserved between species, were identified in *Hevea*, including 15 with their precursors, and 16 species-specific microRNAs\(^\text{11,29-31}\). Approximately 1,000 targets were predicted and only a few targets have been experimentally validated to date\(^\text{13,32}\). All these studies globally analysed gene expression but did not specifically check redox-related gene families.

Laticifers are particularly prone to redox reactions. The latex of this non-photosynthetic tissue represents an interesting model to study how important are transcriptional and post-transcriptional regulations related to redox-related genes. This paper sets out to identify all the members of the most important gene families involved in the production and scavenging of ROS and their expression in latex, based on the new complete reference genome sequence\(^\text{25}\) and a transcriptome for a TPD-susceptible clone\(^\text{26}\). Of the 161 redox-related genes expressed in latex, 27 genes were shown to be targeted by microRNAs using small RNAs and degradome analyses. A working model was proposed for transcriptional and post-transcriptional regulations with respect to the predicted subcellular localization of deduced proteins. To our knowledge, this paper reports on the most complete classification of redox-related genes for a crop species, and reveals new insights into small RNA-mediated post-transcriptional regulations of ROS-scavenging systems.

### Results

**Identification and classification of redox-related genes in *Hevea***. *Hevea* redox-related genes were identified in the rubber tree genome sequence from clone Reyan 7-33-97 using *Arabidopsis thaliana* or *Populus trichocarpa* amino acid sequences from 30 gene families downloaded from the UniProt database according to the procedure described in Fig. 1. *Hevea* genes were compared to eight other species based on a bibliographical analysis (Table 1). This analysis revealed that the redox-related gene families identified mostly dealt with ROS production and scavenging and partial information is available for antioxidant biosynthesis. The number of genes for each species was extracted from several references (Supplemental Table 1).

*Hevea* has a much larger number of redox-related genes (407) compared to *Arabidopsis* (306). This is mainly explained by the absence of genes encoding polyphenol oxidase in *Arabidopsis* when *Hevea* genome harboured 6 genes, and by a smaller number of genes encoding glutaredoxin (43), glutathione S-transferase (51) and peroxidase (73) in *Arabidopsis* compared to *Hevea* (51, 77 and 114, respectively). A phylogenetic analysis was carried for gene families involved in ROS production and scavenging (Supplemental Figs 1–17). This analysis revealed several gene duplications for Grx, GST and Px gene families (Supplemental Figs 7, 8 and 14).
Comparative analysis of published latex transcriptomes. In order to identify redox-related genes expressed in latex, contigs or unigenes annotated as redox-related genes were extracted from the Supplemental Table 2 of recently published latex transcriptome analyses obtained by RNA sequencing technology\textsuperscript{23–28}. For each publication, redox-related contigs or unigenes were assigned to one of the 30 gene families using their initial blastx annotation (Table 2). A small number of contigs (28, 30 and 12) was counted for three studies\textsuperscript{23,24,28} compared to the total gene number found in this work (Table 1) and other transcriptome analyses (912, 77, 231)\textsuperscript{25–27}. The transcriptome published by Wei and collaborators had the largest number of redox-related contigs (234) but a lower coverage (0.37 Gb for all samples)\textsuperscript{27}. This transcriptome was obtained from trees of rubber clone RRIM 600 with long-term latex flow. For several gene families, the number of contigs was larger than the gene number counted in the reference genome. Tang and co-workers published transcriptome data for a mixture of several tissues including latex. Thus, the RNAseq dataset from clone PB 260\textsuperscript{26} was adopted for further analysis for the following reasons: high coverage (6 Gb per sample), largest number of redox-related contigs (912), representation of all gene families, good statistical design with the use of 3 biological replicates, and data from a comparison of latex from healthy and TPD-affected trees.

Transcriptional regulation of redox-related genes and prediction of subcellular localization in laticifers. Of the 407 \textit{Hevea} redox-related genes, 161 unique transcripts were found in latex (Supplemental Table 2). All transcripts were encoded by a unique gene, except for 3 transcripts encoded by two genes harboured by 2 different scaffolds, respectively: CL1895Contig4 (L-galactose dehydrogenase 1 (GDH1) and L-galactose dehydrogenase 2 (GDH2); CL3344Contig2 (glutathione S-transferase U8; GSTU8) and glutathione S-transferase U11 (GSTU11); and CL2806Contig1 (NADPH-dependent thioredoxin reductase 1; NTR1) and NADPH-dependent thioredoxin reductase 3; NTR3). NTR1 and NTR3 were located on scaffold0536_346249 and scaffold0965_30248. GSTU8 and GSTU11 were located on scaffold0702_452766 and scaffold0965_30248. GSTU8 and GSTU11 were located on scaffold0702_452766 and scaffold0965_30248.

| Function                      | Gene family                          | Arabidopsis | Hevea | Manihot | Oryza | Populus | Ricinus | Sorghum | Vitis | Zea |
|-------------------------------|--------------------------------------|-------------|-------|---------|-------|---------|---------|---------|-------|-----|
| **ROS production**            | Respiratory burst oxidase homolog     | 10          | 9     | 11      | 9     | 10      | 9       | 13      | 8     | 18  |
|                               | Polyphenol oxidase                    | 0           | 6     | 1       | 2     | 11      | 1       | 8       | 4     | 6   |
| **ROS scavenging and regulation** | Peroxidase                          | 73          | 114   | —       | 138   | 87      | —       | 140     | —    | —  |
|                               | Catalase                             | 3           | 5     | 10      | 3     | 4       | 2       | 2       | 6     | 3   |
|                               | Superoxide dismutase                 | 8           | 9     | 16      | 7     | 10      | 8       | 5       | 12    | 11  |
|                               | Ascorbate peroxidase                 | 7           | 8     | 19      | 11    | 11      | 10      | 8       | 8    | 16  |
|                               | Glutathione peroxidase               | 8           | 10    | 7       | 6     | 7       | 5       | 6       | 5    | 5   |
|                               | Glutathione reductase                | 2           | 3     | 5       | 3     | 3       | 3       | 2       | 2    | 2   |
|                               | Monodehydroascorbate reductase       | 5           | 6     | 6       | 5     | 3       | 3       | 5       | 3    | 4   |
|                               | Dehydroascorbate reductase           | 4           | 3     | 3       | 2     | 4       | 4       | 2       | 3    | 2   |
|                               | Methionine sulfoxide reductase       | 14          | 9     | —       | 7     | 9       | —       | —       | —    | 6   |
|                               | Peroxiredoxin                        | 10          | 10    | 9       | 11    | 13      | 7       | 6       | 9    | 6   |
|                               | NADPH-dependent thioredoxin reductase| 3           | 3     | —       | 3     | 3       | —       | 3       | 2    | —   |
|                               | Glutathione S-transferase            | 51          | 77    | —       | 84    | 81      | —       | 99      | —    | 72  |
|                               | Glutaredoxin                         | 43          | 51    | —       | 49    | 38      | —       | 32      | 25   | —   |
|                               | Thioredoxin                          | 38          | 54    | —       | 46    | 45      | —       | 29      | 32   | 23  |
| **Ascorbate biosynthesis**    | GDP-L-galactose phosphorylase        | 2           | 3     | —       | 1     | 2       | —       | —       | 2    | 1   |
|                               | GDP-mannose pyrophosphorylase        | 3           | 2     | —       | 3     | —       | —       | 1       | —    | 1   |
|                               | GDP-mannose-3’,5’-epimerase          | 1           | 2     | —       | 2     | 2       | —       | —       | 2    | —   |
|                               | L-galactono-1,4-lactone dehydrogenase| 1           | 1     | —       | 2     | 1       | —       | —       | 1    | 1   |
|                               | Inositol phosphate phosphatase       | 1           | 2     | —       | 1     | 3       | —       | —       | 1    | 1   |
|                               | L-galactose dehydrogenase            | 1           | 3     | —       | 1     | 2       | —       | —       | 2    | 1   |
|                               | L-gulonolactone oxidase              | 7           | 3     | —       | —     | —       | —       | 1       | —    | —   |
|                               | Myo-inositol oxidase                 | 5           | 3     | —       | 1     | —       | —       | 2       | —    | —   |
| **Glutathione biosynthesis**  | Glutamate cysteine ligase            | 1           | 2     | —       | 1     | 2       | —       | —       | 1    | —   |
|                               | Glutathione synthetase               | 1           | 2     | —       | 1     | 2       | —       | —       | 1    | —   |
| **Tocotrienol biosynthesis**  | Tocotrienol cyclase                  | 1           | 1     | —       | 1     | —       | —       | —       | —    | —   |
|                               | Tocotrienol γ-methyltransferase      | 1           | 2     | —       | 1     | —       | —       | —       | —    | —   |
|                               | MPRO/MSBQ methyltransferase         | 1           | 3     | —       | 1     | —       | —       | —       | —    | —   |
|                               | Homogentisate phytyltransferase      | 1           | 1     | —       | 2     | —       | —       | —       | —    | —   |
| **In total**                  |                                      | 306         | 407   | >87     | >403  | >352    | >52     | >360    | >131 | >180 |
GDH1 and GDH2 were located on scaffold1364_78602 and scaffold1364_29743. The phylogenetic analyses revealed a recent duplication of the genes (Supplemental Figs 4, 8 and 11).

Subcellular localization of redox-related genes was performed using WoLF PSORT, CELLO2GO and Plant-mPLoc. The largest number of proteins was predicted in chloroplast. Given that laticifers are non-photosynthetic tissues, chloroplast and plastid predictions were assigned as plastidic proteins. Subcellular localization of latex proteins was predicted as follows: 82 in plastids, 70 in cytosol, 12 in nucleus, 7 in mitochondrion, 2 in extracellular, 1 in vacuole, 2 in peroxisome and 7 non-predicted.

When exploring RNAseq data from latex26, sixty transcripts were abundant (>1000 reads), and twelve of them were very abundant (>5000 reads) for one or other of the conditions. Twenty-nine transcripts were induced and forty-eight repressed in response to ethephon in healthy trees. Nine transcripts were induced in response to ethephon in TPD-affected trees. Four of these genes (PPO2, PrxQ, TrxS12 and TrxS13) showed contrasting regulation: repressed in healthy and overexpressed in TPD-affected trees. For the clarity of this manuscript, gene expression data are presented in Fig. 2 (cf. discussion section).

Small RNA-mediated post-transcriptional regulation of redox-related genes. Redox-related transcripts targeted by microRNAs and ta-siRNAs were searched using CLEAVELAND pipeline13 in the degradome dataset obtained from various tissues (root, leaf, bark, latex, flowers and embryo) and the reference transcriptome for rubber tree clone PB 260. The degradome analysis did not revealed post-transcriptional regulations by ta-siRNA (data not shown). Of the 407 redox-related genes, 13 were targeted by 11 different microRNAs.

Table 2. Annotation of Hevea latex redox-related genes from published latex transcriptomes.

| Reference | Chao 2015 | Li 2015 | Wei 2015 | Li 2016 | Tang 2016 | Montoro 2018 | This study |
|-----------|-----------|---------|----------|---------|-----------|-------------|-----------|
| Topic     | Rubber yield | Rubber yield | Latex flow | Rubber yield | Genome | TPD | Redox |
| Technology| Hiseq2000 | Hiseq2000 | Hiseq2500 | Hiseq2000 | Hiseq2000 | Hiseq2000 | — |
| Throughput| 35 Mb | 4.82 Gb | 0.37 Gb | 16.7Mb | 1.29Gb | 6 Gb | — |
| Clone | CATAS8-79 PR107 | RRIM 600 | RRIM 600 | CATAS7-33-97 | HAIKEN 2 | Reyan7-33-97 | PB 260 | PB 260 |
| Tissue | latex | latex | latex | latex | Mixed tissues | Latex | Latex |

Table 2. Annotation of Hevea latex redox-related genes from published latex transcriptomes.

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Figure 2. ROS production and scavenging systems, and antioxidant biosynthesis in the various latex cell compartments. The gene expression level is represented using RNAseq reads. The data in the three columns originate from healthy trees without ethephon treatment (WH), healthy trees with ethephon treatment (EH) and tapping panel dryness trees with ethephon treatment (ET), respectively. The red arrows represent ROS production or oxidation events. The green arrows represent ROS scavenging reactions or reduction events. Abbreviations are: superoxide radicals (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$), catalase (Cat), peroxidase (Px), ascorbate peroxidase (APx), glutathione peroxidase (GPx), peroxiredoxin (Prx), glutathione (GSH), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione synthetase (GS), γ-glutamylcysteine (γ-EC), L-glutamate (Glu), cysteine (Cys), glycine (Gly), NADPH reductase (NTR), thioredoxin (Trx), methionine sulfoxide (MetO), methionine sulfoxide reductase (MSR), glutaredoxin (Grx), glutathione S-transferase (GST), myo-inositol oxygenase (Miox), L-gulonolactone oxidase (GULO), myo-Inositol (Myo-I), D-Glucuronate (D-
enging pathways coexisting in cytosol (peroxidase, ascorbate peroxidase, glutathione peroxidase, peroxiredoxin microRNAs not yet annotated in the miRBase database (Table 3).

Three microRNAs (miRNAn1 to 3), with cleavage activity in latex, were new for ethephon treatment or TPD occurrence disappeared for genes APX3, GR1, MDHAR2 significantly affected by the new way of calculation. Significant fold changes observed in standard RNA sequencing functionally of mRNAs (Supplemental Tables 3 and 4). The expression of 8 of the 13 targeted transcripts were highly expressed in latex and targeted by miR394.

The expression of the 13 post-transcriptionally regulated genes was recalculated using the reads covering the cleavage site to calculate the expression level. Finally, some genes were more abundant compared to other genes encoding SOD. Unlike Arabidopsis, none of the Hevea cytosolic SOD isoforms was subjected to post-transcriptional regulation by miR398. A mutation in the binding site makes miR398 ineffective. The high expression of the CuZnSOD1 gene might then support the maintenance of SOD activity and a consequent high level of anion superoxide dismutation. To demonstrate the biological relevance of post-transcriptional regulations, the physiological context (type and duration of stress) in which the regulation occurs should be further identified case by case. For example, the cleavage of the chloroplastic CuZnSOD transcript was correlated with the upregulation of miRNA398 expression in response to a salt stress specifically in bark and root.

The second step deals with the decomposition of H₂O₂ to H₂O and O₂ through five hydrogen peroxide scavenging pathways coexisting in cytosol (peroxidase, ascorbate peroxidase, glutathione peroxidase, peroxiredoxin...
| Target Enzyme                          | Contig | Library       | Category | miRNA accession | miRBase annotation | Start position | Stop position | Cleavage site |
|--------------------------------------|--------|---------------|----------|-----------------|--------------------|----------------|---------------|---------------|
| Ascorbate peroxidase 3               | CL1Contig117 | leaf 0        | Pnature12390 | miR535           | 53                 | 73             | 64            |
| Catalase 1                           | CL1Contig10534 | latex 3      | Pyoung21016 | miRNAn1, in progress | 588             | 608            | 599           |
| Catalase 2                           | CL1Contig1382 | latex 3      | Pyoung21016 | miRNAn1, in progress | 421             | 441            | 432           |
| Catalase/Zn superoxide dismutase 2   | CL1553Contig1 | root 4       | acc_420   | miR398b         | 630               | 656            | 646           |
| Glutathione peroxidase 5             | CL449Contig1 | leaf 0       | Pnature37668 | miRNAn3, in progress | 70              | 90             | 81            |
| Glutathione reductase 1              | CL1Contig15684 | leaf 2     | Pyoung83898 | miR394         | 415               | 435            | 453           |
| Glutathione reductase 2              | CL1Contig1556 | leaf 2       | Pyoung83898 | miR394         | 360               | 583            | 571           |
| Methionine sulfoxide reductase A2    | CL372Contig4 | bark 2       | health2164 | miRNAn4, in progress | 210             | 231            | 222           |
| Monodehydroascorbate reductase 1     | CL1Contig7966 | bark 2       | Pnature18863 | miRNAn5, in progress | 149             | 170            | 161           |
| Monodehydroascorbate reductase 3     | CL1250Contig6 | bark 2       | Pyoung84691 | miRNAn6, in progress | 1181            | 1203           | 1194          |
| MPBQ/MSBQ methyltransferase 3        | CL5665Contig1 | leaf 4       | Pyoung169157 | miRNAn7, in progress | 951             | 973            | 962           |
| Myo-inositol oxygenase 2             | CL234Contig10 | flower 2    | Pyoung68471 | miRNAn8, in progress | 401             | 424            | 415           |
| Peroxidase 6                         | CL1Contig8355 | leaf 2       | Pyoung84691 | miRNAn6, in progress | 970             | 990            | 982           |

Table 3. Degradome data analysis with CLEAVELAND pipeline using 161 ROS-related genes, 6 tissue-specific transcriptomes and newly annotated microRNAs.

and catalase). High and steady ROS production in latex cells requires Cat activity, which generally comes into play under stress. A decrease in Cat activity was recorded in TPD-affected trees enabling the general oxidative stress in latex cells35. Cat1 gene was highly expressed in latex and might be the main gene related to the Cat activity. Although post-transcriptional regulation was shown by microRNA miRn1, this microRNA did not efficiently cleave Cat1 transcripts in the tested biological conditions (low number of read ends at the cleavage site in degradation data). For the genes encoding thioredoxins, TrxH5 had the highest level of expression out of the 161 genes expressed in latex. From our knowledge, there is no published information related to the potential role of Prx in latex and further characterization is required. The ascorbate/glutathione cycle, involving in its last lines APx and GPx, is essential in the reduction of H2O2 to H2O and O2. Generation of the ASA and GSH forms reduced by the ascorbate-glutathione cycle involved several enzymes encoded by MDHAR2, DHA2, GR1 and GR2. The ethephon treatment did not transcriptionally activate genes involved in the glutathione/ascorbate cycle. Although some post-transcriptional regulations appeared in the degradome analysis showing that both the GR1 and GR2 transcripts, miR394 did not significantly cleave GR transcripts. APx has a high affinity for H2O2 and can reduce it to H2O in chloroplasts, cytosol, mitochondria and peroxisomes, as well as in the apoplastic space. Of the three genes encoding a cytoplasmic ascorbate peroxidase, the HbAPx1 and HbAPx5 transcripts were the most abundant. Considering the lower expression of these 3 APx genes compared to the plastidic APx4, the cytosolic ASA pathway might have a lower reducing capacity than the plastid pathway, which is obvious since the production of ROS is known to be high in plastids. Of the 23 Hevea genes encoding a GST, 21 were predicted as cytosolic GST. Among them, the GSTU3 and GSTF1 genes were actively expressed in latex cells. As GST plays a central role in the use of the reduction power of GSH to detoxify electrophiles, glutathione might be considered as the most important antioxidant in laticifers.

Glutathione, ascorbate and vitamin E isomers are the major antioxidants in latex22. The glutathione biosynthesis pathway involves two ATP-dependent enzymes: γ-glutamate cysteine ligase (GCL) and glutathione synthetase (GS). Of the two G5 and GCL genes identified in the rubber genome, only one of each was encoded protein predicted to be expressed in latex cytosol (GS1 and GCL2), one GS (GS2) and the two GCL (GCL1 and GCL2) being expressed in plastids. The genes encoding GS2 and GCL2 were significantly over-expressed in response to ethephon. There are four routes for ASA biosynthesis in plant: the L-galactose pathway, the myo-inositol oxygenase pathway, the salvage pathway via L-galactonate, and the L-gulose-pathway. Of these four routes, L-galactose is the major pathway in many plants8,37. The L-galactono-1,4-lactone (L-GalL) biosynthesis pathway occurs in cytosol, which consists of five enzymes (VTC1, GME, VTC2, VTC4 and GDH). All genes encoding these enzymes have homologues expressed in latex cytosol.

There are 4 vitamin E isomers in latex: α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol38,39. Genes involved in the biosynthesis of δ-tocotrienol (VTE1 and VTE2) and γ-tocotrienol (VTE1, VTE31, VTE32 and VTE33) were expressed at moderate or high levels in latex. VTE33 had also a low level of expression related to
Table 4. Comparison of HbmiR398 (acc_420) cleavage site between cytosolic and chloroplastic CuZnSOD isoforms. Arrow indicated the cleavage site observed experimentally for HbCuZnSOD2 by miR398 (Gébelin et al. 2012) and in the degradome analysis. Sequence variations in cytosolic isoforms sequences compared to HbCuZnSOD2 are in bold and highlighted character.

| Gene name          | Sub-cellular localization | mfe kcal/mol | Alignment               |
|--------------------|----------------------------|--------------|-------------------------|
| HbCuZnSOD1         | cytosolic                  | Non functional | mIRNA 23 UAGU-C-CGGGCCGAGACUCU-UGUGU 1 |
|                    |                            |              | Target 364 GUGAUCGGGAGAGUUGGCGCAGAU 390 |
| HbCuZnSOD2         | chloroplastic              | −37.3        | mIRNA 23 UAGU-C-CGGGCCGAGACUCU-UGUGU 1 |
|                    |                            |              | Target 456 GUGAUCGGGAGAGUUGGCGCAGAU 480 |
| HbCuZnSOD3         | cytosolic                  | Non functional | mIRNA 23 UAGU-C-CGGGCCGAGACUCU-UGUGU 1 |
|                    |                            |              | Target 346 GUGAUCGGGAGAGUUGGCGCAGAU 372 |
| HbCuZnSOD4         | cytosolic                  | Non functional | mIRNA 23 UAGU-C-CGGGCCGAGACUCU-UGUGU 1 |
|                    |                            |              | Target 284 GUGAUCGGGAGAGUUGGCGCAGAU 309 |

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To conclude, this study reveals new insights into small RNA-mediated post-transcriptional regulations of ROS-scavenging systems. To our knowledge, this is the first report on a mutation in the miR398 binding site of the CuZnSOD altering the post-transcriptional regulation described in model species. In addition, the literature mentioned microRNA-mediated post-transcriptional regulation on ROS production and scavenging systems. This work paves the way to the study of adaptive mechanisms. Besides, several genetic studies have revealed the involvement of antioxidant compounds in complex traits of several species. In Hevea, the 161 redox-related genes expressed in latex represent candidate genes for the identification of allelic variability. The development of molecular markers and the analysis of genetic variability of antioxidants should support breeding programmes, especially for traits relative to environmental stress.

Methods
Identification and classification of redox-related genes in the Hevea brasiliensis genome and transcriptome. Redox-related genes were identified from both the Hevea reference genome and transcriptome (Fig. 1). An amino acid sequence dataset was created by downloading sequences of thirty redox-related gene families from the UniProt database (http://www.uniprot.org/) using published accession numbers mostly from Arabidopsis, except for the polyphenol oxidase (PPO) family, which is absent in Arabidopsis. Sixteen families were selected for ROS production and scavenging (Table 1). In addition, protein sequences of genes involved in the biosynthesis of three major antioxidants in latex (ascorbate, glutathione, and tocotrienol) were collected. This dataset was blasted against the published Hevea genome and transcriptome. Redox-related contigs were also identified using blastx and GO annotations of the Hevea transcriptome. The two lists of contigs were merged and blasted on the rubber genome to identify unique contigs. Redox-associated genes were classified for each gene family related to ROS production, ROS-scavenging and regulation, and antioxidant biosynthesis (ascorbate, glutathione and tocotrienol).

Phylogenetic analysis of redox-related genes. The full length amino acid sequences of Arabidopsis redox-related protein were aligned with the amino acid deduced sequences from Hevea clone Reyan 7−33−97 genome. Identities of proteins are provided in Supplemental Table 5. The polyphenol oxidase family being absent in Arabidopsis, we used the Populus PPO gene family. This alignment was made by Muscle via Mega 6. Amino acid sequence of Arabidopsis actin 1 or Arabidopsis glutamate cysteine ligase was used as outgroup control. The phylogenetic trees were generated in Mega 6 by Bootstrap method with 500 replications after alignment.

Prediction of the subcellular localization of redox-related proteins. The subcellular location of redox-related genes was predicted with translated sequences using WoLF PSORT (http://www.genscript.com/wolf-psort.html), CELLO2GO (http://cello.life.nctu.edu.tw/cello2go/) and Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). The 3 predictors were successfully tested on subcellular localization prediction. The matching ratio between the prediction result and protein location was calculated according to Xiong’s Supplemental Table 2. The matching ratios from these 3 predictors ranged from 50% to 80%. The prediction of subcellular localization was considered as acceptable when the matching ratio of merged results was above 90%.

Identification of small RNA and target mRNA couples. Degradome data for several Hevea tissues (latex, leaves, male and female flowers, seeds, root and barm and zygotic embryos) were obtained according to a protocol adapted from German. Hevea microRNAs from small RNAseq data published by Gebelin and co-workers were annotated by MITP (https://sourceforge.net/projects/mitp/files/). This pipeline complies to the recommendations set by Axtell and coll, looking from hairpin structures, producing miRNA and miRNA* with up to 3 bulges or 6 unpaired bases between miRNA and miRNA*. The prediction was done with sequences
of 20–22 nt in size from 5 distinct small-RNA-seq libraries as recommended and not based on prediction from genomic sequences only. Degradome data were then analysed using the CLEAVELAND pipeline developed by Addo-Quaye et al. The degradome categories correspond to: category 4: just one read at this position; Category 3: > 1 read, but below or equal to the average depth of coverage on the transcript; Category 2: > 1 read, equal to the average depth of coverage on the transcript when there is > 1 position at maximum value; Category 1: > 1 read, equal to the maximum of the average depth of coverage on the transcript when there is just one position at maximum value.

RNA-seq data mining of cleaved targets. Expression of cleaved transcripts related to redox genes were calculated from the same RNA-seq datasets, with the exact number of reads overlapping the sRNA binding site, by using BEDTOOLS program (2.24.0) to intersect bam files with sRNA binding site coordinates (between Tstart and Tstop) provided by CLEAVELAND outputs. Then, by using R package EdgeR, comprising an over-dispersed Poisson model taking into account both biological and technical variability, differential gene expression analyses of replicated count data were performed by the experimental design allows side-by-side comparison to identify firstly, differentially expressed genes upon ethephon stimulation in the latex of healthy trees, and secondly, differentially expressed genes in the latex of healthy and TPD-affected trees subjected to ethephon stimulation.

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Author Contributions

Y.Z. classed the gene families, predicted subcellular localization and analysed gene expression data. S.W., E.O.A. and S.P. performed the bioinformatics analyses. J.L. and S.W. performed the degradome analysis. C.T. provided the genome dataset. S.H. supervised the bioinformatics analyses. Y.Z., J.L. and P.M. drafted the manuscript. All the co-authors edited and approved the manuscript. P.M. coordinated the project.

Additional Information

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