The Calcium Sensor Calcineurin B-Like Proteins -Calcineurin B-Like Interacting Protein Kinases Is Involved in Leaf Development and Stress Responses Related to Latex Flow in Hevea brasiliensis

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Latex flow in Hevea brasiliensis (the Para rubber tree), the sole commercial source of natural rubber (cis-1,4-polyisoprene, NR), renders it uniquely suited for the study of plant stress responses. Calcineurin B-like interacting protein kinases (CIPK) serving as calcium-sensor protein kinases react with calcineurin B-like proteins (CBL) to play crucial roles in hormone signaling transduction and response to abiotic stress in plant developmental processes. However, little is known about their functions in Hevea. In this study, a total of twelve CBL (HbCBL) and thirty CIPK (HbCIPK) genes were identified from the Hevea genome. Structure and phylogenetic analysis assigned these CIPKs to five groups and CBLs to four groups, and mapped onto fourteen of the eighteen Hevea chromosomes. RNA-seq and qPCR analysis showed that the expressions of HbCBL and HbCIPK genes varied in the seven Hevea tissues examined, i.e., latex (cytoplasm of rubber-producing laticifers), bark, leaf, root, seed, female flower, and male flower. The expressions of two HbCBL and sixteen HbCIPK genes showed upward trends during leaf development. Following ethylene yield stimulation and the latex tapping treatment, both practices invoking stress, the expression levels of most latex-expressed genes were significantly altered. Yeast two-hybrid test revealed interactions for multiple combinations of HbCBLs and HbCIPKs with substantial gene expression in latex or other Hevea tissues. However, all the HbCBL-HbCIPK complexes examined did not recruit HbSOS1 or AtSOS1 to form functional salt tolerance SOS pathway in yeast cells. Taken together, the results suggested a role of the Hevea CBL-CIPK network as a point of convergence for several different signaling pathways in growth, development, and stress responses in relation to latex production.

Keywords: Hevea brasiliensis, calcineurin B-like protein, CBL-interacting protein kinase, gene expression, protein interaction, stress, latex flow
INTRODUCTION

Calcium, functioning as a messenger of plant cells, mediates a multitude of plant responses to external stimuli and regulates a wide range of physiological processes (Gilroy and Trewavas, 2001). Calcium-binding proteins, such as calcineurin B-like (CBL) proteins, represent important relays in plant calcium signaling. These proteins form a complex network with their target kinases, i.e., the CBL-interacting protein kinases (CIPKs) (Gong et al., 2004; Boudsocq and Laurière, 2005; Weinl et al., 2009). The CIPK protein consists of an N-terminal protein kinase domain and a C-terminal autoinhibitory domain known as the NAF or FISL motif, with CBLs interacting with CIPKs through the C-terminal domain that is conserved among different CIPKs (Shi et al., 1999; Guo et al., 2001; Luan, 2009).

Calcineurin B-like interacting protein kinases are extensively involved in plant stress responses. Physiological roles of CBL and CIPK were firstly uncovered in the salt overly sensitive (SOS) pathway, with SOS3 (CBL4) and SOS2 (CIPK24) shown to synergistically up-regulate the activity of the Na+/H+ exchanger SOS1 in Arabidopsis roots, thus leading to Na+ and Cl− hyperaccumulation (Shi et al., 1999; Guo et al., 2001; Luan, 2009). The CIPK protein consists of an N-terminal protein kinase domain and a C-terminal autoinhibitory domain known as the NAF or FISL motif, with CBLs interacting with CIPKs through the C-terminal domain that is conserved among different CIPKs (Shi et al., 1999; Guo et al., 2001; Luan, 2009).

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Natural rubber in *Hevea brasiliensis* is synthesized and stored in the laticifer cells which are differentiated from the cambium and arranged in concentric rings (when viewed in cross section) in the phloem region (Tupy, 1989; Hao and Wu, 2000). The bark of the rubber tree is excised every 2 to 3 days to sever the laticifer rings in a process called tapping to enable the outflow of latex (Tupy, 1989). At each tapping, several tens to a few hundred milliliters of latex per tree are expelled from the laticifers and harvested for sustainable rubber production. Application of ethylene gas or ethephon (2-chloroethyolphosphonic acid, an ethylene generator) to the trunk bark of the rubber tree can significantly increase rubber yield. However, the underlying mechanisms in ethylene stimulation are not yet fully understood, although ethylene signaling and response are assumed to play critical roles (Tang C. et al., 2016). The laticifer network could serve as a useful system for the study of stress and signal transmission.

In this study, the *Hevea* CBL and CIPK genes were identified by genome-wide analysis, and their gene structure, phylogeny and chromosomal distribution were analyzed. Based on RNA-seq data and qRT-PCR analysis, the *HbCIPK* and *HbCBL* gene expression profiles in different *Hevea* tissues and leaf development stages were determined. The influence of ethylene stimulation and the onset of tapping on their expression levels were also analyzed. Furthermore, the interaction relationships between *HbCBL* and *HbCIPK* proteins were detected by yeast two-hybrid, and the salt tolerance SOS pathway in *Hevea* was investigated by yeast complementation test. Our aim was to understand the roles of the CBL-CIPK network in *Hevea* responses to abiotic stress, ethylene-based latex flow stimulation and leaf development.

RESULTS

Genome-Wide Identification of Calcineurin B-Like Interacting Protein Kinases and Calcineurin B-Like Proteins

Family Genes in *Hevea brasiliensis*

BLAST and Hidden Markov Model searches were conducted to identify *H. brasiliensis* CIPKs and CBLs using Arabidopsis, rice and poplar CIPK and CBL protein sequences as queries. A total of twelve CBLs (named *HbCBL*1 to 12) and thirty CIPKs (named *HbCIPK*1 to 30) were identified from the *H. brasiliensis* genome (Tang C. et al., 2016). Detailed information, including protein length, isoelectric point (pI), molecular weight (MW), number of introns and evolution group of identified *HbCBLs* and *HbCIPKs*, is listed in Table 1. The number of amino acid residues of the identified *HbCIPKs* ranged from 328 (HbCIPK22) to 541 (HbCIPK17), and *HbCBLs* ranged from 165 (HbCBL2) to 247 (HbCBL5). Their relative molecular mass ranged from 37.29 kDa (HbCIPK22) to 61.30 kDa (HbCIPK17) for *HbCIPKs* and 19.38 kDa (HbCBL2) to 28.44 kDa (HbCBL5) for *HbCBLs*. The isoelectric points of *HbCBLs* and *HbCIPKs* were between 4.64 (HbCBL6) and 9.21 (HbCBL2), and between 5.47 (HbCIPK28) and 9.51 (HbCIPK22), respectively. It is noteworthy here that the isoelectric point of HbCBL2, at 9.21, was an outlier that was significantly higher than those of the other *HbCBLs* (Table 1). Conserved domain analysis confirmed that most of the *HbCIPKs* identified harbored the Pkinase (PF00069) and NAF (PF03822) domains, while all the *HbCBLs* harbored the calcium-binding EF hand domains (PF13833 and PF13499) which are the hallmark of CBL family.

Phylogenetic and Gene Structure Analysis of the *HbCIPK* and *HbCBL* Family Genes

Phylogenetic analysis of the thirty *HbCIPKs*, together with 103 CIPKs from five other plants, classified the CIPK family into...
TABLE 1 | Characteristics of CIPK and CBL genes in *Hevea brasiliensis*.

| Genes ID | ID | CDS length in bp | Predicted protein | No. of introns | Group |
|----------|----|------------------|-------------------|---------------|-------|
| HbCIPK1  | scaffold0014_83038 | 1,317 | 439 | 6.35 | 49874.40 | 13 | A |
| HbCIPK2  | scaffold0016_914488 | 1,311 | 437 | 9.22 | 49190.93 | 0 | C |
| HbCIPK3  | scaffold0050_2486504 | 1,176 | 392 | 6.01 | 44145.38 | 0 | E |
| HbCIPK4  | scaffold0050_2491974 | 1,302 | 434 | 8.47 | 49318.37 | 1 | C |
| HbCIPK5  | scaffold0050_2493973 | 1,359 | 453 | 9.03 | 51228.77 | 0 | C |
| HbCIPK6  | scaffold0069_353896 | 1,338 | 446 | 6.93 | 50657.28 | 13 | A |
| HbCIPK7  | scaffold0099_255532 | 1,338 | 446 | 7.62 | 50550.11 | 13 | A |
| HbCIPK8  | scaffold0140_117108 | 1,425 | 475 | 7.89 | 53522.25 | 14 | A |
| HbCIPK9  | scaffold0163_468878 | 1,311 | 437 | 8.59 | 49097.59 | 0 | C |
| HbCIPK10 | scaffold0181_102123 | 1,293 | 431 | 8.78 | 48708.09 | 12 | A |
| HbCIPK11 | scaffold0198_1059208 | 1,335 | 445 | 7.89 | 54321.66 | 0 | C |
| HbCIPK12 | scaffold0224_117108 | 1,293 | 431 | 9.22 | 50550.11 | 13 | A |
| HbCIPK13 | scaffold0281_148200 | 1,335 | 445 | 8.72 | 54321.66 | 0 | C |
| HbCIPK14 | scaffold0281_150624 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK15 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK16 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK17 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK18 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK19 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK20 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK21 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK22 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK23 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK24 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK25 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK26 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK27 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK28 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK29 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |
| HbCIPK30 | scaffold0318_1059208 | 1,335 | 445 | 9.11 | 54321.66 | 0 | C |

five clusters (Group A to E, Figure 1A and Supplementary Tables 1, 2). There were eleven HbCIPK members (HbCIPK1, 6–11, 16–17, 27 and 30) in Group A, thirteen (HbCIPK2, 4–5, 12, 15, 18, 22–26, 28–29) in Group C, and two each in the other three groups, i.e., HbCIPK13 and 21 in Group B, HbCIPK14 and 19 in Group D, and HbCIPK3 and 20 in Group E. As expected, CIPKs from *Hevea* generally had closer relationships with those from the two Euphorbiaceae family species, *Manihot esculenta* and *Ricinus communis*, than those from Arabidopsis, rice or poplar, in accordance with plant taxonomic status. Phylogenetic analysis based on predicted amino acid sequences identified some closely related pairs of HbCIPKs, such as HbCIPK9 and 11 in Group A, HbCIPK13 and 21 in Group B, HbCIPK14 and 19 in Group D, and HbCIPK3 and 20 in Group E (Figure 1A). Similar trends are also observed in other plant species (Hu et al., 2015), and closely
related HbCIPK pairs often have similar cellular localization and functions. The exon-intron structures of the thirty HbCIPK genes were determined based on their predicted genomic sequences. As shown in Figure 2A, most HbCIPK members within the same groups shared similar gene structure in terms of intron number, domain localization and exon length. It is worth noting that the members in Group A had eleven to sixteen introns, while those in the four other groups had no intron or only one. A similar intron-rich or poor pattern is also observed in the CIPKs from other plant species, such as Arabidopsis, rice, poplar, cassava, pear, and soybean (Kolukisaoglu et al., 2004; Zhang et al., 2008; Hu et al., 2015; Tang J. et al., 2016; Zhu et al., 2016), reflecting a conserved feature of the CIPK family in gene structure. Considering the rate of intron loss is faster than that of intron gain after segmental duplication (Roy and Penny, 2007), the CIPKs of group A might represent the progenitor genes of the CIPK family.

Phylogenetic analysis put the CBL genes of Hevea and the other five plants into four groups (Group I to IV, Figure 1B), with three, two, two and five members in Group I, II, III, and IV, respectively. This analysis also identified six closely related orthologous pairs between Hevea and cassava (HbCBL9 and McCBL8, HbCBL7 and McCBL7, HbCBL2 and McCBL5, HbCBL3 and McCBL9, HbCBL8 and McCBL1, HbCBL10 and MeCBL6), suggesting the existence of an ancestral set of CBL genes prior to the divergence of Hevea and cassava. The results of gene expression analysis (Figure 3) showed that HbCBL11, 8 and 10 might be the main direction of HcCBLs evolution. Our analysis of genomic organization of the HbCBLs revealed that eight of the twelve members contained seven introns, two contained eight introns, one contained four introns and one contained six introns (Figure 2B). Except for HbCBL2 and HbCBL8, most HbCBL members within the same groups shared very similar gene structures in terms of intron number, domain localization, and exon length.

Chromosomal Distribution and Colinearity Analysis of the HbCIPK and HbCBL Family Genes

The locations of HbCIPK and HbCBL genes in Hevea chromosomes were as shown in Figure 4A. The HbCIPK genes were mapped onto fourteen of the eighteen Hevea chromosomes, while the HbCBL genes were located on seven chromosomes. However, distribution of these two sets of genes on the chromosomes was uneven. There was no HbCIPK or HbCBL members mapped onto chromosomes 6, 11, 13, and 16, whereas the other chromosomes contained one to five members. Chromosome location analysis also found some tandem repeat genes, such as HbCIPK19 and 26, HbCIPK28 and 29, HbCIPK14 and 15, HbCIPK3, 4 and 5, which were located on chromosomes 2, 7, 12, and 17, respectively. The same was true of HbCBL1 and 11, HbCBL5 and 6, HbCBL3 and 4 located on chromosome 5, 8, and 10, respectively. Collinearity analysis found some HbCIPK members might have been amplified by chromosome duplication, such as in the case of HbCIPK26 and 28, HbCIPK24 and 18, HbCIPK11 and 9, HbCIPK13 and 21, HbCIPK6 and 7, HbCIPK20 and 3, HbCBL5 and 8, HbCBL10 and 12, HbCBL4 and 9, HbCBL2 and 7. This was consistent with their clustering patterns shown in the phylogenetic tree (Figures 1A, 2). Chromosomal location and colinearity analysis of CBL and CIPK genes within Hevea (Figure 4A) and across Hevea and three other plant species (Figure 4B) were investigated in order to explore the potential evolutionary relationships. The results revealed a higher homology between H. brasiliensis, M. esculenta and P. trichocarpa than that between P. trichocarpa and A. thaliana. Some HbCIPK and HbCBL genes were collinear with CIPK and CBL genes in P. trichocarpa, A. thaliana and M. esculenta, suggesting their important roles in plant evolution. These results can be useful for subsequent comparative studies of CIPK and CBL genes with known functions.

Expression Analysis of HbCIPK and HbCBL Genes in Different Tissues

To study the expression profiles and deduce the gene function of HbCBL-HbCIPKs, transcriptome analyses were conducted in seven Hevea tissues: latex, bark, leaf, root, seed, and female and male flowers using Solexa sequencing and quantitative PCR as described previously (Xiao et al., 2017). As shown in Figure 3, transcripts of thirteen HbCIPK genes (HbCIPK1, 3–6, 8, 12, 17, 19–20, 22, and 28–29) and six HbCBL genes (HbCBL2, 4–6 and 12) were barely detectable in almost all the tissues examined, indicating the loss of their functions during evolution. In contrast, HbCIPK14, 23, 30 and HbCBL1 were expressed in most of the tissues examined, indicating their functional conservation in the course of evolution. Some of the HbCIPK and HbCBL genes displayed preference expression in specific tissues, such as HbCIPK15, 18 and 26 and HbCBL1 and 3 in latex, HbCIPK13–14 and 23 and HbCBL1 in the root, HbCIPK14 and 30 and HbCBL10 in the bark, HbCIPK9, 14 and 30 and HbCBL8 in the leaf, and HbCIPK13–15 and 21 and HbCBL1 in the seed. It is interesting to note that some of the HbCIPK and HbCBL members showed distinct expression patterns in female and male flowers. For example, the expression levels of HbCIPK2, 9, and 30 and HbCBL10 were much higher in the female flowers than in the male flowers. On the other hand, HbCIPK10–11, 16, 23 and 27 and HbCBL1 and 8 were much higher expressed in the male flowers than the female flowers. The expressions of randomly selected HbCIPK and HbCBL genes including HbCBL3 and 8 and HbCIPK14, 16, 23 and 27 were further investigated by qPCR (Supplementary Figure 1), revealing patterns basically consistent with transcriptome analysis.

Expression Analysis of HbCIPK and HbCBL Genes in Leaf Development

To obtain information on the functions of HbCIPK and HbCBL genes in the course of leaf development, we examined their expression levels by RNA-seq at four progressive leaf stages (bronze, color change, pale-green, and mature). As shown in Figure 5, some of the HbCIPK genes, i.e., HbCIPK13–14, 16, 20–21, 25–26, and 30, were obviously up-regulated during leaf development. Of the HbCBL genes, only HbCBL7 was similarly up-regulated with leaf development. Some HbCIPK and HbCBL genes were up-regulated during the first three leaf stages (bronze,
FIGURE 1 | Phylogenetic analysis of HbCIPK and HbCBL genes in Hevea brasiliensis and five other plant species. Unrooted phylogenetic trees of plant HbCIPK and HbCBL proteins were constructed using the neighbor-joining method with the MEGA 6.0 program. (A) The plant species and their CIPK proteins are as follows: H. brasiliensis, HbCIPKs (30), marked with red dots; A. thaliana, AtCIPKs (26); P. trichocarpa, PtCIPKs (26); O. sativa, OsCIPKs (34); M. esculenta, MeCIPKs (25); R. communis, RcCIPKs (18). (B) The plant species and their CBL proteins are as follows: H. brasiliensis, HbCBLs (12), marked with red dots; A. thaliana, AtCBLs (10); P. trichocarpa, PtCBLs (9); O. sativa, OsCBLs (10); M. esculenta, MeCBLs (9); R. communis, RcCBLs (8).
color change, and pale-green) but down-regulated toward leaf maturity, as seen for HbCIPK2, 10, 19, 23, 27 and HbCBL8. A small number of HbCIPK and HbCBL genes were down-regulated during leaf development, such as HbCIPK11 and HbCBL1 and 10. Some closely related gene pairs exhibited similar expression profiles. For example, HbCIPK2 and 23, HbCIPK28 and 29, HbCIPK13 and 21, HbCIPK9 and 11 had similar expression patterns during leaf development from soft young leaves to fully hardened mature leaves. Nevertheless, there are more pairs of closely related genes showed dissimilar expression patterns. Examples of these were HbCIPK1 and 10, HbCIPK3 and 20, HbCIPK8 and 16, HbCIPK14 and 19, HbCIPK17 and 27, HbCBL1 and 11, HbCBL2 and 7, HbCBL10 and 12. The expressions of HbCIPK14-16, 23, 26 and 27 were further investigated by qPCR (Supplementary Figure 2), all of which were obviously up-regulated during leaf development, consistent with the results of transcriptome analysis.

Expression Analysis of HbCIPK and HbCBL Genes Following Ethephon Treatment

Ethephon is an ethylene generator which is widely used in Hevea to stimulate latex flow, and hence rubber yield. The mechanisms of ethylene signal transduction and yield stimulation are, as yet, poorly understood. Since the CBL-CIPK complex plays an
Expression Analysis of HbCIPK and HbCBL Genes in Response to the Treatment of Latex Tapping

To assess the respective effect of latex tapping on the expression of HbCIPK and HbCBL genes, experiments were performed on previously untapped cultivated trees of the clones PR107 and ReYan8-79, and a number of HbCIPK and HbCBL genes with substantial expression in latex were examined. The first tapping of an untapped rubber tree normally produces very little latex. The latex yield increases progressively with successive tappings at regular intervals to reach a steady output after seven to ten tappings (Tang C. et al., 2010). As shown in Figures 7A,B,
the expressions of HbCIPK27 and HbCBL3 were clearly up-regulated when untapped PR107 and ReYan8-79 trees were first brought into tapping. On the other hand, HbCIPK16, 18, 23, 30 and HbCBL1 were significantly down-regulated from the second tapping onward. The above results suggested that the CIPK-CBL complexes might play a role in tapping-stimulated latex production. The expression patterns of HbCIPK and HbCBL genes in the two Hevea clones were consistent in most cases,
inducing the ways of their response to the tapping treatment being similar under different Hevea genetic backgrounds.

**Interaction Analyses of HbCBL and HbCIPK Proteins**
Calcineurin B-like proteins and calcineurin B-like interacting protein kinases are functionally linked through physical interactions, and participate in plant development various environmental stresses (Luan, 2009). The interaction relationships between the HbCBL and HbCIPK proteins with substantial gene expression in latex or other Hevea tissues were detected by yeast two-hybrid (Y2H), i.e., spotting cos-transformed yeast cells on various types of selective media. As shown in Figure 8A and Supplementary Figure 4, multiple combinations of HbCBL and HbCIPK fusion proteins showed obvious interactions although the strength of interaction varied. HbCBL1 and its closely related homolog, HbCBL10, displayed overlapping strong or moderate interactions with more than half of the HbCIPKs examined. In contrast, HbCBL3 and its close homolog HbCBL8 did not interact with most of the HbCIPKs investigated. Similar overlapping patterns of interaction were observed for the closed related HbCIPK homologs, e.g., HbCIPK11 and 18, and HbCIPK14 and 15. Expression profiles of the genes encoding HbCBL1 and its four strong interaction HbCIPK partners revealed by the Y2H assay (Figure 8A), HbCIPK14, HbCIPK15, HbCIPK16, and HbCIPK26, were examined in different tissues and treatments based on Solexa sequencing (Figure 8B and Supplementary Table 4). The expressions of HbCIPK26 and HbCBL1 displayed a strong correlation in different tissues and treatments, particularly in latex. To validate the results of the Y2H assay, the interactions of HbCBL1-HbCIPK15 (strong interaction) and HbCBL1-HbCIPK30 (weak interaction) were further investigated by the BiFC experiments conducted in N. benthamiana leaves. As shown in Figure 9, the results were consistent with those of the Y2H analysis, with the co-transformation of the HbCBL1-HbCIPK15 pair yielding a stronger green fluorescence than that of the HbCBL1-HbCIPK30 pair.

**DISCUSSION**

The CBL-CIPK network has been reported in Arabidopsis, poplar, cassava, and rice as contributing to plant development and stress response, but has never been reported in Hevea. In this study, HbCBL and HbCIPK genes were identified and the evolutionary relationship, gene structure, chromosomal location, and tissue-specific expression were analyzed. In addition, the expression profiles during Hevea leaf development, following ethylene induced latex flow and initiation of trees into latex tapping have been examined.

Conserved domain analysis showed domains of kinase, NAF, and EF hand harbored in the twelve HbCBL and thirty HbCIPK genes (Figure 2), indicating the Hevea CBL and CIPK families with basic characteristics similar to those in Arabidopsis, poplar and cassava (Yu et al., 2007; Mo et al., 2018). These genome-wide identification results suggested that the number of CIPK genes detected in Hevea was close to that of Populus and cassava, and larger than that of Ricinus. The phylogenetic and amplification patterns of family members showed a clustering of HbCIPK26, 28, and 29 in the phylogenetic tree. Combining the data of chromosomal location, it can be deduced that the gene amplification of HbCIPK28 and HbCIPK29 was due to tandem replication, while HbCIPK26 and HbCIPK28 were amplified through chromosome replication (Figures 1A, 4). The structural analysis suggested that most HbCIPK genes contain one or two exons, although a small number had multiple exons (Group A, Figure 2A). Chromosomal location analysis revealed the twelve HbCBL and thirty HbCIPK genes mapped onto fourteen of the eighteen Hevea chromosomes. However, the distribution of these genes in the chromosomes was uneven, indicative of species evolution and genetic variation (Figure 4).

HbCBL and HbCIPK genes displayed different expression tendencies in different plant tissues (Figure 4). In latex, there were three HbCIPK members, HbCIPK15, 18 and 26 that displayed relatively high expression levels, while only one HbCBL member, HbCBL3, was far more active in latex than the rest, which is consistent with the expression correlation analysis (Figure 10). It is speculated that HbCIPK15, 18 and 26 interacts with HbCBL3 for signal transmission in the latex functioning in the wounding response due to the tapping, latex flow stimulation by ethylene and rubber biosynthesis to regenerate rubber loss through tapping. More than half of the thirty HbCIPK family members were expressed during leaf development, suggesting an important role of the CBL-CIPK complex in the latex growth and development of rubber tree.

Plants have developed unique strategies to adapt to adverse environments, and CBL-CIPK pathways participate in plant responses in this regard through environmental stress signals (Glazebrook, 1999; Luan, 2009). The mechanisms of latex flow upon tapping and ethylene yield stimulation in the rubber tree are not as yet fully understood (Tang C. et al., 2016). Certainly, these horticultural manipulations appear to invoke responses of some HbCBL and HbCIPK genes (Figures 6, 7). Most of the HbCBL and HbCIPK genes involved in these responses, whether up-regulated or down-regulated, might participate in some form of stress responses (Xiang et al., 2007; Hu et al., 2015; Mo et al., 2018). The expression trends in HbCBL3 and HbCIPK7, 18, and 26 suggested that these genes play a role in sustaining the production of latex when trees are tapped routinely, but were negatively regulated by ethephon stimulation. On the other hand, HbCBL1, 10 and HbCIPK11, 14–16, 27, and 30 respond positively to ethephon stimulation, associated possibly with subsequent increased latex output (Figure 6).

The set of HbCBL and HbCIPK genes responded differently when previously untapped trees were first brought into tapping (Figure 7). From the third tapping on, the activity of HbCBL1 could be seen to have declined sharply. This was also the case for HbCIPK16, 18, 23 and 30. On the other hand, bringing the tree into tapping resulted in the expressions of HbCBL3 and HbCIPK26, 27 significantly up-regulated until the 9th tapping, indicating that the different genes had distinct functions when the tree starts latex production (Figure 7). The results also showed the
mechanisms behind initiating latex flow in a previously untapped tree differed from those involved in increasing flow by ethylene stimulation in a tree already subjected to regular tapping.

In Arabidopsis, the SOS pathway is well defined as a salt tolerance signaling pathway that contains three key components, i.e., CPL4/SOS3, CIPK24/SOS2 and the plasma membrane Na\(^{+}/\text{H}^{+}\) antiporter SOS1 (Quan et al., 2007). This pathway is conserved in other plants, e.g., rice, poplar, pear and an Euphorbiaceae relative of Hevea, cassava, and the SOS components from distantly related plants could form inter-species protein complexes and confer salt tolerance to co-transformed yeast cells (Martinez-Atienza et al., 2007; Tang R. et al., 2010; Tang J. et al., 2016; Mo et al., 2018). To determine whether the HbCPL-HbCIPK complexes are involved in the SOS pathway mediated salt tolerance, different combinations of HbCBLs and HbCIPKs were co-transformed with HbSOS1 or AtSOS1 in a yeast mutant strain AXT3K. However, co-expression of any of the three-gene combinations did not confer better salt tolerance to
transformed yeast cells than any of the two-gene combinations (HbCIPKs-HbSOS1 and HbCIPKs-AtSOS1) or single gene (HbSOS1 or AtSOS1) transformed cells (Supplementary Figure 5). In cassava, the MeCBL10-MeCIPK24-MeSOS1 complex forms a functional SOS pathway that confers salt tolerance to co-transformed yeast cells (Mo et al.,...
FIGURE 7 | Expression analyses of HbCIPK and HbCBL genes in latex of trees newly brought into tapping based on qPCR. (A) Expression of HbCBL1, 3, 8, and 10 transcripts in latex from the first, third, fifth, seventh, and ninth tappings (T1, T3, T5, T7, and T9) of previously untapped Hevea trees of the clones PR107 and Reyan8-79. (B) Expression of HbCIPK14, 15, 16, 18, 23, 26, 27, and 30 transcripts in the first, third, fifth, seventh, and ninth tappings (T1, T3, T5, T7, and T9) of previously untapped Hevea trees of the clones PR107 and Reyan8-79. Values are means and standard deviations of three biological replicates. Different letters indicate significant differences with $P < 0.05$. 

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Phylogenetic analysis revealed HbCBL8 and HbCIPK30 as the orthologs of MeCBL10 (named as MeCBL1 in this study) and MeCIPK24, respectively (Figure 1). Unexpected, HbCBL8 and HbCIPK30 revealed no physical interaction (Figure 8), indicating a possible discrepancy in forming an active SOS pathway in Hevea.
In summary, we conducted a genome-wide survey of the \textit{HbCBL} and \textit{HbCIPK} gene families in \textit{Hevea}. A total of twelve \textit{CBL} and thirty \textit{CIPK} genes were identified in the \textit{Hevea} genome database and divided into four and five groups, respectively, by the phylogenetic comparison of homologous genes from \textit{Hevea} and five other plant species. The genes were assigned to the \textit{Hevea} chromosomes and their putative ways of evolution were suggested. Expression analysis among various \textit{Hevea} tissues and phases of leaf development revealed their diversified spatiotemporal expression patterns. Finally, our transcript analysis of \textit{HbCBL} and \textit{HbCIPK} genes following ethylene yield stimulation and the induction of latex tapping, together with the results of multiple \textit{HbCBL} and \textit{HbCIPK} interactions, suggested the involvement of \textit{CBL-CIPK} complexes in responding to many forms of stresses that impact on rubber yield. Our results of genome-wide identification, expression analysis and protein interaction detection provide a foundation for further functional research on the \textit{CBL-CIPK} pathways in \textit{Hevea}. 

**FIGURE 10** | Correlation analyses of \textit{HbCIPK} and \textit{HbCBL} genes based on RNA-seq. Red indicates positive correlation and blue indicates negative correlation.
MATERIALS AND METHODS

Identification of Hevea Calcineurin B-Like Interacting Protein Kinases and Calcineurin B-Like Proteins Genes

Sequences of Arabidopsis thaliana, Populus trichocarpa, and Oryza sativa CBL and CIPK genes were downloaded from GenBank. The genome and protein sequences of Arabidopsis thaliana, Oryza sativa, Populus trichocarpa, Manihot esculenta, and Ricinus communis were downloaded from Phytozome. The Hevea Genome and transcriptome were obtained from GenBank. Local BLAST and Hidden Markov Model searches were conducted to identify Hevea CBLs and CIPKs using Arabidopsis, rice and poplar CBL and CIPK protein sequences as queries to search against the proteome of each species for the candidate CIPKs and CBLs from H. brasiliensis, Arabidopsis thaliana, Oryza sativa, Populus trichocarpa, Manihot esculenta, and Ricinus communis. All putative candidates were manually verified with the InterProScan server to confirm the presence of relevant protein domains. The website ProtParam was used to predict protein isoelectric point and molecular weight.

Phylogenetic, Gene Structure and Chromosomal Location Analyses of Calcineurin B-Like Interacting Protein Kinases and Calcineurin B-Like Proteins Genes

Multiple alignments of the amino acid sequences of CIPK and CBL from Hevea brasiliensis and five other species were set up and phylogenetic trees were constructed with MEGA6.0 by employing the Neighbor-Joining (NJ) method with a bootstrap test for 1,000 replicates. Exon/intron structures of HbCBL and HbCIPK genes were analyzed by comparing the cDNA and their genomic DNA sequences, and chromosomal locations analyzed through mapping the genes to chromosomes, both using the TBtools software (Chen et al., 2020). The collinearity analysis was completed using MCscanX software (Wang et al., 2012).

Expression Analysis Based on Solexa Sequencing

The Solexa sequencing data for various tissues/organs, developmental stages and ethephon stimulation available at the NCBI Sequence Read Archive (SRA) database were used for the expression analysis of Hevea brasiliensis as described previously (Project Accession: PRJNA310171, Supplementary Table 3) (Xiao et al., 2017). The Hevea tissues included (a) latex, bark, leaf, root, seed, female flower and male flower, (b) leaves of four developmental stages (brone, color-change, pale-green and mature), and (c) latex samples collected at 0, 3, 12, and 24 h after ethephon stimulation. Raw RNA-seq reads were processed to trim terminal low-quality bases and adapter sequences via an in-house custom pipeline. The clean reads were then mapped to the Hevea brasiliensis genome using Bowtie2, and RSEM software was used for quantifying transcript abundance with default parameters (Li and Dewey, 2011). The normalized value expression profiles of HbCBL1 and its interaction partner genes were visualized in bar-plots by R. 

RNA Isolation and Quantitative Real-Time PCR

To verify the results obtained by Solexa sequencing and to examine genes expression, qPCR was performed on a number of the Hevea brasiliensis CIPK and CBL genes as described previously (Li et al., 2011). The primer pairs used for the CIPK and CBL genes were listed in Supplementary Table 1. For internal control, the genes RH2b and YLS8 were used as described previously (Li et al., 2011).

Correlation Analysis Based on Gene Expression

To analyze the relationship between CBL and CIPK family members, the expression correlations among family members were analyzed. The input data of correlation analysis was the FPKM values of RNA-seq, included the Hevea (a) tissues latex, bark, leaf, root, seed, female flower and male flower, (b) leaves of four developmental stages (brone, color-change, pale-green and mature), and (c) latex samples collected at 0, 3, 12, and 24 h after ethephon stimulation. R package Corrplot was used for analysis and drawing.

Yeast Two-Hybrid Assay and Yeast Complementation Test

The full length cDNA of eight HbCIPK and four HbCBL genes with substantial expressions in latex were amplified by PCR with their respective primers (Supplementary Table 1) and inserted into the Y2H vectors of pGADT7 and pGBK7, respectively. The pGBK7-HbCBLs and pGADT7- HbCIPKs vectors were co-transformed into the Y2HGold yeast (Saccharomyces cerevisiae) strain and examined for their interactions using the MatchMaker yeast two-hybrid system (Clontech, United States). The detailed protocols were as described (Kleist et al., 2014). To test the functionality of the latex expressed HbCBL-HbCIPK complexes in salt tolerance SOS pathway, complementation test was conducted using the yeast mutant strain AXT3K (4ena1:His3:4ena4,4nha1:LEU2, and 4nhx1:KanMX4) that lacks the main plasma membrane Na+ transporters (Quintero et al., 2011; Zhou et al., 2015). The full length coding regions of HbCBL1/8 and HbCIPK14/15/27/30 were amplified by PCR (Supplementary Table 1) and cloned into the yeast expression

1http://www.ncbi.nlm.nih.gov/genbank
2https://phytozome-next.jgi.doe.gov/
3http://www.ncbi.nlm.nih.gov/nuccore/448814761
4http://www.ebi.ac.uk/Tools/pfa/iprscan/
5https://web.expasy.org/protparam/
6https://cran.r-project.org/web/packages/corrplot/index.html

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4http://www.ebi.ac.uk/Tools/pfa/iprscan/
5https://web.expasy.org/protparam/
vector p414 (Mo et al., 2018), whereas the coding regions of HbSOS1 and AtSOS1 were cloned into the yeast expression vector p416 (Yin et al., 2020). The three plasmids (p414- HbCBLs, p414-HbCIPKs, and p416-HbSOS1 or AtSOS1) were co-transformed into the yeast strain, and the salt tolerance tests were performed according to the detailed process as described previously (Yin et al., 2020).

**Bimolecular Fluorescence Complementation Assay**

Open reading frames (ORFs) of HbCIPK15, HbCIPK30 and HbCBL1 were amplified by RT-PCR and cloned into the pEG100-YFP vectors to construct fusion proteins at the N- and C-termini. Primers are listed in Supplementary Table 1. Each cDNA was under the control of the 35S promoter. For transient expression in *Nicotiana benthamiana* leaves, plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 competent cells for infiltration of 5-week-old *N. benthamiana* leaves. For microscopic observation, the reconstructed green fluorescence protein (GFP) signals of the lower epidermal cells of leaves cut 4 day after infiltration were examined using a Nikon A1RHD25 confocal microscope (Nikon, Japan).

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA310171.

**AUTHOR CONTRIBUTIONS**

CT, XX, and YF conceived and designed the experiments. XX, CM, JS, XLi, XLo, YQ, and YF performed the experiments. XX, JS, and YF analyzed the data. XX and CT wrote the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.743506/full#supplementary-material
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