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Full Length Research Paper

Cloning and molecular characterization of a copper chaperone gene (*HbCCH1*) from *Hevea brasiliensis*

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The cDNA encoding a copper chaperone, designated as *HbCCH1*, was isolated from *Hevea brasiliensis*. *HbCC1* was 589 bp long containing a 261 bp open reading frame encoding a putative protein of 86 amino acids, flanked by a 103 bp 5'UTR and a 225 bp 3'UTR. The predicted molecular mass of HbCCH1 was 9.2 kDa, with an isoelectric point (pl) of 5.13. The *HbCCH1* share the conserved N-terminal metal-binding domain (MXCXXC) and a lysine-rich C-terminus. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that *HbCCH1* was constitutively expressed in all the tested tissues. *HbCCH1* transcripts were accumulated at relatively low levels in the flower, bud and leaves, while *HbCCH1* transcripts were accumulated at relatively high levels in the latex. The transcription of *HbCCH1* in the latex was induced by jasmonate.

Key words: Copper chaperone, *Hevea brasiliensis*, latex.

INTRODUCTION

The rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important industrial crop cultivated for its natural rubber production. Rubber tree is currently propagated by grafting clonal axillary buds onto unselected seedlings to maintain intraclonal heterogeneity for vigour and productivity (Clément-Demange et al., 2007; Hua et al., 2010), because genetic differences in seeding rootstock, stock-scion interaction and mature budded clones exhibit significant intraclonal variability (Clément-Demange et al., 2007). A new type of "selfrooting juvenile clone (JC)" was generated from somatic plant production through embryogenesis issued from rubber-tree anther explants (Wang et al., 1980; Chen et al., 2001, 2002). In a comparative trial between self-rooting JCs and donor clones (DCs), self-rooting JCs had better performance in rubber yield, trunk girth and the laticifer number than those of DCs (Chen et al., 2001, 2002; Hao and Wu, 1996; Yuan et al., 1998; Yang et al., 1994). They may be promising planting materials in future rubber production (Chen et al., 2002).

A great deal of work has been done to reveal the nature and molecular mechanisms underlying the high yield in self-rooting JCs (Chen et al., 2001, 2002; Hao and Wu, 1996; Yuan et al., 1998; Yang et al., 1994; Liang et al., 2009; Lu et al., 2010; Li et al. 2010). However, to our knowledge, molecular mechanism underlying the high yield in self-rooting juvenile clones still remains largely unknown. It is known that rubber biosynthesis takes place only in the laticiferous, where genes are highly expressed in such tissues that may be coded for the enzymes involved in rubber synthesis, and it is more appropriate to study the gene expression in latex (Chow et al., 2007; Venkatachalam et al., 2007). In order to study the genes associated with high yield in self-rooting JCs, latex samples which represent cytoplasmic content of laticiferous cells for RNA isolation, where a subtractive hybriddization between latex from self-rooting JCs and in latex from DCs was employed in a previous study was selected (Liang et al., 2009). One of these differentially expressed clones (designated as HbSSH 20) encoding a

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Abbreviations: CCH, Copper chaperone; **JA,** jasmonate; **ORF,** open reading frame; **RACE,** rapid amplification of cDNA ends; **RT-PCR,** reverse transcriptase polymerase chain reaction; **SOD,** superoxide dismutase; **UTR,** untranslated region.

protein specifying a copper chaperone was obtained in the latex. In this study, the cloning and characterization of the copper chaperone gene (designated as *HbCCH1*) from *Hevea brasiliensis* was reported. This study will contribute to the understanding of the biological function of HbCCH1 in rubber tree.

MATERIALS AND METHODS

Plant material

Hevea brasiliensis were planted at the Experimental Farm of the Chinese Academy of Tropical Agriculture Sciences. Rubber trees were treated with 0.2% ethephon and 0.1% jasmonic acid (JA) according to the Hao method (Hao and Wu, 2000). For latex RNA extraction, rubber trees were tapped using the tapping knife. First, a few drops of latex that contains mostly debris from the plant were discarded. The latex was allowed to drop directly into the liquid nitrogen in an ice kettle. The frozen latex powder was stored at -70℃ or was used immediately. Rubber tree leaves, flower and bud were washed with double-distilled H_2O to remove latex and immediately were frozen in liquid nitrogen. Frozen leaves, flower and bark were stored at -70℃ or were used immediately.

Isolation of RNA

Total RNA was extracted according to Tang's method (Tang et al., 2007). The quality and concentration of the extracted RNA were checked by agarose gel electrophoresis and measured by spectrophotometer (DU-70, Beckman, USA).

Cloning of *HbCCH1*

The full-length cDNA of the copper chaperone was cloned using poly (A)+ RNA from the latex as the template to perform both 3'-RACE reactions based on the cDNA sequence of HbSSH20. 3'-RACE were performed using 3'-Full RACE core kit (TaKaRa, Dalin, China). Primer 3F used for the 3'-RACE was 5'-TGGAAGGTGTGG AATCTTATGACATTG-3'. According to the information of the sequence obtained by 3'-RACE and HbSSH20, the entire coding region of *HbCCH1* was predicted and amplified with a pair of gene-specific primers (F1: 5'-ATGTCTCAGATTGTTGTGCTT-3' and R1: 5'-TTAACATCAAGCAACAGTC AC-3') by RT-PCR with the latex poly (A)+ RNA as the template and was then sequenced. Comparison of DNA and the predicted amino acid sequences with non-redundant database were performed by BLAST analysis.

Multiple alignments and bioinformatic analyses

The nucleotide sequence, deduced amino acid sequence and ORF encoded by *HbCCH1* were analyzed. The sequence comparison was conducted through database search using BLAST program (http://www.ncbi.nlm.nih.gov). Multiple alignments were carried out using the ClustalX software version 1.81 (Thompson et al., 1997).

Southern blot analysis

Genomic DNA was isolated from the rubber-tree leaves by the method described by Dellaporta et al. (1983). Genomic DNA (25 μg/sample) was digested completely with *Eco*RI, *Bam*HI and *Hin*d III overnight, and then separated by electrophoresis in 1.0% agarose gels, blotted onto Hybond-N+ nylon membrane (Amersham Pharmacia, Uppsala, Sweden) and probed with the *HbCCH1* ORF (^{32}P-labeled). Probe labeling and hybridization were performed according to the standard method of Sambrook et al. (1989).

Analysis of *HbCCH1* expression

RT-PCR for the analysis of *HbCCH1* expression was performed by using the total RNA from the rubber tree tissues amplified with *HbCCH1* specific primers P1 (5'-GGTGTGGAATCTTATGACATTG-3') and P2 (5'-TGGTCGCGGCCGAGGTACTAG-3'). Specific primers were designed from the low homology regions of *HbCCH1* coding sequences and the 3'-UTRs. The *ACT* gene was used as an internal control parallel in the reactions amplified with *ACT* specific primers AF (5'-CAGTGGTCGACAACTGGTAT-3') and AR (5'-ATCCTCCAATCCAGACACTGT-3'). The PCR reaction was carried out with 22 cycles of a programmed temperature control of 30 s at 95℃, 30 s at 50℃ and 1 min at 72℃ with a 5 min preheat at 95℃ and a 10 min final extension at 72℃ using *HbCCH1* specific primers for amplification of *HbCCH1*. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

RESULTS

Cloning and characterization of *HbCCH1*

The differentially expressed clones (372 bp, designated as HbSSH20), which showed similarity with other plant copper chaperone genes as revealed by a BLASTX search, was obtained in a previous study (Liang et al., 2009). The BLASTX search identified that HbSSH20 contained a part of ORF of copper chaperone gene, flanked by a 103 bp 5'UTR. The *HbSSH20* was expressed at different levels, with higher levels in self-rooting JCs than in their DCs. Based on the sequence of the fragment, 3'-RACE primers were designed and used in RACE, generating a 442 bp fragment. By alignment and assembling of these two sequences, the full-length cDNA sequence of *HbCCH1* was deduced and amplified by PCR, and was confirmed by sequencing. The full-length cDNA was 589 bp, and it contained a 261-bp ORF, with a 3' UTR of 225 bp downstream of the stop codon and a 5' UTR of 103 bp upstream of the start codon (Figure 1).

The deduced HbCCH1 protein consisted of 186 amino acid residues with a calculated molecular weight of 9.2 kDa and an isoelectric point of 5.13, while BLAST showed that HbCCH1 belonged to the copper chaperone family (Figure 2). The HbCCH1 share the conserved N-terminal metal-binding domain (MXCXXC) and lysine-rich C-terminus, which are common to the majority of copper chaperone (Lin and Culotta, 1995; Pufahl et al., 1997).

Southern blot

To investigate the genomic organization of *HbCCH1* in the rubber tree, the genomic DNA was digested with *Eco*RI, *Bam*H I and *Hin*d III, which were not present in the *HbCCH1* sequence, and was hybridized using the

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                        gacttggagcggccgcccgggcaggttacgcacaagaatcaat  43
caggggggaaaacaaatcaagatttcttctattcttcacttgtattgcacacccataacc  103
ATGTCTCAGATTGTTGTGCTTAAGGTTGGTATGTCATGTGAAGGTTGTGTTGGGGCTGTG  163
 M  S  Q  I  V  V  L  K  V  G  M  S  C  E  G  C  V  G  A  V   20
AAGAGGGTTTTGGGAAAAATGGAAGGTGTGGAATCTTATGACATTGATTTGAAGGAGCAA  223
 K  R  V  L  G  K  M  E  G  V  E  S  Y  D  I  D  L  K  E  Q   40
AAGGTTACAGTGAAAGGAAACGTGCAGCCAGAGGCAGTTCTTCAGACTGTTTCAAAGACC  283
 K  V  T  V  K  G  N  V  Q  P  E  A  V  L  Q  T  V  S  K  T   60
GGGAAGAAGACTACCTTCTGGGAAGCAGAGGCACCTGCAGAGCCCGAAACAAAGCCTGCA  343
 G  K  K  T  T  F  W  E  A  E  A  P  A  E  P  E  T  K  P  A   80
GAAACTGTGACTGTTGCTTGAtgttaattcatatgctttatgaactagtacttcaatgga  403
 E  T  V  T  V  A  *                                           86
tcatagatggtcaaacttaatatttgctgtgatgtattttgccataatttagcacccctta  463
tacgacactcggttgtaagccacaataataacttggaagctctcttagggctgtgtgtat  523
tgttattaatagaaaatgtgctttatggtcgtatgcccaaaaaaaaaaaaaaaaaa      589
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Figure 1. Nucleotide sequence and deduced amino acid sequence of HbCCH1.

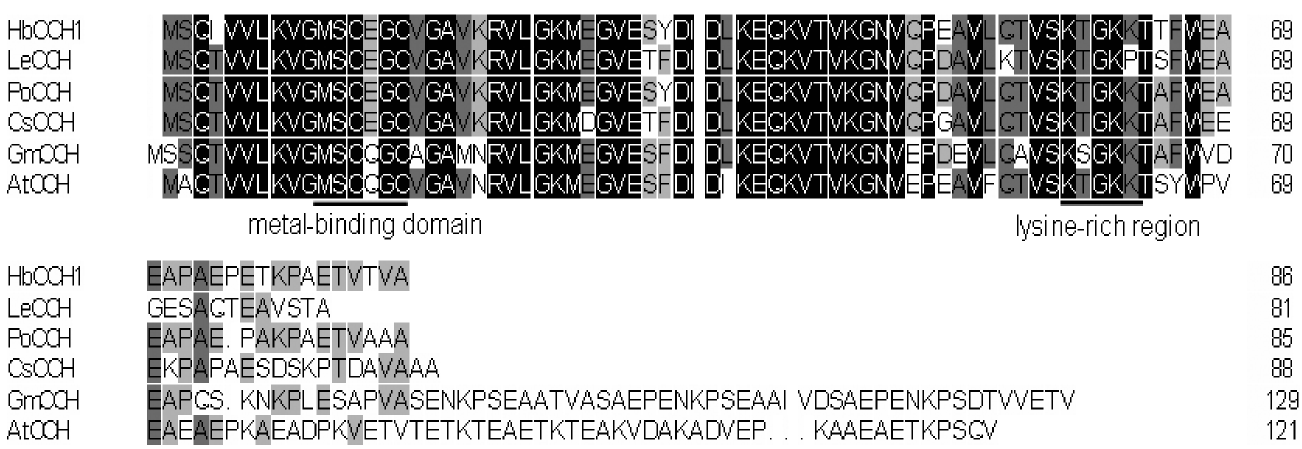

Figure 2. Comparison of the deduced amino acid sequence of HbCCH1 with other plant CCHs. Amino acid residues that were identical in all the six sequences are shaded in dark, while well-conserved residues are shaded in gray. The underlining indicate amino acid residue containing the conserved domain. The CCH1 used in the analysis were retrieved from Genbank, including GmCCH (*Glycine max*, AAF15286), LeCCH (*Lycopersicon esculentum*, AAP06757), AtCCH (*Arabidopsis thaliana*, NP_191183), PoCCH (*Populus alba x Populus tremula* var. glandulosa, AAT12488) and CsCCH (*Citrus* cv. Shiranuhi, ABL67657).

full-length ORF of *HbCCH1* as a probe. The result showed that only one hybridized band was present in each lane (Figure 3), indicating that there was only one copy of the HbCCH1 gene in the rubber tree.

Differential expressions of *HbCCH1* in different tissues

Accumulation of *HbCCH1* transcripts in different tissues was examined using semi-quantitative RT-PCR analysis. The result showed that *HbCCH1* was constitutively expressed in all the tested tissues. As such, *HbCCH1* transcripts were accumulated at relatively low levels in the flower, bud and leaves, while *HbCCH1* transcripts were accumulated at relatively high levels in the latex (Figure 4).

Effects of ethephon and JA on the *HbCCH1* expression in latex

To identify signals that might be involved in the regulation of *HbCCH1* expression during stress, the effects of some

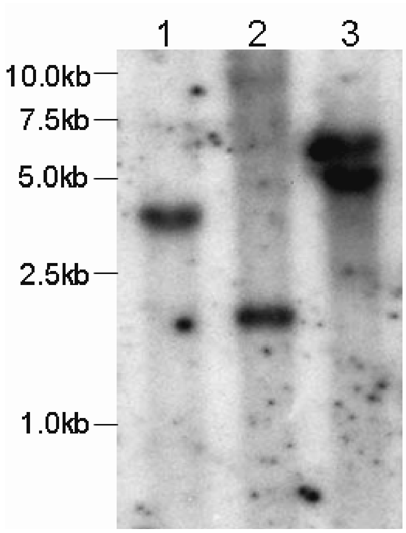

Figure 3. Southern blot analysis of *HbCCH1*. Rubber tree genomic DNA was digested with *Eco*RI (1), *Bam*HI (2) and *Hin*d III (3), followed by hybridization with ^{32}P-labeled HbCCH1 ORF.

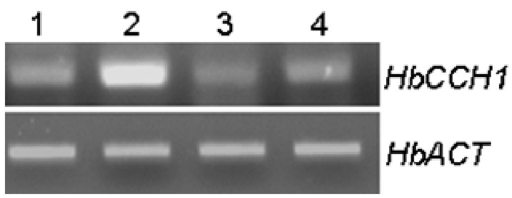

Figure 4. Transcription pattern analysis of *HbCCH1*. Semi-quantitative RT-PCR was performed on RNA derived from various tissues of rubber tree. *Actin* was amplified for normalization. 1, open flowers; 2, latex; 3, leaves; 4, bud.

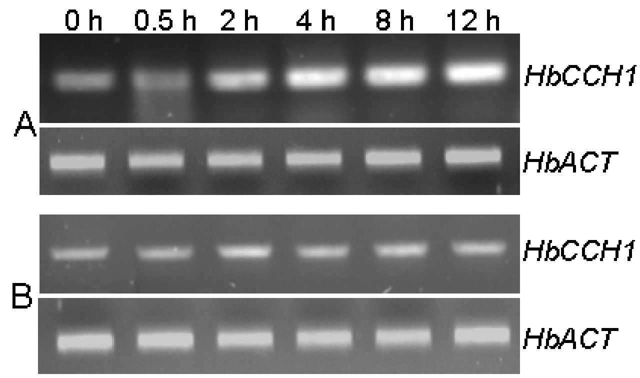

Figure 5. Effects of hormone on the expression of *HbCCH1* in the latex. The RNA extracted from the extruded latex of rubber trees treated with ethephon or JA for 0, 0.5, 2, 4, 8 or 12 h were subjected to RT-RCR assay, while *Actin* was used as the control. A, The *HbCCH1* transcript in latex was induced by JA treatment; B, the *HbCCH1* transcript was not induced upon ethephon treatment.

plant growth regulators were analyzed. Figure 5 shows that the *HbCCH1* transcript was induced by JA, while *HbCCH1* expression was not induced by ethephon.

DISCUSSION

Copper is an important mineral nutrient found in chloroplasts as a cofactor associated with Cu/Zn superoxide dismutase (SOD) (Yruela, 2005). Copper chaperones constitute a family of small Cu^+-binding proteins required for Cu homeostasis in eukaryotes (Puig et al., 2007). Copper chaperones were first characterized in *Saccharomyces cerevisiae* and the three known chaperones are CCS, ATX1 and COX17 (Culotta et al., 1997; Lin and Culotta, 1995; Srinivasan et al., 1998). In *Arabidopsis*, three different members of copper chaperones family (CCH, ATX1 and COX17) were identified and characterized at different levels (Himelblau et al., 1998; Mira et al., 2001; Chu et al., 2005), although CCH genes was cloned and characterized from *Arabidopsis*, tomato, poplar and a few other plant species (Himelblau et al., 1998; Company and Carmen, 2003; Lee et al., 2005). In this study, the cloning and characterization of the gene encoding copper chaperone from rubber tree is reported. The HbCCH1 share the conserved N-terminal metal-binding domain (MXCXXC) and lysine-rich C-terminus, which are common to the majority of copper chaperone (Lin and Culotta, 1995). Mira et al. (2001) reported that the presence of the conserved N-domain in the protein made it possible to function as an antioxidant. *Arabidopsis* CCH has a 47-amino-acid C-terminal extension that is absent in LeCCH and PoCCH (Figure 2); however, HbCCH1 does not have the C-terminal extension identified in *Arabidopsis* CCH. *Arabidopsis CCH* gene is up-regulated by oxidative stress caused by ozone and it complements *ATX1* function in SOD-deficient yeast (Himelblau et al., 1998). PoCCH responds specifically to certain metals and abiotic stresses that induce oxidative damage (Lee et al., 2005). In this study, HbCCH1 was up-regulated by JA (the expression pattern agreed with the known function of JA), which is involved in controlling plant responses to abrasion and pathogen attack (Moons, 2003).

The latex from the rubber tree is expelled from laticifer cells upon bark tapping (Kush, 1994), while the latex yield is mainly limited by the duration of the latex flow, which is controlled by coagulation processes (d'Auzac, 1989; Chrestin et al., 1998). The duration of latex flow is reported to be dependent on the integrity of lutoids (a polydispersed lysosomal vacuome in the laticifer cell). The presence of NAD(P)H oxido-reductase which leads to formation of reactive oxygen species (ROS) has been reported in the lutoids, in that the release of ROS is claimed to be responsible for the peroxidative degradation of lutoid membrane fragility. The damaging of the lutoid membrane leads to latex coagulation processes. However, SOD is present in latex and assists in the maintenance of lutoid membrane integrity (Chrestin et al., 1998). Superoxide dismutases are metallo-enzymes found in most oxygenic organisms with proposed roles in reducing oxidative stress (Cohu et al., 2009).

Copper chaperone is involved in the protection against oxidative stress (Himelblau et al., 1998; Chu et al., 2005; Lee et al., 2005; Puig et al., 2007; Cohu et al., 2009). It was speculated that when HbCCH1 is expressed at different levels, with higher levels in self-rooting JCs than in their DCs, the HbCCH1 may influence latex flow in self-rooting JCs and their DCs, leading to latex yield between self-rooting JCs and their DCs differently. It will be of great interest to further elucidate whether latex flow is regulated by copper chaperone. As a consequence, characterization of HbCCH1 will enable the study of the biological function of HbCCH1 in the rubber tree.

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