Involvement of HbMC1-mediated cell death in tapping panel dryness of rubber tree (Hevea brasiliensis)

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Tapping panel dryness (TPD) causes a significant reduction in the latex yield of rubber tree (Hevea brasiliensis Muell. Arg.). It is reported that TPD is a typical programmed cell death (PCD) process. Although PCD plays a vital role in TPD occurrence, there is a lack of detailed and systematic study. Metacaspases are key regulators of diverse PCD in plants. Based on our previous result that HbMC1 was associated with TPD, we further elucidate the roles of HbMC1 on rubber tree TPD in this study. HbMC1 was up-regulated by TPD-inducing factors including wounding, ethephon and H₂O₂. Moreover, the expression level of HbMC1 was increased along with TPD severity in rubber tree, suggesting a positive correlation between HbMC1 expression and TPD severity. To investigate its biological function, HbMC1 was overexpressed in yeast (Saccharomyces cerevisiae) and tobacco (Nicotiana benthamiana). Transgenic yeast and tobacco overexpressing HbMC1 showed growth retardation compared with controls under H₂O₂-induced oxidative stress. In addition, overexpression of HbMC1 in yeast and tobacco reduced cell survival after high-concentration H₂O₂ treatment and resulted in enhanced H₂O₂-induced leaf cell death, respectively. A total of 11 proteins, rbcL, TM9SF2-like, COX3, ATP9, HbREF/Hevb1, MSSP2-like, SRC2, GATL8, CIPK14-like and STK, were identified and confirmed to interact with HbMC1 by yeast two-hybrid screening and co-transformation in yeast. The 11 proteins mentioned above are associated with many biological processes, including rubber biosynthesis, stress response, autophagy, carbohydrate metabolism, signal transduction, etc. Taken together, our results suggest that HbMC1-mediated PCD plays an important role in rubber tree TPD, and the identified HbMC1-interacting proteins provide valuable information for further understanding the molecular mechanism of HbMC1-mediated TPD in rubber tree.

Keywords: metacaspase, oxidative stress, programmed cell death, rubber tree, tapping panel dryness, yeast two-hybrid assay.

Introduction

Pará rubber tree (Hevea brasiliensis Muell. Arg.), more commonly known as rubber tree, is an economically important tropical tree species used for producing natural rubber, an essential industrial raw material. Latex containing natural rubber is harvested by tapping the bark of rubber tree trunk. In addition, ethephon (an ethylene releaser) is commonly applied to stimulate latex regeneration as well as flow, and consequently increase latex yield (Zhu and Zhang 2009). It is reported that excessive tapping and overstimulation with ethephon can cause oxidative stress inside latex cells and lead to tapping panel dryness (TPD) (Duan et al. 2010, Putranto et al. 2015, Zhang et al. 2017). Tapping panel dryness is a complex physiological syndrome characterized by the partial or complete stoppage of latex flow upon tapping (Chen et al. 2003, Putranto et al. 2015), and it has become one of the most serious threats to natural rubber production. It has been reported that TPD causes 10–40% loss of annual rubber production (Gébelin et al. 2013), with an incidence of 20–50% in almost all rubber-growing regions (de Fay 2011). However, the detailed molecular mechanism underlying...
TPD is largely unknown and currently there is no effective method to treat or prevent TPD.

Numerous studies have been performed to understand the nature and molecular mechanisms of rubber tree TPD. Chen et al. (2003) identified a Myb transcription factor, HbMyb1, closely associated with TPD by differential display RT-PCR, and Peng et al. (2011) further documented that HbMyb1 negatively regulated UV-B irradiation-induced programmed cell death (PCD) in transgenic tobacco. They proposed that TPD in the rubber tree may be a form of plant PCD (Peng et al. 2011). Venkatachalam et al. (2007) reported that most of the genes up-regulated in TPD trees were involved in the PCD process. Li et al. (2010, 2016) separately analyzed the genes differentially expressed between healthy and TPD rubber trees in latex and bark tissues, and found that the TPD-related genes were significantly enriched in PCD, reactive oxygen species (ROS) metabolism and rubber biosynthesis. Additionally, the predicted target genes of TPD-related microRNAs were associated with PCD (Gébelin et al. 2013). Consistent with the aforementioned results, the TPD tree showed typical characteristics of PCD, such as DNA laddering, TUNEL-positive staining of the nucleus in the laticifer cell, nuclear condensation and degradation, etc. (de Fay et al. 2011, Peng et al. 2011). These results suggest a strong correlation between PCD and TPD development, indicating that PCD might play a critical role in TPD occurrence.

The mechanism of PCD induction usually includes an increase in the levels of ROS, which are utilized as mediators of the stress signal (Petrov et al. 2015). The involvement of oxidative stress in rubber tree TPD was recently reviewed by Zhang et al. (2017), and they suggested that environmental and harvesting stresses lead to over-accumulation of ROS in laticifers, which consequently induces TPD occurrence. In TPD trees, the NAD(P)H oxidase and peroxidase activities increased, but the activities of ROS scavenging enzymes (SOD and CAT) decreased (Chrestin 1989). Additionally, the expression of the genes in ROS-scavenging systems was depressed in TPD trees (Li et al. 2010, 2016, Montoro et al. 2018). These changes probably lead to ROS accumulation and burst, whereas the excessive ROS further triggers PCD during the onset of TPD syndrome.

Programmed cell death is a genetically controlled cell suicide process that plays crucial roles in a wide variety of developmental and physiological processes in animals, plants and fungi (Van Hautegem et al. 2015, Huysmans et al. 2017). In animals, caspases are key regulators of PCD (Sueldo and van der Hoorn 2017). However, no orthologous caspases have been identified in plants. Proteins structurally similar to caspases in plants are metacaspases, a family of cysteine proteases (Uren et al. 2000). Although metacaspases have no caspase activities like animal caspases, they are essential for cell death regulation in plants (Bonneau et al. 2008). In the last decade, the biological functions of metacaspases have been well characterized in Arabidopsis. AtMC1 and AtMC2 act as positive and negative regulators of hypersensitive response-related cell death, respectively (Coll et al. 2010); AtMC4/AtMCP2d is a positive regulator of biotic and abiotic stress-induced PCD (Watanabe and Lam 2011); AtMC8 positively modulates PCD induced by H2O2 and UVC (He et al. 2008); AtMC9 is known to function in xylem cell death (Bollhoner et al. 2013). Additionally, several metacaspases have been found to function in different types of PCD in other plant species, including Populus PttMC13 and PttMC14 (Bollhoner et al. 2018), Norway spruce mctl-Pa (Bozhkov et al. 2005), Nicotiana benthamiana NbMCA1 (Hao et al. 2007), pepper Camc9 (Kim et al. 2013), and wheat TaMCA1 (Hao et al. 2016) and TaMCA4 (Wang et al. 2012). However, up to now, no metacaspases from rubber tree have been functionally characterized.

Protein–protein interactions play an important role in the biological function and regulation of metacaspases. Most recently, Lema Asqui et al. (2017) demonstrated that AtSerpin1 blocked AtMC1 self-processing by interacting with AtMC1 and then inhibited AtMC1-mediated cell death. In addition, AtMC1 can interact with AtLSD1 (Coll et al. 2010), a negative regulator of cell death (Dietrich et al. 1997). Huang et al. (2015) have also shown that OsMC1 interacted with OsLSD1 and OsLSD3, while OsMC3 only interacted with OsLSD1. In soybean, the interaction between metacaspase GmMcn11 and Rps1-k-2 is essential for race-specific resistance to Phytophthora sojae (Baskett 2012). Identifying the potential interaction partners with metacaspases and elucidating the functional consequences of these potential protein networks will help to reveal the complexity of regulations that have evolved to control PCD in plants (Lam and Zhang 2012).

Our previous study showed that rubber tree contains nine metacaspase family genes, and that HbMC1 is associated with TPD (H.Liu et al. 2016). In the present study, we further investigated HbMC1 expression in the bark tissues of rubber trees with different degrees of TPD and characterized its function in oxidative stress tolerance and PCD by overexpressing it in yeast (Saccharomyces cerevisiae) and tobacco (N. benthamiana). In addition, a yeast two-hybrid assay was performed to screen the proteins interacting with HbMC1. Our results provide valuable information for understanding the molecular mechanisms underlying HbMC1-mediated PCD during the onset of TPD in rubber tree.

**Materials and methods**

*Plant materials and treatments*

Seven-year-old virgin trees of Reyan 7-33-97 planted at the experimental farm of Chinese Academy of Tropical Agricultural
Hao and Wu
Putranto et al. (2015)
First-strand cDNA was synthesized with PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa) according to the manufacturer's instructions. The thermal cycle was as follows: 95 °C for 1 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 20 s. All primers were designed by the online software Primer3web version 4.1.0. (http://bioinfo.ut.ee/primer3/). The primers used for qRT-PCR analysis of HbMC1 (GenBank accession No.: KU188281) were 5′-AGGGAGTCCTCAGTCCGGATT-3′ and 5′-CGGAAGTAGAAAGACAGA-3′ (H.Liu et al. 2016). The rubber tree HbUBC4 gene was used as the internal control with the primer pair 5′-TCTTATGAGGCGGAGTGC-3′ and 5′-CAA GAACCGACTTGGAGG-3′ (Li et al. 2011). The relative expression was calculated using the 2−ΔΔCT method, in which CT indicates cycle threshold (Schmittgen and Livak 2008).

Overexpression of HbMC1 in yeast and oxidative stress tolerance assays
The full-length coding sequence (CDS) of HbMC1 was amplified with the cDNA from rubber tree bark tissues using the specific primers (F: 5′-GGAATATTGATATCCTGGCCTG-3′ and R: 5′-CCGCTGAGTCCAAAGAAGTTTTTT-3′, EcoRI and Xhol restriction enzyme sites underlined) and inserted into the yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA) between the EcoRI and Xhol restriction sites to create pYES2-HbMC1. The pYES2-HbMC1 and pYES2 plasmids were separately introduced into yeast (S. cerevisiae) strain INVSc1 according to the small-scale yeast transformation protocol described in the pYES2 user manual (Invitrogen). The transformants were screened by growing on synthetic complete medium deficient in uracil (SC-U) plates, supplemented with 2% glucose. Positive colonies were further verified by PCR. For pYES2-HbMC1 transformants, T7 (5′-TAATAACGACCTATAGCCGG-3′) and HbMC1-R primers were used. For pYES2 transformants, T7 and pYES2-R (5′-TATTTAAGCCCTTGGAGCTCC-3′) primers were used. To investigate the expression of HbMC1 in transgenic yeast, yeast cells containing pYES2 (control) or pYES2-HbMC1 were cultivated in induction medium (SC-U medium supplemented with 2% galactose) for 24, 36 or 48 h to induce the expression of HbMC1. After that, total RNA from yeast cells was isolated using Yeast Total RNA Isolation Kit (Sangon Biotech, Shanghai, China). The quality and quantity of the isolated RNA were determined as described above. First-strand cDNA was synthesized with PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa) according to the manufacturer's instructions. The expression of HbMC1 was examined by semi-quantitative RT-PCR. The yeast ACT1 was used as the reference gene to normalize the amount of mRNA in each reaction with the primer pair 5′-ATGTGCCAGGAAGAACCCC-3′ and 5′-TACCAGCGGATTCGACCC-3′ (Teste et al. 2009). The amplified products were analyzed by electrophoresis in 1.0% agarose gel.

For comparison of growth curves, yeast cells containing pYES2-HbMC1 or pYES2 were grown in liquid induction medium at 30 °C for 36 h with shaking at 200 rpm to induce HbMC1 expression. The cultures were centrifuged at 1500g for 10 min and the cell pellets were diluted to a final optical density at 600 nm (OD600) of 0.2 with induction medium containing 0, 2, 2.5 or 3 mM H2O2. Cultures were incubated at 30 °C for 36 h with shaking at 200 rpm. Cell density was monitored by measuring the OD600. A spotting assay was performed to ascertain the function of HbMC1 in oxidative stress response in yeast. Yeast cells containing pYES2-HbMC1 or pYES2 (control) were grown in liquid induction medium at 30 °C for 36 h with shaking (200 rpm). The cultures were centrifuged at 1500g for 10 min.
and the cell pellets were re-suspended in sterile water to OD$_{600}$ = 1.0. After fivefold serial dilutions, 5 μl aliquots of each dilution were spotted onto the solid induction medium containing 0, 2, 2.5 or 3 mM H$_2$O$_2$, and then incubated at 30°C for 4 days. Each experiment was carried out in triplicate.

For comparison of cell survival after oxidative stress, yeast cells containing pYES2-HbMC1 or pYES2 were cultivated in liquid induction medium at 30°C for 36 h with shaking (200 rpm) to induce the expression of HbMC1. After incubation, the cultures were centrifuged at 1500g for 10 min and the cell pellets were adjusted to OD$_{600}$ = 1.0 with sterile water containing 0 (control) or 5 mM H$_2$O$_2$. After 12 h of treatments, samples were fivefold serially diluted and 5 μl aliquots of each dilution were spotted on solid SC–U medium (supplemented with 2% glucose). All of the plates were incubated at 30°C for 4 days. Each experiment was carried out in triplicate.

**Generation of HbMC1 transgenic tobacco plants**

To investigate the function of HbMC1 in plants, the full-length CDS of HbMC1 was amplified with the cDNA from rubber tree bark tissues using the specific primers (F: 5′-CATCCATGGCCA TCTTCTGCGTGCCTAT-3′ and R: 5′-TAAGGTGACCAACGTA GAAACGAGAACATGC-3′). Ncol and BsrI restriction enzyme sites were underlined and inserted into the plant binary expression vector pCAMBIA1301 under the control of CaMV35S promoter. The construct was introduced into tobacco (N. benthamiana) by Agrobacterium tumefaciens-mediated leaf disc transformation as described by Rajput et al. (2014). The transgenic plants (T$_0$) were selected on Murashige and Skoog (MS) medium plates with 30 mg l$^{-1}$ hygromycin and further identified by genomic DNA PCR using CaMV35S promoter-specific primer (5′-AGCGA CAATCCCACATCCTGC-3′) together with gene-specific reverse primer. The progeny of these transgenic plants (T$_1$, seeds) were used for segregation analysis of hygromycin resistance, and the lines segregating 3:1 for hygromycin resistance were selected for further experiments. Hygromycin-resistant plants of these T$_1$ lines were self-fertilized to obtain T$_2$ seeds. T$_2$ seeds were also subjected to segregation analyses. Lines producing 100%-resistant plantlets (homozygous lines) were selected to obtain T$_3$ seeds. The relative expression levels of HbMC1 were analyzed in the T$_1$ transgenic lines and confirmed in the T$_3$ homozygous lines by qRT-PCR. The first fully expanded leaf from the top was harvested from transgenic and wild-type (WT) tobacco plants (4-week-old). Three independent biological replicates were collected for each line, and the leaves of each biological replicate were collected from three plants. All samples were immediately frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was synthesized with PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa) according to the manufacturer’s instructions. qRT-PCR was performed as described above. The N. benthamiana EF1α gene was used as the internal control with the primers (F: 5′-TTGCTGCTTTACCCCTGTGG-3′ and R: 5′-TCGAGCAAGAGAGA TGGGAC-3′) (Rajput et al. 2014). After expression and segregation analysis, three independent T$_3$ homozygous lines (L1, L5 and L9) were selected and used for further studies.

**Oxidative stress tolerance analysis**

Seeds of WT, L1, L5 and L9 (T$_3$ generation) were surface-sterilized and sown on solid MS medium. After 4 days, the same size germinated seeds were transferred to plastic pots filled with a mixture of peat, vermiculite and soil (1/1/1, v/v/v), and then after seedlings were cultivated for 6 weeks in a growth chamber under conditions: 25/23°C light/dark temperature regime, 16 h light/8 h dark cycle and 200 μmol m$^{-2}$ s$^{-1}$ light intensity. To evaluate the tolerance of HbMC1 transgenic lines to oxidative stress, leaf discs detached from the third fully expanded leaves of transgenic and WT tobacco plants were floated on 200 mM H$_2$O$_2$ solution or distilled water as control, and then placed in a growth chamber under the above-mentioned conditions. Each treatment was repeated three times, with 20 leaf discs per replicate. After 2 days of treatment, the chlorophyll contents of leaf discs were measured as described previously (Zhang and Kirkham 1996).

We further compared the differences in seedling growth between the WT and transgenic lines under H$_2$O$_2$-induced oxidative stress; seeds of WT and transgenic T$_3$ lines were sterilized and sown on solid MS medium and allowed to germinate and grow in a growth chamber under the same conditions described above. After 7 days, the uniform seedlings were then transferred to solid MS medium containing 0 (as control group) or 4 mM H$_2$O$_2$. Each treatment consisted of three replicates and each replicate contained five seedlings. After 4 weeks of growth under the above-mentioned conditions in the growth chamber, the fresh weights and plant heights of the seedlings were measured.

**H$_2$O$_2$-induced cell death assay**

Five-week-old plants of WT and transgenic lines cultivated under the conditions mentioned above were used for H$_2$O$_2$-induced cell death assay. For H$_2$O$_2$ treatment, the third fully expanded leaves detached and incubated in 50 mM H$_2$O$_2$ solution. Detached leaves incubated in distilled water were used as a control group. Nine leaves from each line were performed for each treatment. After 1 day of incubation in the growth chamber under the above-described conditions, leaf samples were stained with trypan blue to visualize dead cells. Trypan blue staining was performed as described by Bowling et al. (1997). Photographs of representative stained leaves were taken with a digital camera. Quantitative evaluation of the percentage of trypan blue-stained leaf area was performed using the Leaf Doctor app developed by Pethybridge and Nelson (2015).
Construction of normalized cDNA library

A normalized cDNA library was constructed from various tissues of rubber tree, including root, leaf, stem tip, latex, bark and flower. Isolation, quality and quantity determination of total RNA were performed as mentioned above. The normalized cDNA library was constructed using the Make Your Own Mate & Plate™ library system (Clontech, Mountain View, CA, USA) according to the manufacturer’s protocol. In brief, equal amounts of DNase I-treated total RNA from various tissues were pooled and used for first-strand cDNA synthesis. The double-stranded cDNA was synthesized and amplified by long-distance PCR using Advantage 2 Polymerase Mix (Clontech). The double-stranded cDNA was normalized using the Trimmer-Direct cDNA Normalization kit (Evrogen, Moscow, Russia) and subsequently purified with CHROMA SPIN TE-400 columns (Clontech) according to the manufacturer’s instructions. The purified double-stranded cDNA and the linearized pGADT7-Rec AD cloning vector (Clontech) were co-transformed into yeast strain Y187 following the library-scale transformation protocol (Yeast Transformation System 2 Manual, Clontech). The titer, capacity and recombination rate of the yeast two-hybrid cDNA library were evaluated according to the Make Your Own ‘Mate & Plate™’ Library System User Manual (Clontech). The length of the inserted fragments of the library was evaluated by PCR amplification of 24 independent colonies with Matchmaker Insert Check PCR Mix 2 (Clontech) according to the manufacturer’s protocol. The amplified products were analyzed by electrophoresis in 1.0% agarose gel.

Auto-activation and toxicity tests of bait plasmid

The full-length CDS of HbMC1 was amplified by PCR with the primers (F: 5’-CCATGCGCGTGCATGCTATGGTG-3’ and R: 5’-GTCGACCCGAAAGTTAAGGAAGCAGC-3’, NcoI and SalI restriction enzyme sites underlined). The PCR product was purified, double-digested with NcoI and SalI, and then inserted into the pGBK7 bait vector, which was double-digested with the same restriction enzymes. The pGBK7-HbMC1 bait plasmid was subsequently transformed into yeast strain Y2HGold using the Yeastmaker™ Yeast Transformation System 2 kit (Clontech) according to the user manual. The toxicity and auto-activation of bait protein were tested according to the user manual of the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech).

Yeast two-hybrid screening and confirmation of the interactions

Yeast two-hybrid screening using yeast mating was carried out as previously described (Ramalingam et al. 2015). The putative interacting clones were first selected on DDO/X/A (SD/-Leu/−Trp supplemented with 40 μg ml⁻¹ X-α-Gal and 200 ng ml⁻¹ Aureobasidin A) agar plates. Blue colonies were then patched out onto higher stringency QDO/X/A (SD/-Ade/-His/-Leu/−Trp supplemented with 40 μg ml⁻¹ X-α-Gal and 200 ng ml⁻¹ Aureobasidin A) agar plates. Yeast colony PCR using the Matchmaker™ Insert Check PCR Mix 2 (Clontech) was performed to estimate each insert size of the potential positive prey plasmids. Rescue and isolation of prey plasmids were carried out as described by Zhao et al. (2017). The putatively positive prey plasmids were sequenced using T7 and 3′ AD primers (5′-TAATACGACTCCTATAGGG-3′ and 5′-AGATTTCCAGATGCACGAG-3′). The sequences obtained were blasted against the Transcriptome Shotgun Assembly (TSA) and whole-genome shotgun contigs (wgs) database of rubber tree at NCBI to get the full-length sequences of the corresponding genes. Gene prediction was carried out using the online program FGENESH at Softberry (http://www.softberry.com). Functional annotation and analysis of the deduced amino acid sequences of the positive clones were performed using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Gene Ontology (http://www.geneontology.org) and UniProt (https://www.uniprot.org) databases.

Statistical analysis

All data were expressed as the mean ± SD of three biological replicates. Statistical analyses were carried out using IBM SPSS Statistics v22.0. Duncan’s multiple range test or Student’s t-test was performed to determine significant differences at P < 0.05 and P < 0.01.

Results

HbMC1 is closely associated with rubber tree TPD

Our previous study showed that HbMC1 may participate in TPD occurrence in the rubber tree (H.Liu et al. 2016). To further elucidate the role of HbMC1 in rubber tree TPD, we systematically analyzed its expression in the bark tissues of rubber trees with different degrees of TPD or treated with TPD-inducing factors. As shown in Figure 1A–C, HbMC1 expression firstly increased and then decreased in the bark tissues with the treatments of TPD-inducing factors, including H₂O₂, ethephon and wounding. The highest expression of HbMC1 was at 4 h after H₂O₂ and wounding treatments (Figure 1A and C), while it was at 8 h under ethephon treatment (Figure 1B). Being consistent with the aforementioned results, HbMC1 expression in the bark tissues of TPD rubber trees was significantly higher than that of...
healthy ones. In addition, the expression of HbMC1 was significantly increased in the bark tissues along with TPD severities (Figure 1D), suggesting a positive correlation between HbMC1 expression and TPD severity. Taken together, these results indicate that HbMC1 is closely associated with rubber tree TPD.

Overexpression of HbMC1 in S. cerevisiae enhances H2O2-induced cell death and reduces oxidative stress tolerance

Previous studies suggested that ROS over-accumulation in laticifers plays a crucial role in TPD occurrence in rubber tree (Putranto et al. 2015, Zhang et al. 2017). Our expression analysis indicated a potential involvement of HbMC1 in rubber tree TPD. Therefore, we further determined the biological role of HbMC1 in oxidative stress using a yeast (S. cerevisiae) heterologous expression system. HbMC1 was inserted into the yeast expression vector pYES2 and transformed into the S. cerevisiae strain INVSc1. The results from PCR and semi-quantitative RT-PCR analysis confirmed that HbMC1 was introduced into yeast and expressed in the presence of galactose (see Figure S1 available as Supplementary Data at Tree Physiology Online). After culture in induction medium for 36 h, equal numbers of yeast cells transformed with pYES2 or pYES2-HbMC1 were cultured in liquid induction medium supplemented with different concentrations of H2O2. As shown in Figure 2A, the growth of yeast cells was markedly inhibited by H2O2-induced oxidative stress, and the inhibition effect was much more significant as H2O2 concentrations increased. Compared with pYES2-transformed yeast cells, the growth inhibition in yeast cells overexpressing HbMC1 was more severe. Under different concentrations of H2O2, the pYES2-transformed yeast cells exhibited better growth than the yeast cells transformed with pYES2-HbMC1. To further substantiate our findings, we performed growth assays on solid induction medium plates containing different concentrations of H2O2. The phenotypes observed on plates were fully consistent with the cell density (OD600) measured in liquid medium. Under H2O2 treatment, more clones were found in pYES2-transformed yeast cells than HbMC1-overexpressing yeast cells after dilutions of 5^-3 and 5^-4 (Figure 2B). Taken together, these results suggested that overexpression of HbMC1 reduced oxidative stress tolerance in S. cerevisiae.

We also compared the cell survival after high-concentration H2O2 treatment. As shown in Figure 3A, the pYES2-transformed yeast cells showed better survival than yeast cells transformed with pYES2-HbMC1 after treatment with 5 mM H2O2 for 12 h. The number of surviving clones after 25-fold dilution was counted. In control group, there was no significant difference in numbers of clones between pYES2-HbMC1 and pYES2-transformed yeast cells, while the surviving clone number of the yeast cells overexpressing HbMC1 was significantly less than that of pYES2-transformed yeast cells after H2O2 treatment (Figure 3B). The clone number of pYES2-transformed yeast cells was more than sixfold greater than that of yeast cells overexpressing HbMC1 after H2O2 treatment (Figure 3B). These results indicated that overexpression of HbMC1 increased cell death induced by H2O2 in S. cerevisiae.

Overexpression of HbMC1 in tobacco reduces tolerance to oxidative stress

To further understand the function of HbMC1 in planta, HbMC1 driven by CaMV35S promoter was introduced into tobacco (N. benthamiana) by Agrobacterium-mediated leaf disc transformation. Seven independent T0 HbMC1-transgenic lines were obtained using hygromycin selection and confirmed by PCR with CaMV35S forward plus gene-specific reverse primer pairs (Figure 4A). Among these lines, three lines (L1, L5 and L9) showed a 3:1 segregation ratio for hygromycin resistance in the T1 generation, suggesting that the three lines may contain a single copy of HbMC1. The three lines displayed different expression levels of HbMC1 in both T1 and T3 generations, whereas no HbMC1 expression was observed in WT (Figure 4B). The homozygous T3 seeds of these three transgenic lines were used for further analyses.

To evaluate oxidative stress tolerance of transgenic plants, leaf discs of HbMC1-transgenic and WT plants were floated independently on H2O2 solution under illumination. After 2 days of H2O2 treatment, the leaf discs of the three transgenic lines were more yellowish than that of the WT (Figure 5A). The chlorophyll content of leaf discs was measured, and no significant difference was found between HbMC1-transgenic lines and WT in the control group. However, the three transgenic lines exhibited more reduction in chlorophyll content compared with WT after 2 days of H2O2 treatment (Figure 5B), suggesting that HbMC1 overexpression accelerated H2O2-induced leaf senescence and reduced tolerance to oxidative stress.
We also assayed the growth responses of WT and HbMC1-transgenic seedlings to H$_2$O$_2$-induced oxidative stress. As shown in Figure 6A, oxidative stress (4 mM H$_2$O$_2$) dramatically inhibited the growth of WT and transgenic seedlings. Nevertheless, the growth inhibition of transgenic seedlings was much more than that of the WT seedlings. Under unstressed conditions, the seedling fresh weights were similar between WT and transgenic seedlings, ~250 mg. Under H$_2$O$_2$-induced oxidative stress, the seedling fresh weight of WT was 169.5 mg, which was about 65% of the unstressed plants. The seedling fresh weight of the transgenic lines was <78.0 mg (<32% of the unstressed plants), which was significantly lower than that of WT (Figure 6B). In addition, a dramatic difference in plant height between the transgenic and WT seedlings was observed under oxidative stress (Figure 6A and C). The average plant height of the transgenic lines ranged from 1.9 to 2.3 cm, whereas the average plant height of WT was 3.2 cm, which was significantly higher than that of the transgenic lines (Figure 6C). These results indicate that overexpression of HbMC1 in tobacco inhibits plant growth under H$_2$O$_2$-induced oxidative stress, suggesting that HbMC1 is a negative regulator of plant tolerance to oxidative stress.

**Overexpression of HbMC1 enhances H$_2$O$_2$-induced cell death in tobacco**

It has been reported that metacaspases play important roles in regulating different types of PCD in plants (Fagundes et al. 2015, Kabbage et al. 2017). To examine whether HbMC1 functions in oxidative stress response via regulating PCD, we compared the differences in leaf cell death between WT and transgenic lines after oxidative stress treatment. The detached leaves from WT and three HbMC1-overexpressing lines were treated with 0 (the control group) or 50 mM H$_2$O$_2$ for 24 h, and then stained with trypan blue to visualize dead cells. As shown in Figure 7A, little blue staining was observed in the leaves of the control group, and no differences were observed between the WT and transgenic lines. H$_2$O$_2$ treatment enhanced cell death, and much more leaf cell death was observed in the three HbMC1-overexpressing lines as compared with WT. The percentage of trypan blue-stained leaf area in WT was only 30.8%, while it was while it was separately 52.1%, 68.5% and 72.4% in L1, L9 and L5, which are significantly higher than in the WT after oxidative stress (Figure 7B). In addition, there is a significant positive correlation between HbMC1 expression and the percentage of trypan blue-stained leaf area (Pearson correlation coefficient $r = 0.955$, $P < 0.05$). These results indicate that overexpression of HbMC1 in tobacco enhances cell death under H$_2$O$_2$-induced oxidative stress.

**Identification of proteins that interact with HbMC1**

To screen proteins interacting with HbMC1, a yeast two-hybrid cDNA library derived from various tissues of rubber tree was constructed in this study. The capacity and titer of the established library were 1.3 × 10$^6$ and 9.4 × 10$^7$ cfu ml$^{-1}$, respectively. The average length of inserted cDNA fragments was ~1 kb (see Figure S2 available as Supplementary Data at Tree Physiology Online), and the recombination rate was 93.3%. To examine whether proteins that interact with HbMC1 could be identified, the pGBK7 vector was co-transformed with the cDNA library containing HbMC1 into the yeast strain Y2HGold and tested for auto-activation and toxicity. The pGBK7-HbMC1 transformed yeasts did not display blue on SD/Trp/X-α-Gal plates and could not grow on SD/–Trp/X-α-Gal plates.
Gal/AbA plates (see Figure S3 available as Supplementary Data at Tree Physiology Online), suggesting that the pGBKT7-HbMC1 had no auto-activation activity. Furthermore, the growth of colonies transformed with either pGBKT7-HbMC1 or the pGBKT7 empty vector in SD/–Trp liquid medium showed no significant differences, indicating that the bait plasmid had no toxicity to yeast strain Y2HGold. These results suggested that the pGBKTT7-HbMC1 bait plasmid could be used as bait for further yeast two-hybrid screening.

To identify the proteins interacting with HbMC1, Y2HGold cells containing pGBKTT7-HbMC1 were mated with Y187 strain containing the library plasmid. After mating and growth on two different selection plates (DDO/X/A and QDO/X/A), 47 blue clones were identified as potential positive HbMC1 interactors. After isolating, sequencing and analyzing these positive prey plasmids, we found that they matched 11 TSA sequences of rubber tree (Table 1). To validate the screening results, each of the 11 prey plasmids was co-transformed with pGBKTT7-HbMC1 plasmid into yeast strain Y2HGold and then tested on the QDO/X/A plates. As shown in Figure 8, all co-transformants of bait and prey plasmids displayed blue colonies as the positive control (pGBKTT7-53 and pGADTT7-T co-transformants), whereas the negative controls (pGBKTT7-Lam and pGADTT7-T co-transformants or pGBKTT7-HbMC1 and pGADTT7-T co-transformants) had no colony growth on QDO/X/A plates. The results confirmed the genuine interactions between bait and prey proteins.

Gene prediction and BLASTP results indicated that the 11 interactive proteins are rbcL, TM9SF2-like, COX3, ATP9, DRP, REF/Hevb1, MSSP2-like, SRC2, GATL8, CIPK14-like and STK (Table 1). Functional annotation revealed that these proteins are involved in diverse molecular and biological processes, including signal transduction, rubber biosynthesis, stress response, autophagy, ATP synthesis, carbohydrate metabolism and others (Table 1), indicating that these proteins that interacted with HbMC1 are closely associated with important regulating pathways underlying TPD, including ROS metabolism, PCD and rubber biosynthesis.

**Discussion**

Rubber tree is the main commercial source of natural rubber. As an important industrial material, natural rubber is an elastomer with excellent physical and chemical properties that cannot be fully matched by synthetic rubber. The demand for natural rubber has continuously increased due to the fast development of the world economy. Tapping panel dryness, a complex physiological disorder caused by overexploitation, is a serious threat to natural rubber yields. Some researchers suggested that PCD might occur in TPD rubber trees (Chen et al. 2003, Venkatachalam et al. 2007, de Fay 2011, Peng et al. 2011, Gébelin et al. 2013, Putranto et al. 2015, Li et al. 2010, 2016). Metacaspases are critical regulators of various PCD in plants. Previously, we performed a genome-wide identification and expression analysis of the metacaspase gene family in rubber tree and found that HbMC1 expression was significantly increased in the latex of TPD trees as compared with that of the healthy ones (H.Liu et al. 2002).
In the present study, we further found that *HbMC1* expression in the bark tissues was induced by wounding, H$_2$O$_2$ and ethephon, which are key factors leading to TPD (Putranto et al. 2015, Zhang et al. 2017). In addition, a positive correlation was observed between the expression of *HbMC1* in the bark tissues and TPD severity. These results indicate that *HbMC1* is closely associated with rubber tree TPD occurrence.

Oxidative stress-activated PCD provides a dramatic example of a metacaspase-mediated process that is evolutionarily conserved from protozoa to plants (Tsiatsiani et al. 2011). Several metacaspase genes have been reported to be involved in oxidative stress-induced cell death. For example, *YCA1*, the only metacaspase in *S. cerevisiae*, is required for H$_2$O$_2$-induced PCD (Madeo et al. 2002). Interestingly, the *Arabidopsis AtMC1* (*AtMC1b*), *AtMC5* (*AtMC2b*) and *AtMC8* (*AtMC2e*) are able to complement the cell death function of *YCA1* (Watanabe and Lam 2005, He et al. 2008). The wheat *TaMCA1* also has a function similar to *YCA1*, but it can only partially complement the function of *YCA1* (Hao et al. 2016). Moreover, the pepper *Camc9* was demonstrated to be involved in the production of ROS during pathogen-induced cell death (Kim et al. 2013). It is reported that ROS are key inducers of PCD in plants (De Pinto et al. 2012). The onset of rubber tree TPD is accompanied by excessive production of ROS (Zhang et al. 2017), and the typical characteristics of PCD were detected in TPD rubber trees (Peng et al. 2011). Therefore, we speculate that *HbMC1* may function in TPD occurrence via regulating ROS-induced PCD. In accordance with our speculation, both yeast and tobacco overexpressing *HbMC1* reduced tolerance to H$_2$O$_2$-induced oxidative stress. Overexpression of *HbMC1* in tobacco resulted in reductions in seedling fresh weight, plant height and leaf chlorophyll content under oxidative stress (Figures 5 and 6). The reductions appeared to increase with increasing *HbMC1* expression. However, these negative correlations did not reach statistical significance. Compared with yeast transformed with the empty vector pYES2, the cell death rate of yeast harboring pYES2-*HbMC1* increased obviously after high-concentration H$_2$O$_2$ treatment. Moreover, transgenic tobacco overexpressing *HbMC1* also showed an increased level of cell death induced by H$_2$O$_2$. Additionally, a significant positive correlation was detected between *HbMC1* expression and the cell death rate. Taken together, these results demonstrate a positive role of *HbMC1* in mediating cell death induced by oxidative stress and establish an interrelation between *HbMC1*-mediated cell death and TPD occurrence.

*HbMC1* is a member of type I metacaspases, which contain a lesion simulating disease 1 (LSD1) zinc finger domain (H.Liu et al. 2016). In the present study, we further found that *HbMC1* expression in the bark tissues was induced by wounding, H$_2$O$_2$ and ethephon, which are key factors leading to TPD (Putranto et al. 2015, Zhang et al. 2017). In addition, a positive correlation was observed between the expression of *HbMC1* in the bark tissues and TPD severity. These results indicate that *HbMC1* is closely associated with rubber tree TPD occurrence.

Oxidative stress-activated PCD provides a dramatic example of a metacaspase-mediated process that is evolutionarily conserved from protozoa to plants (Tsiatsiani et al. 2011). Several metacaspase genes have been reported to be involved in oxidative stress-induced cell death. For example, *YCA1*, the only metacaspase in *S. cerevisiae*, is required for H$_2$O$_2$-induced PCD (Madeo et al. 2002). Interestingly, the *Arabidopsis AtMC1* (*AtMC1b*), *AtMC5* (*AtMC2b*) and *AtMC8* (*AtMC2e*) are able to complement the cell death function of *YCA1* (Watanabe and Lam 2005, He et al. 2008). The wheat *TaMCA1* also has a function similar to *YCA1*, but it can only partially complement the function of *YCA1* (Hao et al. 2016). Moreover, the pepper *Camc9* was demonstrated to be involved in the production of ROS during pathogen-induced cell death (Kim et al. 2013). It is reported that ROS are key inducers of PCD in plants (De Pinto et al. 2012). The onset of rubber tree TPD is accompanied by excessive production of ROS (Zhang et al. 2017), and the typical characteristics of PCD were detected in TPD rubber trees (Peng et al. 2011). Therefore, we speculate that *HbMC1* may function in TPD occurrence via regulating ROS-induced PCD. In accordance with our speculation, both yeast and tobacco overexpressing *HbMC1* reduced tolerance to H$_2$O$_2$-induced oxidative stress. Overexpression of *HbMC1* in tobacco resulted in reductions in seedling fresh weight, plant height and leaf chlorophyll content under oxidative stress (Figures 5 and 6). The reductions appeared to increase with increasing *HbMC1* expression. However, these negative correlations did not reach statistical significance. Compared with yeast transformed with the empty vector pYES2, the cell death rate of yeast harboring pYES2-*HbMC1* increased obviously after high-concentration H$_2$O$_2$ treatment. Moreover, transgenic tobacco overexpressing *HbMC1* also showed an increased level of cell death induced by H$_2$O$_2$. Additionally, a significant positive correlation was detected between *HbMC1* expression and the cell death rate. Taken together, these results demonstrate a positive role of *HbMC1* in mediating cell death induced by oxidative stress and establish an interrelation between *HbMC1*-mediated cell death and TPD occurrence.

*HbMC1* is a member of type I metacaspases, which contain a lesion simulating disease 1 (LSD1) zinc finger domain (H.Liu et al. 2016). The LSD1 zinc finger domain has been reported to participate in protein interactions (Kaminaka et al. 2006). To identify proteins interacting with *HbMC1*, we screened a rubber...
tree cDNA library by yeast two-hybrid system with HbMC1 as the bait. A total of 11 proteins were identified to be interacted with HbMC1. The screened proteins provide useful information for further research on the molecular mechanisms of HbMC1-mediated TPD in rubber tree. However, the interactions detected by the yeast two-hybrid system are preliminary results, and further research is needed to confirm these interactions via independent methods, such as bimolecular fluorescence complementation, co-immunoprecipitation, pull-down assays, etc. Our future work will focus on verifying these interactions to better understanding the molecular mechanisms of HbMC1-mediated TPD in rubber tree.

Among the screened proteins, rubber elongation factor rubber elongation factor (REF), also known as Hevb1, has been proposed to be involved in rubber biosynthesis or quality (Berthelot et al. 2014a, 2014b, Wadeesirisak et al. 2017). Rubber elongation factor interacts with HRBP (HRT1-REF bridging protein) to form the ternary protein complexes (HRT1-HRBP-REF) on rubber particles, which is required for rubber biosynthesis (Yamashita et al. 2016). Under normal circumstances, REF binds and inserts into the rubber particle membrane, where rubber biosynthesis occurs (Berthelot et al. 2014b). However, REF was reported to accumulate in the cytosol of TPD rubber tree (Sookmark et al. 2002). The interaction between HbMC1 and REF probably makes REF release from the rubber particles into the cytosol, which may decrease the efficiency of rubber synthesis in rubber tree. Sucrose is the primary substrate for rubber synthesis, and an efficient sucrose supply is required for rubber biosynthesis (Tang et al. 2010). Interestingly, two proteins related to carbohydrate metabolism, MSSP2-like and GATL8, were identified to interact with HbMC1, which may impair the sucrose supply in rubber tree. Moreover, two stress-related proteins, SRC2 and DRP, were identified to interact with HbMC1. It has been reported that Arabidopsis AtSRC2 functions in cold stress responses by enhancing AtRbohF-mediated ROS production (Kawarazaki et al. 2013). Additionally, the pepper SRC2-1 was demonstrated to play an important role in cell death induced by hypersensitive reaction and defense response by interacting with PcINF1 and SGT1 (Z.Q.Liu et al. 2015, 2016). DRP shows high homology to Arabidopsis LEA27 (At2g46140). Dang et al. (2014) reported that overexpression of LEA27 in yeast enhanced cell survival during desiccation. TM9SF2-like belongs to the TM9SF family, which is highly conserved through

Figure 7. Comparison of H2O2-induced leaf cell death between HbMC1-overexpressing lines and WT. (A) Histochemical detection of leaf cell death induced by oxidative stress. Leaves excised from 5-week-old seedlings of WT and transgenic lines were treated with 50 mM H2O2 or distilled water (control) for 1 day and subsequently stained with trypan blue. (B) The percentage of trypan blue-stained leaf area. Values are the means ± SD (n = 9). Asterisks indicate significant differences compared with the WT. **P < 0.01 (Student’s t-test).

Table 1. List of HbMC1-interacting proteins identified by the yeast two-hybrid assay.

<table>
<thead>
<tr>
<th>GenBank accession No. (rubber tree)</th>
<th>Gene name</th>
<th>Function annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JR366851.1 ARK37416.1 rbcL (Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit)</td>
<td>Photosynthesis; reductive pentose-phosphate cycle</td>
<td></td>
</tr>
<tr>
<td>JT922991.1 XP_021638748.1 TM9SF2-like (Transmembrane 9 superfamily member 2-like)</td>
<td>Immune response, autophagy</td>
<td></td>
</tr>
<tr>
<td>JT350026.1 COX3 (Cytochrome c oxidase subunit 3)</td>
<td>Aerobic electron transport chain</td>
<td></td>
</tr>
<tr>
<td>JT927960.1 ATP9 (ATP synthase FO subunit 9)</td>
<td>ATP synthesis coupled proton transport</td>
<td></td>
</tr>
<tr>
<td>GDFU0104566.1 XP_021669261.1 DRP (Desiccation-related protein At2g46140-like isoform X1)</td>
<td>Water stress and hypersensitive response</td>
<td></td>
</tr>
<tr>
<td>JT363984.1 XP_021653602.1 REF/Hevb1 (Rubber elongation factor protein)</td>
<td>Rubber biosynthesis, rubber elongation</td>
<td></td>
</tr>
<tr>
<td>JT344765.1 XP_021686568.1 MSSP2-like (Monosaccharide-sensing protein 2-like)</td>
<td>Sugar (and other) transporter</td>
<td></td>
</tr>
<tr>
<td>JT943681.1 XP_021650566.1 SRC2 (Protein SRC2 homolog)</td>
<td>Response to cold/defense response</td>
<td></td>
</tr>
<tr>
<td>GDFU01043253.1 XP_021690397.1 GATL8 (Galacturonosyltransferase 8-like)</td>
<td>Pectin biosynthesis/glycan metabolism</td>
<td></td>
</tr>
<tr>
<td>GDHB01004041.1 XP_021646124.1 CIPK14-like (CBL-interacting serine/threonine-protein kinase 14-like)</td>
<td>Signal transduction</td>
<td></td>
</tr>
<tr>
<td>JT932487.1 XP_021635630.1 STK (Probable serine/threonine-protein kinase At1g54610)</td>
<td>Signal transduction</td>
<td></td>
</tr>
</tbody>
</table>
evolution. The human TM9SF proteins, TM9SF3 and TM9SF4, are associated with tumor development (Oo et al. 2014, Lozupone et al. 2015). Moreover, TM9SF1 and TM9SF4 were proved to play important roles in regulating autophagy (He et al. 2009, Sun et al. 2018). Autophagy is a highly conserved process essential for cell survival under stress conditions (Sun et al. 2018). Minina et al. (2013) demonstrated that the metacaspase mcII-Pa is the upstream regulator of autophagy in the vacuolar cell death pathway. HbMC1 may modulate autophagy during the execution of cell death through interacting with TM9SF2-like in TPD rubber trees.

Li et al. (2010) reported that ROS production and scavenging, ubiquitin proteasome pathway (UPP), PCD and rubber biosynthesis pathways might play important roles in rubber tree TPD. Consistent with the aforementioned results, Gébelin et al. (2013) found that the target genes of TPD-related microRNAs are closely associated with rubber biosynthesis, ROS-scavenging systems, and PCD. Based on our previous speculation about rubber tree TPD (Li et al. 2016), we integrate our present results as following: overexploitation (excessive tapping as well as overstimulation with ethephon) induces excessive ROS production, which alters the balance between ROS production and scavenging and results in ROS accumulation. Meanwhile, HbMC1 is up-regulated by TPD-inducing factors including ROS, tapping and ethephon stimulation, etc. As toxic molecules, the excessive ROS further induces PCD and UPP in rubber tree. HbMC1 up-regulation results in stress-induced PCD and further affects ROS metabolism, stress tolerance, sucrose supply and rubber biosynthesis by interacting with multiple proteins (REF, MSSP2-like, SRC2, DRP, TM9SF2-like, etc.), which finally reduces latex biosynthesis in laticifers. To protect itself against further damage, rubber tree initiates PCD and latex yield decreases (or even final stoppage of latex flow), resulting in TPD occurrence.

Conclusions

In summary, the expression of HbMC1 in bark tissues was not only induced by TPD-inducing factors but also correlated with TPD severity. Further studies demonstrated that overexpression of HbMC1 in yeast cells and tobacco plants decreased tolerance to H$_2$O$_2$-induced oxidative stress, and HbMC1 was a positive regulator of cell death induced by H$_2$O$_2$. In addition, 11 proteins (rbcL, TM9SF2-like, COX3, ATP9, DRP, HbREF/Hevb1, MSSP2-like, SRC2, GATL8, CIPK14like and STK) associated with important regulation pathways of rubber tree TPD including PCD, ROS metabolism and rubber biosynthesis were identified to interact with HbMC1. Taken together, HbMC1 is involved in TPD occurrence and development probably by regulating ROS-induced PCD process in rubber tree.

Supplementary Data

Supplementary Data for this article are available at Tree Physiology Online.

Conflict of interest

None declared.

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