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Research article

Identification and functional characterization of *HbOsmotin* from *Hevea brasiliensis*

Zheng Tong [a], Yong Sun [a], Dan Wang [a], Limin Wang [a], Ling Li [c], Xueru Meng [a], Weiqiang Feng [a, b], Eve Syrkin Wurtele [d], Xuchu Wang [a, b, c, *]

[a] Institute of Tropical Biosciences and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan 571101, China
[b] College of Agriculture, Hainan University, Haikou, Hainan 570228, China
[c] Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA
[d] Center for Metabolic Biology, Iowa State University, Ames, IA 50011, USA

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ABSTRACT

Latex in the laticiferous cell network of *Hevea brasiliensis* tree is composed of cytoplasm that synthesizes natural rubber. Ethylene stimulation of the tree bark enhances latex production partly by prolonging the duration of latex flow during the tapping process. Here, we identified an osmotin-like cDNA sequence (*HbOsmotin*) from *H. brasiliensis* that belongs to the pathogenesis-related 5 (PR-5) gene family. The HbOsmotin protein is present in the lutoids of latex in *H. brasiliensis*, whereas in onion epidermal cells, this protein is predominantly distributed around the cell wall, suggesting that it may be secreted from the cytoplasm. We investigated the effects of exogenous ethylene on *HbOsmotin* transcription and protein accumulation in rubber latex, and further determined the protein function after osmotic stress in *Arabidopsis*. In regularly tapped trees, *HbOsmotin* expression was drastically inhibited in rubber latex after tapping, although the expression was subsequently recovered by ethylene stimulation. However, in virgin plants that had never been tapped, exogenous ethylene application slightly decreased *HbOsmotin* expression. *HbOsmotin* overexpression in *Arabidopsis* showed that *HbOsmotin* reduced the osmotic stress tolerance of the plant, which likely occurred by raising the water potential. These data indicated that *HbOsmotin* may contribute to osmotic regulation in laticiferous cells.

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1. Introduction

Hevea brasiliensis is a unique commercial source of natural rubber (Hébant, 1981). During rubber tree maturation, articulated networks of laticifer vessels form in the inner bark of the rubber tree, and these vessels accumulate rubber latex (Hagel et al., 2008; Hébant, 1981). After the tree is tapped, rubber latex flows out from the bark until the latex coagulates, which finally plugs the latex vessels (d'Auzac, 1989; Yeang, 2005). Thus, after tapping, fresh latex quickly flows out from the vessels and then gradually slows until it finally stops. The major intrinsic factors that limit the latex yield are the rate and duration of the latex flow (d'Auzac, 1989). Latex flow is controlled by the turgor pressure level in the laticiferous tissues, which drives the flow, and inhibits by the coagulated rubber in latex, which gradually forms after tapping and subsequently plugs the latex vessel to stop the latex flow (Buttery and Boatman, 1964; d'Auzac, 1989; Tungngoen et al., 2009; Yeang, 2005). The regeneration of rubber latex after each tapping is critical for the latex yield in subsequent tapping (Wang et al., 2015).

Lutoids, vacuole-like organelles in laticifers, are involved in the formation of coagulated rubber (Hao et al., 2004; Southorn, 1968). Coagulated rubber is formed by rubber particle aggregation (RPA) (Milford et al., 1969; Wititsuwannakul et al., 2008) or by the formation of a protein network with meshes containing the RPA (Hao et al., 2004). Lutoids contain soluble proteins, notably hevein, chitinase, and glucanase, the main components of the protein networks (Hao et al., 2004; Wang et al., 2013). When lutoids rupture, they release lots of cations, acid hydrolases, oxidoreductases, hevein, and lectin, which likely cause the RPA (Subroto et al., 1996; Wititsuwannakul et al., 2008).

Treating rubber tree bark with ethephon, an ethylene releaser, markedly increased the production of fresh latex because it

* Corresponding author. Institute of Tropical Biosciences and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan 571101, China.
E-mail address: xchwanghainan@163.com (X.C. Wang).

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prolongs the latex outflow (d'Auzac and Ribaillier, 1969). Ethylene stimulation is speculated to decrease RPA, increase laticifer turgor pressure, stimulate the production of small rubber particles, and/or increase the general regenerative metabolism of rubber biosynthesis (Zhu and Zhang, 2009). We propose that turgor pressure, coagulated rubber formed by RPA, and rubber regeneration all participate in controlling latex flow. Turgor pressure and coagulated rubber have immediate effects on promoting and inhibiting latex flow respectively, and rubber regeneration has a long-term effect on increasing latex flow. Together, these three factors contribute to short- and long-term latex formation and rubber production.

Our previous proteomics study which, focused on the response of lutoid proteins to ethylene stimulation showed that ethylene induces an osmotin-like protein (OLP) (Supplementary Fig. S1, S2, and Table S1). This OLP belongs to the pathogenesis-related 5 (PR-5) family, which is involved in plant defense against pathogens (Vigers et al., 1991). PR-5-like proteins are also present in fungi and animals, and despite their dramatic diversification in organisms, PR-5 proteins appear to share well-defined domains (Liu et al., 2010; Shatters et al., 2006). Thaumatin from *Thaumatococcus daniellii* (de Vos et al., 1985), osmotin from tobacco (Singh et al., 1987), NP24 from tomato (King et al., 1988), and zeamatin from maize (Vigers et al., 1991) are well-studied proteins in the PR-5 family that were identified before the family was established. Subsequently identified members of the PR-5 family include thaumatin-like protein (TLP), OLP, or zeamatin-like protein (ZLP); however, the differences among these subclasses are not clearly defined. Osmotin is an abundant cationic multifunctional protein adapted to environments of low osmotic potential (Abdin et al., 2011). OLPs are found in many fruits, seeds and vegetative organs, where they appear to have antifungal activity (Abad et al., 1996; Yun et al., 1998). OLPs confer tolerance to biotic stress through the permeabilization of fungal membranes and dissipation of the membrane potential in fungal cells (Abad et al., 1996; Yun et al., 1998). OLPs have also been reported to confer tolerance to abiotic stress in plants (Goel et al., 2010; Husaini and Abdin, 2008), although the exact mechanism is unclear.

Here, we cloned an *OLP* cDNA sequence, *HbOsmotin* from the latex of *H. brasiliensis*, analyzed the effects of tapping and ethylene treatment on *HbOsmotin* expression, verified *HbOsmotin* function during osmotic stress, and determined the possible roles of *HbOsmotin* in natural rubber latex production.

2. Materials and methods

2.1. Plant material and treatments

The latex samples were collected from mature rubber plants (~8-year-old, *H. brasiliensis* Mull. Arg., clone RY 7-33-97) grown in an experimental farm at the Chinese Academy of Tropical Agricultural Sciences in Danzhou City, Hainan Province of China. A flow chat describing the treatments and latex sample collections is shown in Supplementary Fig. S3. Initially (day 0, D0), the trees were divided into two groups. Sixty trees were tapped with a shallow cut that did not hurt the laticifer layer, such that latex did not flow out (Wounding-only group, WG); the other 30 trees were tapped and latex was collected (Tapping group, TG), latex from 15 trees was used as D0 for WG, and latex from the other 15 trees was used as D0 for TG. On the first day after day 0 (day 1, D1), half the trees of each group were brushed with ethephon (3%, v/v) at the site of the tapping cut, while the other half of the trees were brushed with ddH_2O as control. On the third day (D3), half of the trees from WG (15 ethephon-treated and 15 controls) and all the 30 trees (15 ethephon-treated and 15 controls) from TG were tapped and latex was collected. On the fifth day (D5), the remaining untapped trees from WG and all the 30 trees from TG were tapped and latex was collected. The latex from five trees was collected together as one replication for each treatment, and all the treatments have three replications. All latex was collected in ice-chilled glass beakers before analysis.

2.2. Cloning of HbOsmotin

RNA was extracted from the latex of mature rubber plants (*H. brasiliensis* Mull. Arg., clone RY 7-33-97) by using a high sodium dodecyl sulfate (SDS) buffer (100 mM Tris-HCl, 300 mM LiCl, 10 mM EDTA-Na_2, and 10% SDS) as extraction buffer (1: 1 mixed with latex during use), phenol-chloroform and chloroform as extraction solvents, and 8 M LiCl as RNA precipitator. First strand cDNA was synthesized using 1 μg of total RNA and the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA). A pair of degenerate primers, RS (5′-GATGCTTAyA GyTAyCCyCA rGATGA-3′) and SS (5′-TArCTrTAAG CATCnGGrCA CCT-3′), was designed according to the homologous sequence regions of the *OLPs* from *Vitis riparia* (AF178653.1), *Sambucus nigra* (AF378573.1), *Ficus awkeotsang* (DQ277011.1), *Ricinus communis* (DQ285656.1), *Camellia sinensis* (DQ444296.1), and *Populus deltoides* (GU129189.1) to amplify the corresponding region of *HbOsmotin*. The PCR products were purified with the AxyPrep™ DNA gel extraction kit (Axygen, USA), ligated into pMD18T (Takara, Japan), transformed into *Escherichia coli* strain JM109, and then sequenced (Life Biological Technology, China). The 3'- and 5'-terminal sequences of *HbOsmotin* were cloned referring to the sequenced region by SMART™ RACE cDNA Amplification Kit (Clontech, USA). The complete cDNA sequence of *HbOsmotin* was then submitted to GenBank under the accession number KC509437.1 (http://www.ncbi.nlm.nih.gov/nuccore/KC509437.1). Multiple sequence alignments of OLPs and TLPs were assembled using the EBI ClustalW server (http://www.ebi.ac.uk/clustalw/) and were visualized using BioEdit software (Ibis Biosciences, Carlsbad, USA). Phylogenetic and molecular evolution analyses were conducted using MEGA software version 5.0 (Tamura et al., 2011).

2.3. Southern blot analysis of HbOsmotin

Southern blots were conducted using the DIG Application Manual for Filter Hybridization (Roche, Switzerland). The *H. brasiliensis* genomic DNA was extracted from the leaves of rubber trees (*H. brasiliensis* Mull. Arg., clone RY 7-33-97) and then digested with *Bam*HI, *Eco*RI, *Hind*III, and *Xba*I. Ten microgram of each digested product was electrophoresed on a 0.8% agarose gel at 1 V/cm overnight and then transferred onto a positively charged nylon membrane (Roche, Switzerland) through capillary transfer by 20×SSC for approximately 12 h. UV crosslinking of the DNA to the filter was performed at a strength of 90,000 μJ m^{-2} (SCIENTZ, China). The cross-linked filter was hybridized with a 747 bp digoxigenin (DIG) PCR labeled probe for the *HbOsmotin* ORF region. The primers for the DIG labeled PCR were "TCCATCAACC ATGAGTAACT TCAAC" and "TCAACATCTT TTTCTCTGAC CATTTC". Hybridization was performed at 42 °C, and the probe was washed at 60 °C. DNA blots were visualized using a LAS4000mini (GE Healthcare, Uppsala, Sweden).

2.4. Real-time RT-PCR (qRT-PCR) analysis of HbOsmotin

RNA was extracted from the latex samples (Section 2.1) using the high SDS extraction buffer (Section 2.2; Supplementary Fig. S4A). First strand cDNA synthesis was performed using 1 μg total RNA and the RevertAid First Strand cDNA Synthesis Kit (Thermo Inc., USA) in a reaction volume of 25 μL. The cDNA (1 μL)

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