Jasmonate signalling in regulation of rubber biosynthesis in laticifer cells of rubber tree (*Hevea brasiliensis* Muell. Arg.)

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The highlights of this manuscript are as follows,

Enhanced natural rubber biosynthesis is associated with the activation of a specific jasmonate signalling module in the laticifer of rubber trees.

Abstract

Rubber trees are the major source of worldwide natural rubber. Rubber-containing latex is obtained from the laticifer cells of rubber tree (*Hevea brasiliensis* Muell. Arg.) via regular tapping. However, little is known about the positive feedback regulation of rubber biosynthesis, a typical isoprenoid metabolic process in laticifer cells, by tapping-caused loss of latex. Here, we demonstrated the crucial role of jasmonate signalling in this feedback regulation. The endogenous jasmonate levels, the expression levels of rubber biosynthesis-related genes and the efficiency of *in vitro* rubber biosynthesis were significantly higher in laticifer cells of regularly tapped trees than those of virgin trees. Application of methyl jasmonate had similar effects to latex exploitation in up-regulating the rubber biosynthesis-related genes and enhancing rubber biosynthesis. The specific jasmonate signalling module (COI1-JAZ3-MYC2) in laticifer cells of rubber tree was further identified. Its activation was associated with the enhanced natural rubber biosynthesis via up-regulating the expression of a farnesyl pyrophosphate synthase gene and a small rubber particle protein gene. The increase in the corresponding proteins, especially the farnesyl pyrophosphate synthase likely contributes to the increased efficiency of rubber biosynthesis. To our knowledge, this is the first study to reveal a jasmonate signalling pathway in regulating rubber biosynthesis in laticifer cells. The identification of the specific jasmonate signalling module in laticifer cells of rubber tree may provide a clue for genetic improvement of rubber yield potential.

Key words: *Hevea brasiliensis* Muell. Arg.; jasmonate signalling; laticifer cell; isoprenoid metabolism; rubber biosynthesis; secondary metabolism

Introduction

Major progresses in elucidating the jasmonate signalling pathway in model plants have been achieved. These findings include the characterization of endogenous bioactive jasmonate as (+)-7-iso-jasmonoyl-L-isoleucine (Ile-JA) (Fonseca *et al.,* 2009a) and its receptor as COI1 (Katsir *et al.,* 2008; Mach, 2009; Yan *et al.,* 2009), the COI1-JAZ co-receptor (Sheard *et al.,* 2010), the COI1 as a component of an SCF (Skp/Cullin/F-box) E3 ubiquitin ligase (Xu *et al.,* 2002; Devoto *et al.,* 2002) and its targets as jasmonate ZIM-domain (JAZ) proteins (Chini *et al.,* 2007; Thines *et al.,* 2007; Yan *et al.,* 2007), and MYC2 as a crucial transcription factor for activating many jasmonate-responsive genes (Lorenzo *et al.,* 2004; Dombrecht *et al.,* 2007). The results demonstrate the importance of the COI1-JAZ-MYC2 core module in jasmonate signalling (Fonseca *et al.,* 2009b). Recently, no-TIFYJAZ proteins (Thireault *et al.,* 2015) and other transcription factors were reported to positively or negatively regulate jasmonate responses (Fonseca *et al.,* 2014; Sasaki-Sekimoto *et al.,* 2014; Zhou and Memelink, 2016). The core module COI1-JAZ-MYC2 is conserved in plants (Thines *et al.,* 2007; Shoji *et al.,* 2008) and mediates activation of the biosynthesis of several secondary metabolites, such as nicotine in tobacco (Shoji and Hashimoto, 2011), tanshinone in *Salvia miltiorrhiza* (Shi *et al.,* 2016a) and anthocyanin in *Arabidopsis* (Niu *et al.,* 2011; Qi *et al.,* 2011; Xie *et al.,* 2016) and apple (Chen *et al.,* 2017). Nevertheless, little is known about this core signalling module in tissues specific for secondary metabolite biosynthesis.

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is the major source of natural rubber worldwide. The rubber biosynthesis is a typical isoprenoid metabolic process and occurs in the laticifer, a specific tissue for rubber biosynthesis and storage in rubber tree (Hao and

Wu, 2000). Rubber biosynthesis consists of the general mevalonate (MVA) pathway converting pyruvic acid into isopentenyl pyrophosphate (IPP) as well as the 2C-methyl-d-erythritol 4-phosphate (MEP) pathway converting pyruvic acid and glycerate 3-phosphate into IPP. The specific integration of isopentenyl pyrophosphate (IPP) units into prenyl chain is initiated with the aid of initiators, such as FPP (Adiwilaga *et al.,* 1996; Cornish *et al.,* 1999). Among these processes, the reactions that are catalyzed by hydroxymethylglutaryl-CoA reductase (HMGR), 1-deoxy-d-xylulose 5-phosphate synthase (DXS), 1-Deoxy-d-xylulose 5-phosphate reductoisomerase (DXR), farnesyl pyrophosphate synthase (FPS) and *Hevea* rubber transferase (HRT) together with small rubber particle protein (SRPP) or rubber elongation factor (REF) are critical, considering that these steps are involved in the synthesis of MVA and MEP, and FPP and the integration of IPP units into the prenyl chain, which determines the efficiency of rubber biosynthesis (Oh *et al.,* 1999).

The laticifer that is directly related to rubber production is the secondary laticifer in the trunk bark. The secondary laticifer is a single-cell type tissue and consists of laticifer cells that are differentiated from the fusiform initials of vascular cambia in rubber tree (Hao and Wu, 2000). For rubber production, latex is exploited by tapping (i.e., cutting the trunk bark every 2 d in general). Natural latex is the cytoplasm of laticifer cells and contains numerous rubber particles, where the rubber is synthesized and stored. Although it is well known that latex exploitation enhances latex regeneration, including rubber biosynthesis in the laticifer cells (Paardekooper, 1989), little is known about the positive feedback regulation of rubber biosynthesis. Ethylene appears to be a key regulator of the feedback regulation considering that ethrel (an ethylene releaser) application increases rubber yield per tapping (Kush, 1990). The available data showed that the ethylene-induced increase in rubber yield per tapping is primarily due to the prolonged duration of latex flow (Coupé and Chrestin, 1989) as well as up-regulation of the genes related to sucrose allocation (Tang *et al.,* 2010), water transportation (Tungngoen *et al.,* 2009), glycolysis and C3 carbon fixation (Liu *et al.,* 2016). These effects are directly related to the energy regeneration, turgor pressure maintenance and supply of carbon building blocks, which

are required for the biosynthesis of rubber and other organic metabolites in laticifer cells. Alternatively, jasmonate signalling has been suggested to control the positive feedback regulation of rubber biosynthesis *per se* based on its pivotal roles in activating secondary metabolite biosynthesis (Peng *et al.,* 2009; Tian *et al.,* 2010; Zhao *et al.,* 2011). Although orthologs of the COI1, MYC and JAZ genes in *Arabidopsis* have been cloned and characterized from laticifer cells in rubber tree (Peng *et al.,* 2009; Tian *et al.,* 2010; Zhao *et al.,* 2011; Hong *et al.,* 2014; Pirrello *et al.,* 2014), there is still a lack of direct evidence showing the presence of the core module of jasmonate signalling and its regulation on rubber biosynthesis.

In the present study, we examined whether the COI1-JAZ-MYC2 signalling pathway is present in laticifer and used to regulate rubber biosynthesis. We observed that latex exploitation raised the level of endogenous jasmonates, up-regulated most rubber biosynthesis genes tested, and enhanced rubber biosynthesis in laticifer cells. Application of methyl jasmonate also had the similar effects to latex exploitation. We further identified an HbCOI1-HbJAZ3-HbMYC2 module in laticifer cells and demonstrated it mediated latex exploitation-induced up-regulation of the rubber biosynthesis-related genes *HbFPS1* and *HbSRPP1*. The increase in the level of HbSRPP1 and especially HbFPS1 should contribute to the increased efficiency of rubber biosynthesis.

Materials and methods

Plant materials and treatments

Eight-year-old virgin trees and tapped trees as well as the epicormic shoots described by Hao and Wu (2000) of the rubber tree clone CATAS7-33-97 were grown at an Experimental Farm of the Chinese Academy of Tropical Agricultural Sciences in Danzhou City, Hainan Province, P.R. China. The tapped trees were regularly tapped for one-year from the seven-year-old virgin trees in a downward half spiral every 3 d without ethylene stimulation. After the tapped trees have been currently tapped for 60 times, a total of

thirty virgin trees and thirty tapped trees with same circumference were selected for determining the content of endogenous jasmonates, the efficiency of rubber biosynthesis and rubber biosynthesis-related gene expression in the latex collected 2 d after the last tapping. The latex samples that were collected from ten of the thirty virgin trees and thirty tapped trees were respectively pooled as one sample for one of the three biological experiments. Thereafter, all the thirty tapped trees were rested from tapping for 15 d and then nine of which were respectively treated with 0.07% methyl jasmonate, 0.5% ethrel, and as control, without any treatments. One day after the treatments, latex samples were collected from the trees that were rested from tapping. The samples from three of the nine trees for each treatment were pooled as one sample in three biological experiments to determine the efficiency of *in vitro* rubber biosynthesis. For investigating the effect of JA on the rubber biosynthesis-related gene expression, 120 epicormic shoots were treated with 0.07% methyl jasmonate as described by Hao and Wu (2000) and the other 120 epicormic shoots without any treatment were used as control. Latex samples from the treated sites were collected by cutting the stem bark of the first and second extension units (Hao and Wu, 2000; Zhang *et al.,* 2015) at 4 h, 12 h, 1 d, and 5 d after the treatment. Latex samples at each time interval were collected from 30 epicormic shoots and pooled into one sample from each of 10 epicormic shoots. The latex samples from the control shoots were accordingly collected and pooled. Tobacco (*Nicotiana benthamiana*) was sowed and grown at 25^{o}C with a 16 h /8 h light/dark photoperiod in an illumination incubator for approximately six weeks and then used for BiFC and transient expression analysis.

Sequence analysis and phylogenetic tree construction

The deduced amino acid sequence of the HbMYC2 or HbJAZ3 protein was aligned with other homologous proteins using the Clustal X 2.05 program and then edited by Genedoc software. Phylogenetic tree construction was performed by Clustal X 2.05 and MEGA4.0 software.

Transcriptional activation analysis in yeast cells

The full-length coding region and different truncated derivatives of *HbMYC2* were separately cloned into the bait vector pGBKT7 (Supplementary Figures S1-S8+Tables S1-S2.pdf) and transformed into the yeast strain Y2HGold for transcriptional activation according to the standard protocols of the manufacturer (Clontech, USA). The transformed strains were then streaked onto SD/-Trp or SD/-Ade-Trp-His plates, and transcriptional activation was determined by the colony growth and blue color after the α-galactosidase reaction on the defective medium (SD/-Ade-Trp-His/X-α-gal).

Subcellular localization and BiFC assays

For subcellular localization, the coding regions of *HbMYC2* and *HbJAZ3* were separately cloned into the pCambia1302 vector and fused with the *EGFP* gene. This recombinant plasmid and control GFP plasmids were introduced into *A. tumefaciens* GV3101 and infiltrated into onion epidermal cells for fluorescence detection according to recently described methods (Li *et al.,* 2016). For BiFC assays in tobacco (*Nicotiana benthamiana*) leaves and onion epidemic cells, the coding sequence of *HbJAZ3* was cloned into pSPYNE(R)173 described in Waadt *et al.* (2008). *HbMYC2* as well as *HblMYC1* and *HblMYC2* were cloned into pSPYCE(MR). The human lamin C gene from the pGBKT7-Lam vector was cloned and inserted into the pSPYNE(R)173 for the negative control use, while the murine p53 gene from the pGBKT7-53 vector was cloned and inserted into the pSPYNE(R)173 for the positive control use. The SV40 large T-antigen gene was cloned from the pGADT7-T vector and inserted into the pSPYCE(MR) for both the negative and positive controls use. The corresponding combined plasmids were introduced into the *Agrobacterium tumefaciens* (strain GV3101) and confirmed by RT-PCR with the specific primers. The BiFC experiments were designed according to Kudla and Bock (2016) and also performed in onion epidemic cells refer to the methods described previously by Li *et al.* (2016). The

pSPYNE(R)173-HbJAZ3 and pSPYCE(MR)-HbMYC2 constructs were separately introduced into *A. tumefaciens* GV3101 and coinfiltrated in combination with pSPYNE(R)173-HbJAZ3 and pSPYCE(MR)-HbMYC2 at an optical density at 600 nm = 0.6 to 0.7. The negative controls without *HbJAZ3* or *HbMYC2* were performed in the same way. After cultivation for 2 to 4 d, the transformed tobacco leaves were cut approximately in a square and put into the 10 μg/ml DAPI solution (Solarbio, Beijing, China) within 15 min and then washed with ddH_2O at least two times. The fluorescences of YFP and DAPI in transformed tobacco leaves and onion epidemic cells were recorded using confocal microscopy.

Yeast one/two-hybrid assays

For yeast one-hybrid assays, the *HbFPS1* promoter and *HbSRPP1* promoter were cloned into the pHIS2.1 vector (Clontech), generating pHIS-*pHbFPS1* and pHIS-*pHbSRPP1*, respectively (Wang *et al.,* 2013; Guo *et al.,* 2014). The yeast-one library was constructed according to the instructions of the manufacturer (Clontech) and described previously (Wang *et al.,* 2013; Guo *et al.,* 2014). The yeast-one library strain Y187 was transfected with the bait vector pHIS-*pHbFPS1* and then cultivated on SD/-His/-Leu/-Trp medium supplemented with 20 mM 3-AT for the selection of transformants at 30^{o}C for 3 d.

For confirmation of the *HbFPS1* or *HbSRPP1* promoter-binding proteins, HbMYC2 was cloned into pGADT7-Rec2. The pGADT7-HbMYC2 and pHIS-*pHbFPS1* or pHIS-*pHbSRPP1* vector was co-transformed into the yeast strain Y187. pGADT7-Rec53 and p53-His were used as positive controls (CK+) and p53-His as a negative control (CK-). Transformed clones were grown on SD/-His/-Leu/-Trp medium with 20 mM (for *pHbFPS1*) or 42.5 mM 3-AT (for *pHbSRPP1*) for 3 d at 30^{o}C.

For yeast two-hybrid assays, the full-length coding region of *HbMYC2* was cloned into the pGADT7 vector (Clontech) with specific primers (Supplementary Figures S1-S8+Tables S1-S2.pdf). *HbJAZ3* and *HbCOI1* were respectively cloned into the pGADT7 and pGBKT7 vectors. *HbJAZ8.0a*, *HbJAZ8.0c* and *HbJAZ10.0a* were respectively cloned into pGBKT7

vector. Detection of protein-protein interactions between HbMYC2 and JAZs or JAZs and HbCOI1 was performed in SD/-His/-Leu/-Trp-Ade/X-α-gal medium (DDO/X medium, -4+X) or SD/-His/-Leu/-Trp-Ade/X-α-gal (-4+X) medium containing 0 μM or 50 μM COR.

EMSAs

Coding sequence of *HbMYC2* was cloned into the pET-30a vector fused with the His tag. Recombinant His-HbMYC2 protein was expressed and affinity-purified from *E. coli* (BL21) using Ni^+ affinity resin (GE). The EMSA was performed with an Electrophoretic Mobility Shift Assay kit (Invitrogen, USA) following the manufacturer's instruction as described previously (Wang *et al.,* 2013; Guo *et al.,* 2014).

Transient dual-luciferase assays

The coding regions of *HbMYC2* and *HbJAZ3* were respectively cloned into the effector vectors pGreenII-62SK and pCAMBIA2301 vector (CAMBIA). The empty vector pGreenII-62SK was used as negative control. The inhibitory effect of HbJAZ3 on HbMYC2 activity on the transcription of *HbFPS*1 and *HbSRPP1* was determined by transient dual-luciferase assays. The reporter vectors (pGreen II-0800-LUC containing *pHbFPS1* or *pHbSRPP1*) and the effector vector (p35S::HbMYC2) were transferred into *Agrobacterium tumefaciens* (strain GV3101). Overnight cultures of *Agrobacterium* were collected by centrifugation, resuspended in the infiltration buffer (10 mM MES, 150 mM acetosyringone, and 10 mM $MgCl_2$), and incubated at room temperature for 4 h before infiltration. The *Agrobacterium* strain containing the reporter was used alone or mixed with the *Agrobacterium* strains harboring the effectors or the empty vector control. *Agrobacterium* suspension in a 5 ml syringe was carefully press-infiltrated manually onto healthy leaves of 6-week-old *N. benthamiana*. After culture for 3 d, the infected area was harvested for total protein extraction. The supernatant of total proteins was treated with the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's

manual, and the fluorescent values of LUC and REN were detected with a GloMaxR-Multi+Detection System (Promega). The LUC value was normalized to that of REN. Three biological repeats were measured for each combination.

qRT-PCR

The indicated latex samples with different treatments were used for quantitative real-time PCR analysis of the expression levels of the studied genes in the present work. Total RNAs were extracted using a Total plant RNA isolation kit (Tiangen, China) and reverse transcribed using M-MLV reverse transcriptase (Fermentas, USA). Quantitative real-time PCR (qRT-PCR) was performed with the CFX384 real-time PCR system (Bio-Rad, USA) using the SYBR Prime Script RT-PCR kit (TaKaRa, Japan). The relative gene expression level was calculated using the 18S-rRNA gene as the internal normalization controls. The qRT-PCR experiments were performed with each sample measured in triplicate. Primers used for real-time PCR analysis are listed in Supplementary Figures S1-S8+Tables S1-S2.pdf.

JA determination

The extraction and quantification of JA from the latex samples were refered to Tian *et al.* (2015) and modified in sample preparation. Approximately 3 g of latex samples was dripped into 20 ml of 80% methanol containing 0.01% (w/v) sodium diethyldithiocarbamate as an antioxidant. [9,10-$^{2}H_{2}$]Dihydro-JA (250 ng) was added as internal standards for JA determination. The admixture was stirred overnight at 4 ^{o}C and then centrifuged. After the methanol phase was collected, the rubber lump was extracted two times with 20 ml of cold (4^{o}C) 80% methanol. The organic phase was combined, adjusted to pH 8.0 with ammonia and condensed to the aqueous phase (approximately 5 ml) in a rotary film evaporator (Heidolph Laborata 4000 efficient, Germany), frozen at -20^{o}C, followed by three rounds of melting and thawing. After centrifugation at 1200 rpm

for 20 min, 1 mg of polyvinylpolypyrrolidone was added to the collected supernatant. The combination was homogenized at 4.5 m/s for 10 s and filtered. The filtrate was collected and adjusted to pH 2.5-3.0 with 2 M aceticacid and then extracted three times by mixing the filtrate with 5 ml of ethyl acetate. The organic layers were combined, adjusted to pH 8 with ammonia and evaporated to dryness in a rotary film evaporator. The dried extracts were dissolved in 5 ml of 0.1 M acetic acid and passed through a C_{18}column (Sep-Pak® Classic C18, Part No. WAT051910,Waters Corporation, Milford, Massachusetts USA) that had been pre-washed with 5 ml 100% methanol and pre-equilibrated with 5 ml 0.1 M acetic acid. The column was eluted with5 ml of 17% (v/v) acid methanol (100% methanol: 0.1 M acetic acid = 17:83), followed in turn by 5 ml of 60% acid methanol (100% methanol: 0.1 M acetic acid = 60:40) for JA. JA fractions were collected and evaporated to dryness (40^{o}C) after being adjusted to pH 8 with ammonia. The methyl esterification was carried out by dissolving the residue in 0.5ml of methanol and adding 3 ml of ethereal diazomethane. The excess diazomethane was removed under a stream of oxygen-free nitrogen gas. The methylated samples were re-dissolved in ethyl acetate for analysis by GC-MS as described by Tian *et al,* (2015). All analyses were performed with three biological replicates.

In vitro rubber biosynthesis assay

The reaction system was described by Archer *et al.* (1963) and modified in the present study. In detail, 25 µl fresh latex was added into a 400 µl reaction system containing 10 µM $MgCl_2$, 10 µM DTT, 5 µM ATP, and 0.736 µM ^{13}C-MVA (mevalonolactone-2-^{13}C) (Sigma Aldrich, Lot# MBBB5201 V) in 0.1 M PBS buffer (pH 7.8). The reaction was carried out at 30°C for 8 h with 70 rpm constant shaking. Thereafter, the rubber was extracted according to the method described by Rattanapittayaporn *et al.* (2004). The total volume of 400 µl saturated NaCl solution was added into the reaction solution and mixed thoroughly, and an equal volume (800 µl) of extractant (toluene/hexane, 1:1, v/v) was added to the mixture and subjected to centrifugation at 12,000 rpm for 30 min. The upper organic

phase was collected and mixed with equal volume of 1-butanol to dissolve out the rubber overnight. The organic phase was removed after centrifugation at 12,000 rpm for 30 min, and the remained rubber was dried. Approximately 0.5 mg dried rubber sample was embedded into a tin can (Elemental Microanalysis, 6×4 mm, BN261080) and subjected to the detection of the $^{13}C/^{12}C$ ratio using a stable isotope mass spectrometer (GV Instruments, IsoPrime JB312) and elemental analyzer (Thermo, FLASH EA1112 Series). The value of APC (atom percentage) is equal to the percentage of the content of ^{13}C in the total carbon ($^{13}C + ^{12}C$) content in the rubber sample. All analyses were performed with three biological replicates.

Latex collection for Western blotting assay

The previous 5 min outflow of latex of ten virgin rubber trees and ten tapped trees after tapping was collected with an equal volume of isotonic solution used in the i*n vitro* rubber biosynthesis efficiency assay. Then, an equal volume of the above diluted latex from each virgin rubber tree or tapped tree was mixed to represent the virgin tree sample or tapped tree sample and was store on ice for subsequent use. Three replicates were performed. The latex samples were centrifuged at 4^{o}C at a speed of 12,000 rpm for 10 min. The middle layer suspension liquid was filtered by a 0.45 μm filter membrane, and the filtrate was collected. SDS-PAGE and western blot were performed as described by Towbin *et al.* (1979) and Shi *et al.* (2016b) with some modification. The loading amount was respectively 20 μl and 6 μl for HbFPS1 and HbSRPP1 protein detection. The identification of FPS and SRPP proteins recognized by specific polyclonal antibodies (1:1000 dilution for anti-FPS1, 1:2500 dilution for anti-SRPP1) was respectively detected using the Enhanced Chemiluminescence (ECL) Kit and BCIP/NBT Kit from PIERCE (Pierce, USA).

Statistical analysis

Statistical analysis was carried out with SPSS Statistics 17.0 by analysis of variance (ANOVA), and the statistical significance was evaluated using the Student's t-test (two group comparisons between treated samples and control samples) described previously (Wang *et al.,* 2013; Guo *et al.,* 2014; Li *et al.,* 2016). Means were considered significantly different based on T-test threshold value corresponding to the *P* value ($P < 0.05$ and $P < 0.01$) which are indicated by asterisks (*) when $T_{0.975}(4) > 2.7764$, or indicated by (**) when $T_{0.990}(4) > 3.7469$.

Results

Latex exploitation-induced increase in the level of endogenous jasmonates, up-regulation of rubber biosynthesis genes and enhancement of rubber biosynthesis

The endogenous jasmonates in the latex from virgin trees and regularly tapped trees were determined by GC-MS. The level of endogenous jasmonates in the regularly tapped trees was approximately six times that of virgin trees (Fig.1A). The levels of expression of rubber biosynthesis-related genes in the latex were also different between the virgin trees and the regularly tapped trees. Most of the genes in the MVA pathway (Fig.1B), including *HbHMGR1*, *HbMVK1*, *HbPMK1*, *HbMVD1*, *HbMVD2*, *HbIPPI1*, *HbFPS2*, *HbREF1*, *HbCPT6* (also designated *HbHRT2*), *HbCPT7* and *HbCPT8* (*HbHRT1*), were significantly up-regulated in regularly tapped trees in comparison with that in virgin trees (Fig. 1C). Most of the up-regulated genes in the regularly tapped trees were also up-regulated in the latex from the epicormic shoots at 12 h or 1 d after being treated with methyl jasmonate (Fig.1D). The *HbCMK*, *HbMCS1*/*HbMDS1* and *HbMCS2/HbMDS2* in the MEP pathway (Supplementary Figures S1) were significantly up-regulated while those including *HbDXS1*, *HbDXS2*, *HbDXR*, *HbCMS1/HbMCT1*, *HbCMS2/HbMCT2* and *HbHDR* were moderately up-regulated in the regularly tapped trees in comparison with the virgin trees (Supplementary Figures S1). These genes were also significantly up-regulated in the epicormic shoots at 1 d after being treated with 0.07% methyl jasmonate (Supplementary Figures S1).

The efficiency of *in vitro* rubber biosynthesis in the regularly tapped trees was significantly higher than that in the virgin trees that had never been tapped (Fig.1E). It decreased significantly when the regularly tapped trees were rested from tapping for half a month (Fig.1E). The level of the efficiency of *in vitro* rubber biosynthesis significantly decreased 1 d after the trees resting from tapping were applied with 0.5% ethrel (an ethylene releaser) while significantly increased 1 d after being applied with 0.07% methyl jasmonate (Fig.1E).

Identification of the HbCOI1-HbJAZ3-MYC2 module in laticifer cells

To dissect the jasmonate signalling in the laticifer cells of rubber trees, a gene homologous to *AtJAZ3* and *SmJAZ3* was cloned and characterized. It was designated as *HbJAZ3* (GenBank No. KJ911911) with preferential expression in latex in contrast to the bark tissues (Supplementary Figure S2). Its open reading frame (ORF) was 1164 bp in length and encoded a predicted protein of 387 amino acids with an MW of 41.0 kDa and a pI of 8.77. The HbJAZ3 possesses a ZIM domain and a Jas domain that were conserved in the JAZ proteins from other plant species (Supplementary Figure S3). It was clustered closely with AtJAZ3 and SmJAZ3 in the phylogenetic tree (Fig.2A). Subcellular localization analysis revealed that the green fluorescence from the HbJAZ3-GFP was only detected in the nuclei, which was confirmed by DAPI staining (Fig.2B). Using yeast two-hybrid assays, we showed that the yeast cells harboring HbJAZ3 and HbCOI1 could survive in the selective medium when the medium contained 50 μM coronatine (COR) (Fig.2C, Supplementary Figure S6), suggesting that HbJAZ3 was the target of SCF^{COI1} in response to a burst of endogenous jasmonates.

To identify the downstream partners of HbJAZ3 in jasmonate signalling, we used HbJAZ3 as bait to screen a cDNA library from latex by yeast two-hybrid assays. A positive clone was obtained after several cycles of verification on the selective medium (Fig.3A). It contained a single plasmid harboring an inserted fragment of 2495 bp in length. The fragment contained an ORF of 2046 bp in length, a 72 bp 5'UTR region and a 377 bp 3'UTR

region. The ORF encoded a MYC-type protein of 681 amino acids with an MW of 74.58 kDa and a pI of 5.70. This protein shared high similarity with AtMYC2 upon BLASTP analysis. Therefore, the fragment was designated as *HbMYC2* (GenBank No. KM507201) in the present study. HbMYC2 shared highly conserved motifs with the AtMYC2, NtMYC2a, SmMYC2a, and AaMYC2 transcription factors in an amino acid alignment (Supplementary Figure S4) and was clustered into the MYC2 ortholog subgroup in the phylogenetic tree (Fig.3B). As a potential transcription factor, the transcriptional activation of HbMYC2 and its N-terminal and C-terminal regions was assessed in yeast. Only the full-length protein and N-terminal region of residues 1-377 could activate the expression of the *HIS* and *MEL1* genes in yeast cells, resulting in survival of the yeast cells in the selective media lacking histidine (Fig.3C). Subcellular localization analysis revealed that the green fluorescence from HbMYC2-GFP was only detected in the nuclei, which was confirmed by DAPI staining (Fig.3D).

The interaction between HbJAZ3 and HbMYC2 was further confirmed by yeast two-hybrid assays. As described, only the yeast cells harboring *HbJAZ3* and *HbMYC2* and the positive control could grow and exhibit a blue color on QDO/X-α-gal plates, suggesting that HbJAZ3 interacted with HbMYC2 (Fig.3A).Their interaction was further verified *in planta* by bimolecular fluorescence complementation (BiFC) assays. The tobacco cells (Fig.4A) or the onion epidermal cells (Supplementary Figure S7) harboring HbJAZ3-cYFP and HbMYC2-nYFP exhibited a bright yellow color in the sites that were specifically stained by DAPI, similar with the YFP signal in the cells harboring the yeast positive control p53 and SV40-T. By contrast, the bright yellow color was not detected in the tobacco cells (Fig.4A) or the onion epidermal cells (Supplementary Figure S7) harboring either HbJAZ3-cYFP or nYFP as well as either HbMYC2-nYFP or cYFP, and the HbJAZ3-cYFP and HblMYC1 or HblMYC2, or the yeast negative control Lam and SV40-T, although DAPI staining of the nuclei was observed (Supplementary Figure S7), suggesting that the bright yellow color were specific and positive. *HbCOI1*, *HbJAZ3* and *HbMYC2* were all significantly up-regulated in laticifer of the regularly tapped trees compared with those of the virgin trees (Fig.4B). The expression of the three genes in the epicormic shoots was significantly

up-regulated at 12 h or 1 d after being treated with 0.07% methyl jasmonate (Fig.4C) The positive response of the three genes to JA and the interaction between HbCOI1 and HbJAZ3 in a COR-dependent manner and between HbJAZ3 and HbMYC2 in a COR-independent manner demonstrated the presence of an HbCOI1-HbJAZ3-MYC2 module in the laticifer cells of rubber tree.

Effect of HbJAZ3 on the HbMYC2-activated transcription of HbFPS1 and HbSRPP1

To identify possible transcriptional factors involved in JA signalling regulating rubber biosynthesis, a yeast one-hybrid screening of the latex cDNA library was performed using the promoter of the key rubber biosynthesis gene *HbFPS1*. A fragment harbored in a positive clone was identical to *HbMYC2*. The interaction of the full-length HbMYC2 with the *HbFPS1* promoter was revealed by the yeast one-hybrid assay (Fig.5A).The physical binding of HbMYC2 to the *HbFPS1* promoter was further verified by *in vitro* EMSAs. *HbFPS1* promoter fragments were more and more retarded with increased amounts of the purified HbMYC2 protein (Fig.5B).

Considering the presence of a conserved G-box ('CANNTG') in the *HbSRPP1* promoter (Supplementary Figures S1-S8+Tables S1-S2.pdf), the potential interaction of this promoter with HbMYC2 was tested by yeast one-hybrid. The yeast cells harboring HbMYC2 and the *HbSRPP1* promoter survived on the selective medium (Fig.5C), suggesting the interaction occurred. The physical binding of HbMYC2 to the *HbSRPP1* promoter was verified by *in vitro* EMSAs. *HbSRPP1* promoter fragments were retarded along with increased amounts of the purified HbMYC2 protein (Fig.5D).

A dual-luciferase assay system (Fig.5E) was used to assess the effect of the physical binding of HbMYC2 to either the promoter of *HbFPS1* or the promoter of *HbSRPP1* as well as the effect of HbJAZ3. The luciferase (LUC) activity in the tobacco leaves harboring *HbMYC2* and *Luc* that was driven by either the promoter of *HbFPS1* or the promoter of *HbSRPP1* was significantly higher than that in the tobacco leaves harboring *Luc* alone,

while it was significantly decreased when HbJAZ3 was co-expressed (Fig.5 F-G). There was no obvious difference in the *Luc* expression in the tobacco leaves when expressed *HbJAZ3* gene alone in comparison with the empty vector control (Fig.5 F-G). These data demonstrated that both *HbFPS1* and *HbSRPP1* were positively regulated by HbMYC2, while HbJAZ3 repressed the HbMYC2-activated expression of either *HbFPS1* or *HbSRPP1*.

Effect of latex exploitation on the level of HbFPS1 and HbSRPP1

Given that the expression levels of *HbFPS1* and *HbSRPP1* were higher in the regularly tapped trees than that in the virginal rubber trees (Fig.1D), we further detected the difference in the level of HbFPS1 and HbSRPP1 between the virgin trees and regularly tapped trees. Considering the HbSRPP1 is more abundant than the HbFPS1, a mount of 20 µl and 6 µl of rubber particle filtrates were differentially loaded for respectively detecting the HbFPS1 and HbSRPP1 proteins (Fig. 6A). The western blotting analysis revealed that the level of HbFPS1 protein was significantly higher in the regularly tapped trees as compared with the virginal trees (Fig.6B, C). Although it seemed no difference in the level of HbSRPP1 in the SDS-PAGE profiles between the regularly tapped trees and the virgin trees (Fig. 6D), western-blot analysis showed the level of HbSRPP1 in the regularly tapped trees was also significantly higher than that in the virgin trees (Fig. 6E, F). These results indicated that the increase in the transcripts was positively correlated with the increase in their corresponding proteins.

Effect of HbEIN3 on the HbMYC2-activated transcription of HbFPS1 and HbSRPP1

Considering that the *in vitro* rubber biosynthesis was enhanced by exogenous methyl jasmonate while inhibited by ethrel application (Fig.1E), one member of EIN3 family, HbEIN3, on the HbMYC2-activated transcription of HbFPS1 and HbSRPP1 was assessed in the transient luciferase (LUC) activity detection system (Fig.7A). HbEIN3 had little influence on the expression of the *HbFPS1* and *HbSRPP1*, although

ET-responsive elements are present in the promoter sequence of either the*HbFPS1* or *HbSRPP1* (Supplementary Figures S1-S8+Tables S1-S2.pdf). It had also no influence on the HbMYC2-activated expression of *HbFPS1* and *HbSRPP1* (Fig.7B,C). Therefore, the mechanism for the antagonistic effect of JA and ET on rubber biosynthesis (Fig.1E) remains to be elucidated.

Discussion

The jasmonate signalling has been well dissected with the aid of forward genetics in herbaceous model plants (Xie *et al.,* 1998; Chini *et al.,* 2007; Wasternack and Song, 2017; Zhu and Napier, 2017) and revealed a COI1-JAZ-MYC2 module that seems to be conserved among plant species (Fonseca *et al.,* 2009b; Shoji and Hashimoto, 2011; Shen *et al.,* 2016; Zhou *et al.,* 2016). However, the jasmonate signalling in a specific tissue is not elucidated clearly to date. The secondary laticifer in rubber tree is a single cell type tissue that is composed of laticifer cells. There are no plasmodesmata between the laticifer cells and their surrounding phloem parenchyma cells (de Faÿ *et al.,* 1989). Therefore, the easily obtained latex by tapping is the pure cytoplasm of laticifer cells. As a specific tissue for rubber biosynthesis (Kush, 1994), the secondary laticifer is enriched in the transcripts of rubber biosynthesis-related genes (Chow *et al.,* 2007). These structural and molecular characteristics endow the laticifer cells with a suitable model for investigating the transcriptional regulation of secondary metabolism.

Rubber biosynthesis in laticifer cells is a typical isoprenoid metabolism and is carried out by the MVA pathway as well as the MEP pathway (Seetang-Nun *et al.,* 2008; Chow *et al.,* 2012; Tang *et al.,* 2016; Deng *et al.,* 2016). Most of rubber biosynthesis-related genes in MVA pathway and MEP pathway are up-regulated in the regularly tapped trees. These genes are down-regulated in regularly tapped trees that are rested from tapping for a short time (Tian *et al.,* 2013) and in TPD (tapping panel dryness)-affected trees that are rested from tapping for a long time (Liu *et al.,*

2015). The up-regulated expression of rubber biosynthesis-related genes should be associated with the high level of endogenous JA in the latex from the regularly tapped trees, since the expression pattern of rubber biosynthesis-related genes caused by exogenous methyl jasmonate is similar to that in regularly tapped trees. The level of endogenous JA in the laticier cells of regularly tapped trees is several times more than that of virgin trees two days after tapping (mechanical wounding). As the mechanical wounding-caused a burst of endogenous JA in bark tissues occurs within 4 h (Tian *et al*., 2015), the maintenance of high level of endogenous JA in the regularly tapped trees 2 d after being tapped may not be contributed to the mechanical wounding *per se*. It should mainly be ascribed to the changes in the turgor pressure of laticifer cells. Available data show that change in turgor pressure is critical for JA biosynthesis (Creelmen and Mullet, 1997), and the turgor pressure of laticifer cells is ten times that of the atmosphere before tapping and significantly decreases within the latex drainage areas, even resulting in a detectable decrease in the trunk diameter after tapping (Pakianathan *et al.,* 1989). The high level of endogenous JA is also positively correlated to the enhanced rubber biosynthesis in the regularly tapped trees because the exogenous methyl jasmonate is effective in enhancing the *in vitro* rubber biosynthesis.

A jasmonate signalling module, HbCOI1-HbJAZ3-HbMYC2, in the specific laticifer tissue of rubber trees is identified for the first time. HbJAZ3 interacts with HbCOI1 in a coronatine-dependent manner. It also interacts with HbMYC2 in a coronatine-independent manner and inhibits the HbMYC2-activated transcription of *HbFPS1* and *HbSRPP1*. The cascade of HbCOI1-HbJAZ3-HbMYC2-HbFPS1/HbSRPP1 in laticifer cells directly links the jasmonates with rubber biosynthesis regulation. It is the first time to discover the key rate-limiting enzyme FPS-encoding gene is the regulating target for the MYC2-type transcriptional factor in the conserved MVA pathway. This finding will enlarge our understanding of the jasmonate signalling in plants' isoprenoid metabolism regulation. The *HbFPS1* and *HbSRPP1* transcripts are respectively the most abundant in the laticifer cells of rubber tree among the FPS and

SRPP gene families (Guo *et al.,* 2015a; Tang *et al.,* 2016), suggesting their significant and indispensable roles in rubber synthesis. HbFPS1 is essential for incorporating two IPP molecules and one DMAPP molecule into one FPP molecule, an initial primer for initiating rubber biosynthesis (Cornish *et al.,* 1999). It shares 91% similarity or 83% identity with TkFPS1 from *Taraxacum kok-saghyz* Rodin (Supplementary Figure S8). Over-expression of *TkFPS1* resulted in a high increase of 7.48% or an average increase of 3.92% in rubber content in transgenic plants (Cao *et al.,* 2016). HbSRPP1 plays a positive role in rubber biosynthesis (Oh *et al.,* 1999; Yamashita *et al.,* 2016; Brown *et al.,* 2017). Its orthologs also play a vital role in rubber biosynthesis in *T. kok-saghyz* (Collins-Silva *et al.,* 2012) and *T. brevicorniculatum* (Hillebrand *et al.,* 2012), although silencing the homolog of *HbSRPP1* had little effect on rubber biosynthesis in *Lactuca sativa* (Chakrabarty *et al.,* 2015). The increased levels of HbFPS1 and HbSRPP1 (Fig.6) may be partly ascribed the enhanced rubber biosynthesis in the regularly tapped trees compared with the virgin trees. HbJAZ3-mediated repression of HbMYC2-activated expression of both *HbFPS1* and *HbSRPP1* suggests possible transgenic improvement of rubber yield potential of rubber tree since silencing its ortholog, SmJAZ3, resulted in tanshinone accumulation in *S. miltiorrhiza* (Shi *et al.,* 2016a).

It has long been believed that rubber biosynthesis in rubber tree is positively regulated by ethylene signalling since application of ethrel (an ethylene releaser) significantly raises the rubber yield per tapping. This effect is primarily due to the prolonged duration of latex flow (Coupé and Chrestin, 1989) in addition to the enhanced sucrose allocation (Tang *et al.,* 2010), water transportation (Tungngoen *et al.,* 2009), glycolysis and C3 carbon fixation (Liu *et al.,* 2016). By contrast, ethylene signalling does not have a major role in activating rubber biosynthesis *per se* as ethylene application has little effect on up-regulating the genes related to IPP biosynthesis and IPP integration into rubber (Oh *et al.,* 1999; Zhu and Zhang, 2009; Liu *et al.,* 2016). It has little effect on or even decreases the abundance of REF and SRPP proteins (Tong *et al.,* 2017). In the present study, we provide evidence that

ethylene inhibits rubber biosynthesis. The antagonistic effect between JA signalling and ethylene signalling on rubber biosynthesis remains to be elucidated. Although EIN3-MYC2 complex mediates the crosstalk of ET signalling and JA signalling in resistant reaction in *Arabisopsis* (Song *et al.,* 2014), the latex preferentially expressed HbEIN3 (KR013139) (Yang *et al*., 2015) had no evident influence on the HbMYC2-activated transcription of HbFPS1 and HbSRPP1 (Fig.7B,C), indicating that the crosstalk of ET signalling and JA signalling on rubber biosynthesis regulation may not be associated with the transcriptional regulation on the *HbFPS1* and *HbSRPP1* genes. Alternatively, the transcriptional regulation of *HRT2* gene may be associated with the crosstalk because HbEIN3 could bind to the promoter and regulate the expression of *HRT2* gene (Yang *et al*., 2015). Although HbMYC2 could not bind the promoter of *HRT2* gene (data not shown), it could not be excluded other transcription factors which may be associated with JA signalling (Guo *et al*., 2018). Just as WD-repeat/bHLH/MYB transcriptional complexes are essential for JA-regulated anthocyanin biosynthesis in *Arabidopsis* (Qi *et al.,* 2011), the rubber biosynthesis-related genes may be regulated by multiple transcriptional factors. In addition to positive regulation by HbMYC2 in the present study, *HbSRPP1* is also negatively regulated by HbWRKY1 (Wang *et al.,* 2013) and HbMADS4 (Li *et al.,* 2016). Ethylene up-regulates *HbWRKY1* expression and down-regulates *HbSRPP1* expression while methyl jasmonate significantly up-regulates *HbSRPP1* and has little effect on the expression of *HbWRKY1* (Wang *et al.,* 2013). Both ethylene and methyl jasmonate up-regulate the expression of *HbMADS4* (Li *et al.,* 2016). HMGR is the key rate-limiting enzyme in MVA (Goldstein and Brown, 1990). The transcripts of *HbHMGR1* are the most abundant in laticifer cells among the five HMGR gene members (Tang *et al.,* 2016). *HbHMGR1* is positively regulated by HbCZF1, a CCCH-type zinc finger protein (Guo *et al.,* 2015b). *HbCZF1* is significantly up-regulated by methyl jasmonate, but its expression is little influenced by ethylene (Guo *et al.,* 2015b).

Taken together, the dramatic changes in the turgor pressure of laticifer cells after latex exploitation result in a burst of endogenous JA. The increased JA up-regulates the rubber biosynthesis-related genes, *HbFPS1* and *HbSRPP1*, by the degradation of repressor HbJAZ3 via the 26S and the release of HbMYC2. The up-regulation of *HbSRPP1* and *HbFPS1* results in the increase in HbSRPP1 and especially HbFPS1 proteins. The increased HbSRPP1 and HbFPS1 are partially ascribed to the increased efficiency of *in vitro* rubber biosynthesis. In addition to HbMYC2, other transcriptional factors such as HbCZF1, HbWRKY1 and HbMADS4 may integrate into the JA signalling and mediate the latex exploitation-activated rubber biosynthesis in the laticifer cells of rubber trees.

Data availability

The full-length cDNA sequences of *HbMYC2* and *HbJAZ3* were deposited in NCBI database with the accession no. KM507201 and KJ911911, respectively. The accession no. of *HbFPS1*, *HbSRPP1*, *HbCOI1* and *HbEIN3* were AY349419, HQ640231, EU136026 and KR013139, respectively.

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Figure Legends

Figure 1 Changes in several physiological parameters under different conditions. (A) Effect of tapping on endogenous JA levels in laticifer cells. (B) The rubber biosynthesis-related genes tested (C) and (D). Effect of tapping (C) and exogenous methyl jasmonate (D) on the expression of rubber biosynthesis-related genes. (E) Determination of the efficiency of *in vitro* rubber biosynthesis in the latex from the virgin trees (virgin), regularly tapped trees (tapped), the trees resting from tapping without any treatment (rested), treated with

ethrel (ET) and methyl jasmonate (JA). Data are the mean (±SD) of three biological replicates. Asterisks represented Student's *t* test significance (**P* < 0.05, ***P* < 0.01).*Enzyme abbreviations*: HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MVD, mevalonate diphosphate decarboxylase; IPPI, isopentenyl diphosphate isomerase; FPS, farnesyl diphosphate synthesis; CPT, *cis*-prenyltransferase; HRT, *Hevea* rubber transferase ; SRPP, small rubber particle protein; REF, rubber elongation factor.

Figure 2 HbJAZ3 identification. (A) Phylogenetic tree was constructed by using the neighbor-joining method with 1000 replications after ClustalX2 alignment analysis of HbJAZ3with other JAZ proteins including AtJAZ1 (NP_564075), AtJAZ2 (Q9S7M2), AtJAZ3 (AEE76017), AtJAZ4 (NP_001117450), AtJAZ5 (ANM58475), AtJAZ6 (NP_565043), AtJAZ7 (AEC08997), AtJAZ8 (NP_564349), AtJAZ9 (Q8W4J8), AtJAZ10 (NP_974775), AtJAZ11 (NP_001190007), AaJAZ1 (AJK93412), AaJAZ2 (AJK93413), AaJAZ3 (AJK93414), AaJAZ4 (AJK93415), SmJAZ1 (AGC73980), SmJAZ3 (AHK23660), SmJAZ9 [protein sequences in Shi *et al.* (2016a)], NtJAZ1 (BAG68655), NtJAZ2 (BAG68656), NtJAZ3 (BAG68657), HbJAZ1 (ADI39634), HbJAZ2 (AIY25007), HbJAZ3 (KJ911911), HbJAZ7 (AIY2500), HbJAZ8 (AIY25009), HbJAZ9 (AIY25010), HbJAZ10 (AIY25011), and HbJAZ11 (AIY25012). The evolutionary distance is indicated with the bar. (B) Subcellular localization of HbJAZ3 in onion epidermal cells.The nuclei of the onion epidermal cells were visualized by DAPI staining. (C) Interaction of HbCOI1 with HbJAZ3 in yeast cells in the presence of 50 μM coronatine (COR).

Figure 3 HbMYC2 identification. (A) Screening of HbMYC2 by HbJAZ3 using yeast two hybrid assay. (B) Phylogenetic tree was constructed by using the neighbor-joining method with 1000 replications after ClustalX2 alignment of HbMYC2 (AJC01627) and other bHLH proteins, including NtMYC2a (ADU60100), NtMYC2b (ADU60101), SmMYC2a (AIO09733), SlMYC2 (AGZ94899), JcMYC2 (XP_012076236), RcMYC2 (XP_002519814), AtMYC2

(Q39204), AtMYC3 (Q9FIP9), AtMYC4 (O49687), AaMYC2 (AKO62850), MaMYC2a (XP_009384727), MaMYC2b (XP_009413229), TcJAMYC (ACM48567), SmMYC2b [protein sequences in Zhou *et al.* (2016)], AtbHLH13 (Q9 LNJ5), HblMYC1 (ADK56287), and HblMYC2 (ADK91082). The evolutionary distance is indicated with the bar. (C) Activating activity of full-length HbMYC2 and its N-terminal derivative (1-377 aa) for the transcription in yeast cells. The schematic diagram represents for the constructs of N-terminal and C-terminal of the HbMYC2 in the transcriptional activation assay in yeast. (D) Subcellular localization of HbMYC2 in onion epidermal cells. The nuclei of the onion epidermal cells were visualized by DAPI staining.

Figure 4 Identification of an HbCOI1-HbJAZ3-HbMCY2 module. (A) Interaction of HbJAZ3 with HbMYC2 in tobacco (*Nicotiana benthamiana*) by bimolecular fluorescence complementation (BiFC) assays. The empty pairs (n-YFP and c-YFP) were used as negative controls. YFP fluorescence was detected 3 d after infiltration under a confocal microscope. Scale bar=20 μm. (B-C) Up-regulated expression of *HbCOI1*, *HbJAZ3* and *HbMYC2* upon tapping (B) and methyl jasmonate (C).

Figure 5 Transcriptional regulation of *HbFPS1* and *HbSRPP1*. (A) Activation of the *HbFPS1* promoter in yeast by HbMYC2 protein. (B) The physical binding of the HbMYC2 protein to the promoter of *HbFPS1* by electrophoretic mobility shift assays (EMSA). (C) Activation of the *HbSRPP1*promoter in yeast by HbMYC2 protein. (D) The physical binding of the HbMYC2 protein to the promoter of *HbFPS1* by EMSA. (E) The schematic diagram of the constructs for a dual-luciferase assay. (F-G) Assay of the transient transcriptional activity of HbMYC2 on *HbFPS1* (F) and *HbSRPP1* (G) without or with the expressed HbJAZ3 protein. Data are the mean (±SD) of three biological replicates. Asterisks represented Student's t test significance (**P*< 0.05, ***P*< 0.01).

Figure 6 Changes in the levels of HbFPS1 and HbSRPP1 in the latex from the regularly tapped trees and virgin trees. (A and D) The SDS-PAGE profiles of latex from the regularly tapped trees (T) and virgin trees (V). Twenty microliters latex was loaded per lane in (A), and 6 µl latex was loaded per lane in (D). (B and E) Western blotting analysis of the duplications with polyclonal antibodies raised against the HbFPS1 (B) and HbSRPP1 (E). (C and F) Relative quantitative analysis of HbFPS1 (C) and HbSRPP1 (F). M, protein standards. Arrow head, HbFPS1 in b and HbSRPP1 in e. Data are the mean (±SD) of three biological replicates. Asterisks represented Student's t test significance (*P < 0.05, **P< 0.01).

Figure 7 Effect of HbEIN3 on the regulation of HbMYC2-activated *HbFPS1* and *HbSRPP1* expression. Effect of HbEIN3 on the regulation of HbMYC2-activated *HbFPS1* and *HbSRPP1* expression. (A) The schematic diagram of the constructs for a dual-luciferase assay. (B-C) Analysis of the transient transcriptional activity of HbMYC2 on *HbFPS1* (B) and *HbSRPP1* (C) without or with the expressed HbEIN3 protein. Data are the mean (±SD) of three biological replicates. Asterisks represented Student's t test significance (*P< 0.05, **P< 0.01).

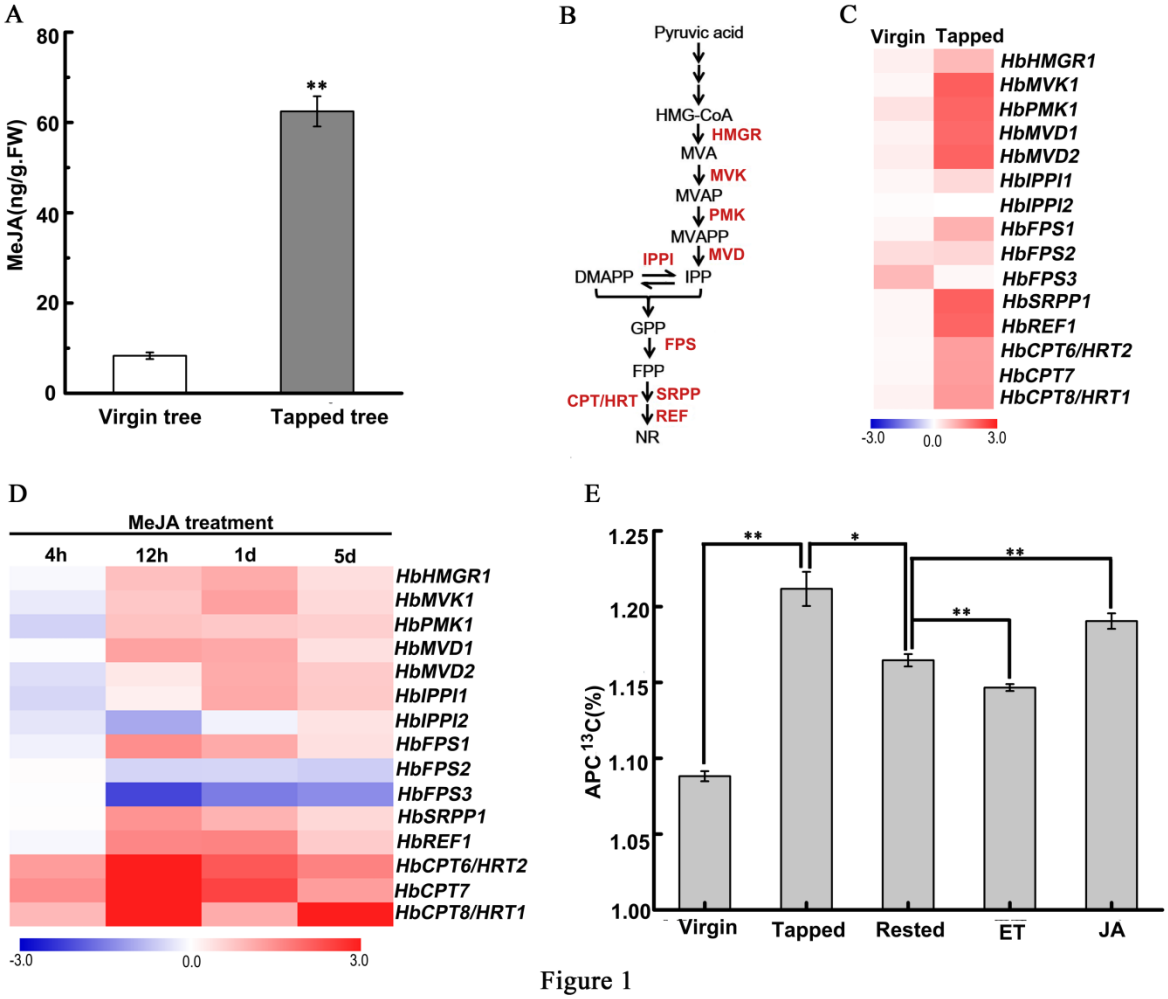


Figure 1

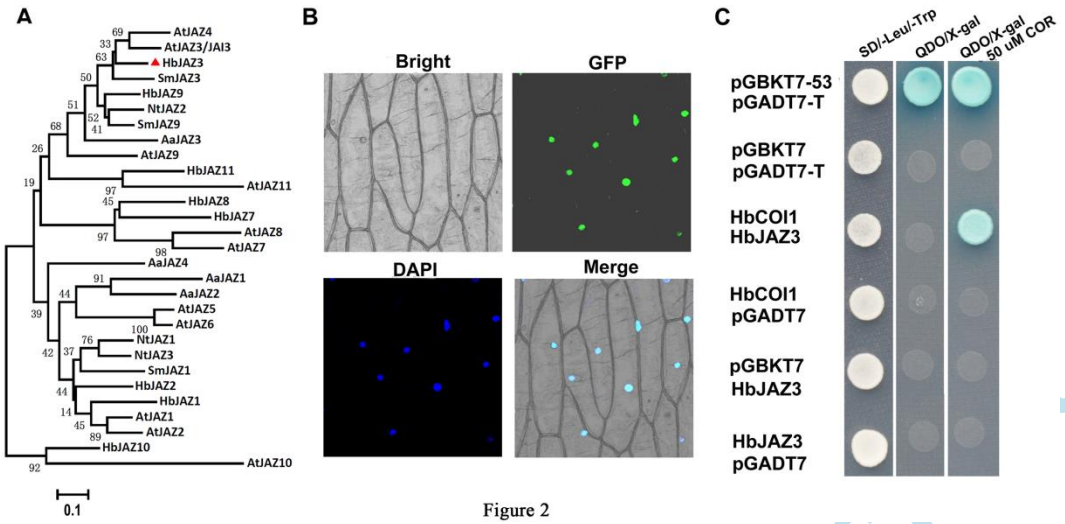


Figure 2

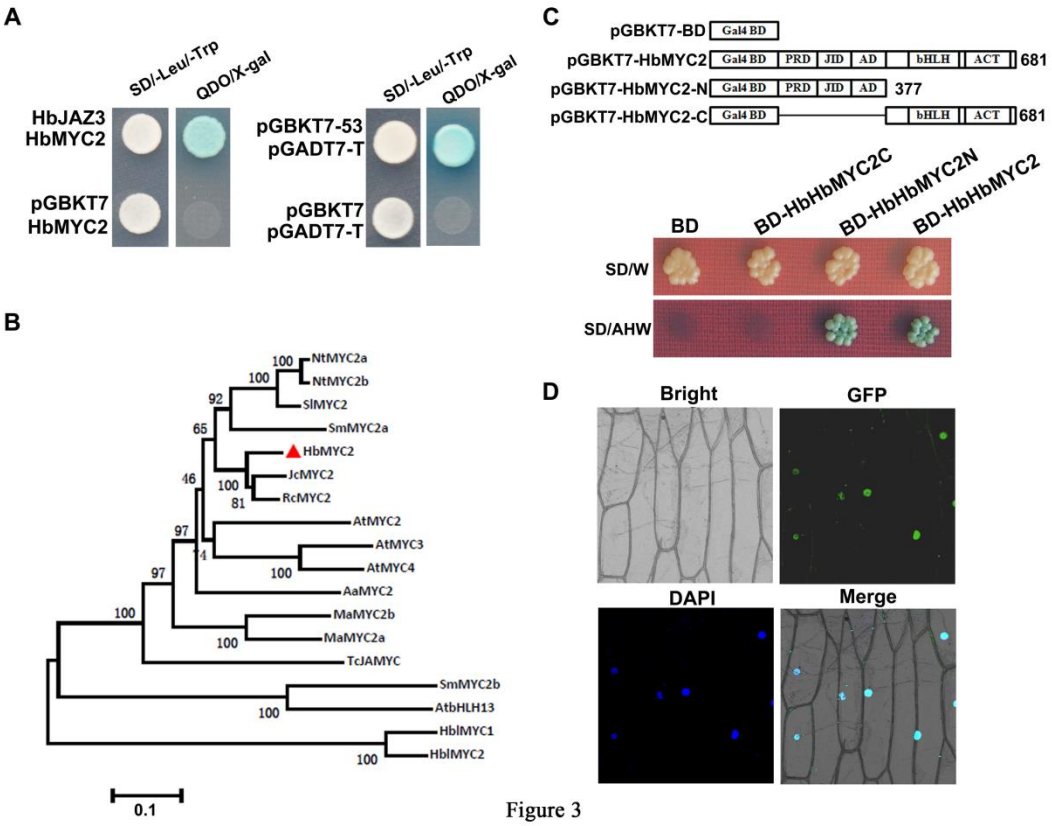
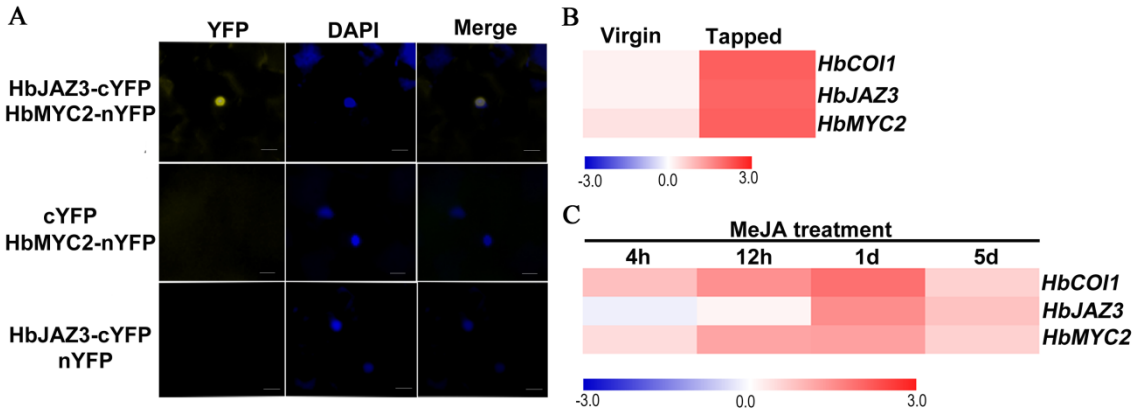


Figure 3



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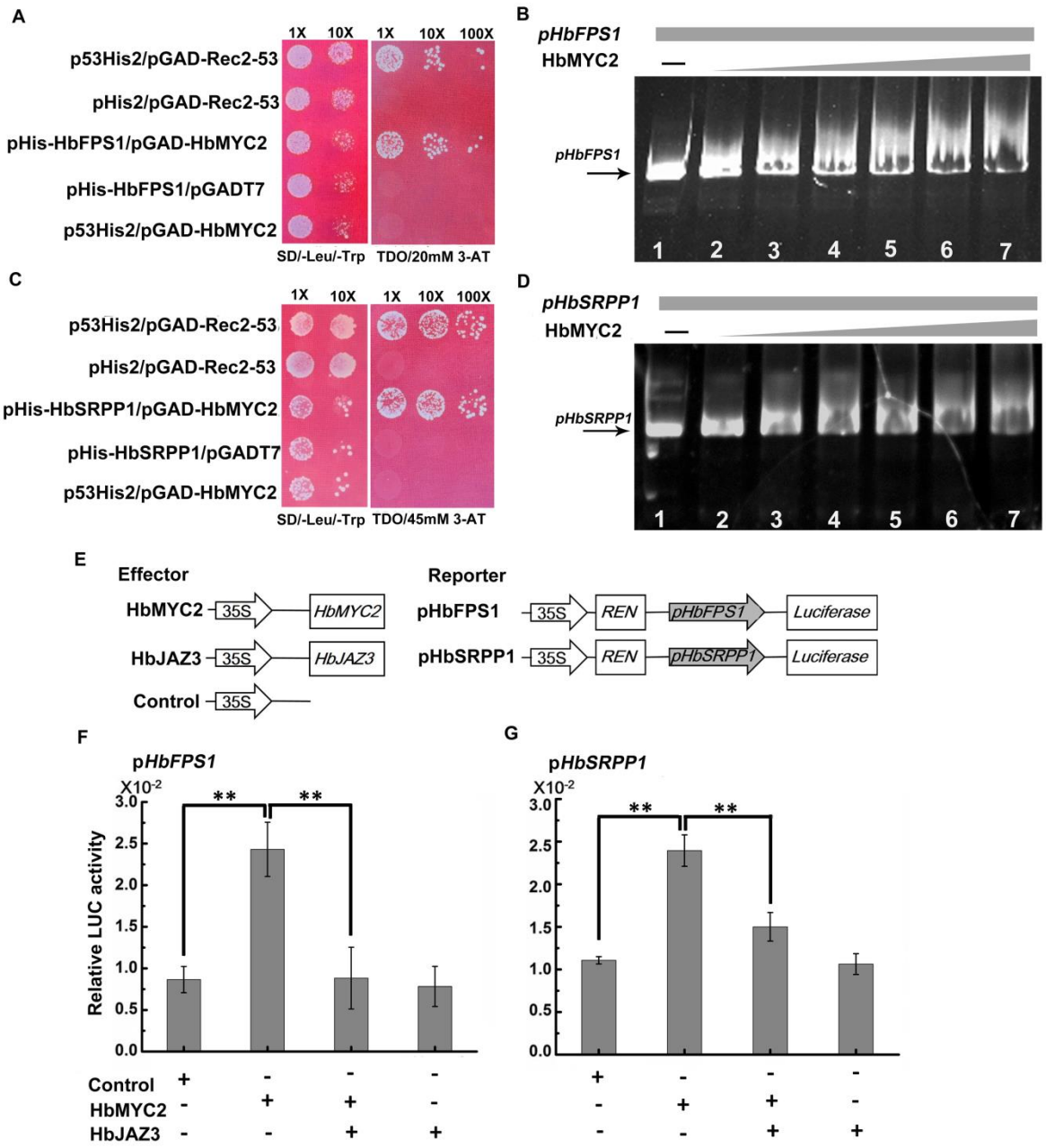

Figure 5

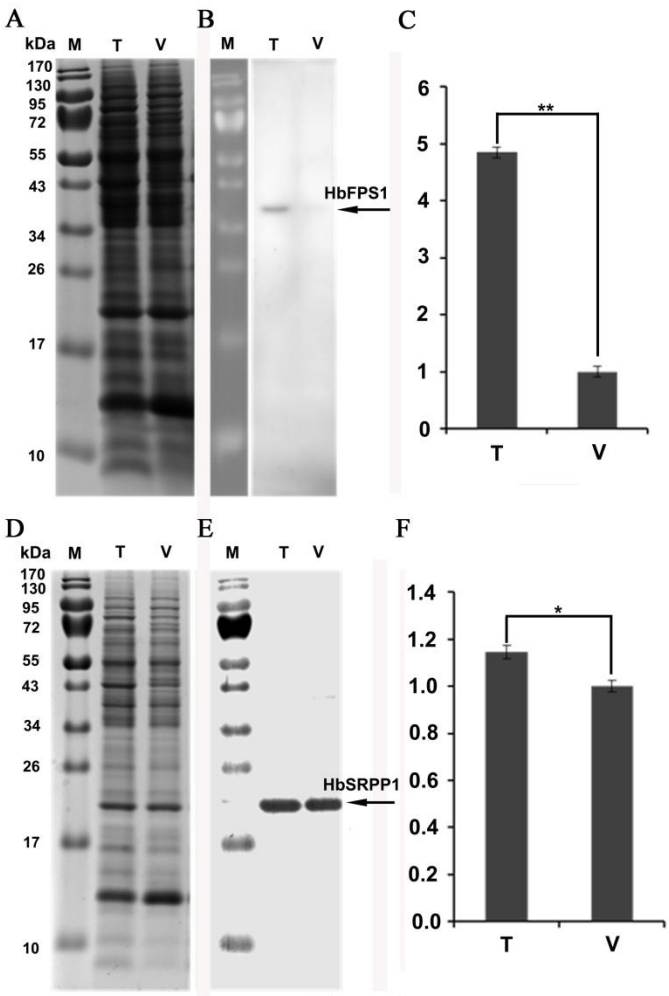

Figure 6

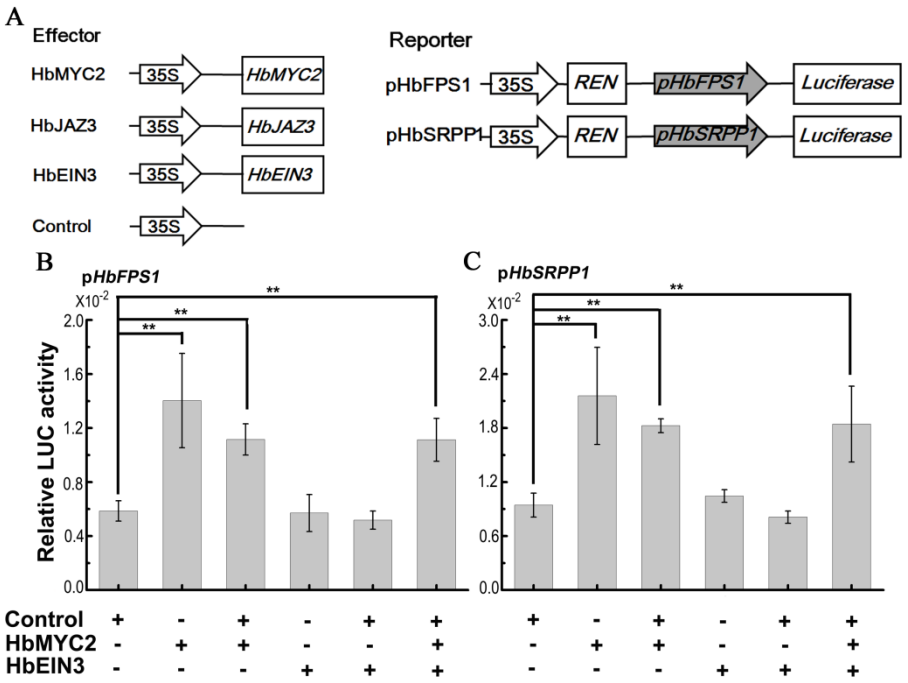

Figure 7