Transcriptome analysis of *Hevea brasiliensis* in response to exogenous methyl jasmonate provides novel insights into regulation of jasmonate-elicited rubber biosynthesis

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Received: 28 November 2017 / Revised: 7 March 2018 / Accepted: 27 March 2018 / Published online: 13 April 2018
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Abstract The phytohormone methyl jasmonate (MeJA) is known to trigger extensive reprogramming of gene expression leading to transcriptional activation of many secondary metabolic pathways. However, natural rubber is a commercially important secondary metabolite and little is known about the genetic and genomic basis of jasmonate-elicited rubber biosynthesis in rubber tree (*Hevea brasiliensis*). RNA sequencing (RNA-seq) of *H. brasiliensis* bark treated with 1 g lanolin paste containing 0.02% w/w MeJA for 24 h (M2) and 0.04% w/w MeJA for 24 h (M4) was performed. A total of 2950 and 2850 differentially expressed genes in M2 and M4 compared with control (C) were respectively detected. Key genes involved

in 2-C-methyl-D-erythritol 4-phosphate, rubber biosynthesis, glycolysis and carbon fixation (Calvin cycle) pathway were found to be up-regulated by MeJA treatment. Particularly, the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in MVA pathway was down-regulated by MeJA treatment, but the expression of farnesyl diphosphate synthase (FPS) and cis-prenyltransferase (CPT, or rubber transferase) in rubber biosynthesis pathway were up-regulated by MeJA treatment. Up-regulation of critical genes in JA biosynthesis in response to MeJA treatment exhibited the self-activation of JA biosynthesis. In addition, up-regulated genes of great regulatory importance in cross-talk between JA and other hormone signaling, and of transcriptional regulation were identified. The increased expression levels of FPS and CPT in rubber biosynthesis pathway possibly resulted in an increased latex production in rubber tree treated with MeJA. The present results provide insights into the mechanism by which MeJA activates the rubber biosynthesis and the transcriptome data can also serve as the foundation for future research into the molecular basis for MeJA regulation of other cellular processes.

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12298-018-0529-0>) contains supplementary material, which is available to authorized users.

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Keywords Rubber tree · Methyl jasmonate · Rubber biosynthesis · RNA sequencing · Transcriptome

Introduction

Although over 2500 species of higher plants have been reported to produce natural rubber, rubber tree (*Hevea brasiliensis*) is the only one source of commercial natural rubber (Cornish 2001; Whalen et al. 2013). With high-yielding cultivars developed and high-yielding practices with ethephon (a ethylene generator) stimulation adopted,

rubber tree is extensively cultivated in tropical areas as a cash tree. Rubber is produced in the cytoplasm of specialized cells or vessels (laticifers) and stored in small subcellular rubber particles. The fluid cytoplasm of laticifers is also known as latex. Upon tapping the soft bark tissues, the latex vessels are opened, the milky rubber-containing latex flows out and is periodically harvested (d'Auzac 1989; Hagel et al. 2008). Natural rubber, an important industrial and strategic material, is used extensively in many applications and products for its outstanding physical properties. In addition, the rubber wood can be used to manufacture furniture and floor after latex collected for 20 years or so.

The rubber molecule (*cis*-1,4-polyisoprene) is a high-molecular weight polymer composed of isoprene units derived from isopentenyl diphosphate (IPP) in the *cis*-configuration (Cornish 2001; Whalen et al. 2013). As the building blocks of the rubber, IPPs has been known to derive from the mevalonic acid (MVA) pathway (Hepper and Audley 1969; Skilleter and Kekwick 1971; Kekwick 1989). However, there is increasing evidence of crosstalk between the cytosolic MVA pathway and another metabolic pathway of IPP, the plastidial 1-dexoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DEX/MEP) pathway and exchange of common isoprenoid precursors to an extent (Rodríguez-Concepción et al. 2013). The MEP-derived isoprenoid precursors might be exported to the cytosol by a plastidial proton symport system (Bick and Lange 2003) and be involved in rubber biosynthesis (Ko et al. 2003; Seetang-Nun et al. 2008; Chow et al. 2007, 2012). The sequential condensation of the IPPs at the priming farnesyl pyrophosphate (FPP) into natural rubber (*cis*-1,4-polyisoprene) is catalyzed by *cis*-prenyltransferase (CPT) (Cornish and Xie 2012). CPT has been intensively investigated in rubber tree and other plants (Asawatratanakul et al. 2003; Brasher et al. 2015; Qu et al. 2015; Schmidt et al. 2010; Spanò et al. 2015; Takahashi et al. 2012).

Free-acid jasmonic acid (JA) and JA derivatives including methyl jasmonate (MeJA) collectively referred to as jasmonates (JAs), are vital lipid-derived cellular regulators involved in diverse developmental processes and plant responses to biotic and abiotic stresses (Wasternack 2015; Wasternack and Hause 2013). Especially, JAs have been reported to transcriptionally activate secondary metabolism. Generally, secondary metabolites are both important for plant in responses to development and environmental cues, and some of them can be used by human as pharmaceuticals and other industrial materials (Cheong and Choi 2003; Pauwels et al. 2009; De Geyter et al. 2012). In rubber tree, exogenous JA and linolenic acid treatment has been reported to stimulate secondary laticifer differentiation (Hao and Wu 2000; Shi and Tian

2012; Tian et al. 2003). Moreover, natural rubber is a commercially useful secondary metabolite and exogenous applications of JAs was reported to increase latex production in a small-scale field experiment (Duan et al. 2004), but the related molecular mechanism remains elusive.

As a powerful tool for quantitatively examining gene expression changes across the transcriptome, RNA sequencing (RNA-Seq) has been applied to understand diverse biological processes in plant (Jamaluddin et al. 2017; Jasrotia et al. 2017; Spyropoulou et al. 2014; Sun et al. 2013; Wang et al. 2017). In the present study, transcriptome analysis of rubber tree in response to exogenous MeJA was conducted using Illumina RNA sequencing technique. Our objective was to identify MeJA-responsive genes in rubber tree, emphasizing the genes involved in IPP, rubber and JA biosynthesis, and the genes in phytohormone signal transduction and transcriptional regulation, which may shed light on the underlying molecular mechanism of MeJA activation of rubber biosynthesis.

Materials and methods

Plant material

Bark tissues were collected from the rubber trees of clone RY8813 at the Experimental Farm of the Chinese Academy of Tropical Agriculture Science (Baodaoxincun, Danzhou, Hainan Province, China). The trees were planted in 1993 and latex was harvested firstly in 2001. The tapping system was s/2 d/3 (half spiral tapped every 3 days) with 1.5%-ethephon treatment one day before tapping at intervals of 15 days.

On the 9th of April 2015 (the date for the start of tapping this year), eighteen trees of equal size in the same plot were chosen and classified into three groups (each with six trees). Sampling technique was modified with the methods described in the related literatures (Hao and Wu 2000; Shi and Tian 2012; Tian et al. 2003; Zeng et al. 2003). The stem surface from the tapping line to 5 cm below was applied with 1 g lanolin paste containing 0, 0.02 or 0.04% w/w MeJA (thus with 0, 2 mg or 4 mg MeJA in 1 g lanolin paste respectively) after the dead epidermis cuticle of treatment site was scraped with a sharp knife. After the lanolin paste application, the treatment area was covered with polyethylene membrane. Bark samples were collected from three groups 24 h after MeJA treatments, respectively. The samples were immediately frozen in liquid nitrogen and shipped on dry ice to BGI Life Tech Co., Ltd (Shenzhen, China) for Illumina sequencing.

RNA extraction, cDNA library construction and sequencing

Total RNAs were isolated from a pool of six biological replicates of the bark samples via the TRIzol® Reagent (Invitrogen) according to the protocol in the manufacturer's instructions. RNA integrity was examined by a 2100 Bioanalyzer (Agilent Technologies). Three cDNA libraries (with no replicates), C (treatment with 1 g lanolin paste containing 0% w/w MeJA for 24 h as control), M2 (treatment 1 g lanolin paste containing with 0.02% w/w MeJA for 24 h) and M4 (treatment with 1 g lanolin paste containing 0.04% w/w MeJA for 24 h), were constructed using the mRNA-Seq 8 sample prep Kit (Illumina) following the manufacturer's instructions.

After digestion of DNA with DNase I, 20 µg of total RNAs were purified to obtain the poly(A) mRNA molecules using poly-T oligo-attached magnetic beads. Then, the samples were fragmented into small pieces in a Thermomixer (Eppendorf, German) using divalent cations at 94 °C for 5 min and converted into the first and second-strand cDNA with the SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA). End repair and adenylation of 3' ends were performed on the resulted cDNA. Subsequently, the double stranded cDNA was purified using the QIAquick PCR Purification Kit (QIAGEN). Next, the resulting cDNA fragments were ligated with Illumina paired end adapters and used to generate cDNA libraries. Lastly, the quality of the cDNA libraries was examined by an Agilent Technologies 2100 Bioanalyzer prior to sequencing with Illumina HiSeq 4000 (Illumina Inc., San Diego, CA, USA).

Alignment, gene expression analysis and gene annotation

In order to precisely evaluate the expression levels of genes, all the RNA-seq reads were mapped against the rubber tree sequencing genome recently released (Tang et al. 2016) using TopHat on default settings (Trapnell et al. 2009). The gene expression was quantified as the count of all clean reads mapped to the sequenced genome. The expression levels of each gene were normalized by the fragments per kb per million reads (FPKM) (Mortazavi et al. 2008). The FPKM value was calculated for each protein-coding gene by Cufflinks (<http://cufflinks.cbc.umd.edu>) using default parameters. Differential gene expression between the different samples was determined using Cuffdiff. In this study, we used the $FDR \leq 0.001$ and the ratio (the absolute $\log_2$ -fold change) larger than 2 as the threshold to judge the significance of gene expression differences. $\log_2$ fold changes were re-calculated between C and M2, and between C and M4, using the formula of

$\log_2(M2 + 5)/(C + 5)$ and $\log_2(M4 + 5)/(C + 5)$, respectively. Rubber tree genes were annotated via Blastp against peptide sequences of a minimal set of the TAIR10 release of the Arabidopsis genome (<http://www.arabidopsis.org/>).

Quantitative real-time PCR analysis

Six DEGs such as *JAZ1*, *MYC2*, *OPR3*, *HDR*, *ERF1* and *AUX2* were randomly selected to confirm the DGE results using real time quantitative PCR (qRT-PCR). The RNA samples were extracted as in the RNA sequencing and the qRT-PCR was performed according to the method described by Liu et al. (2015). The reactions of three independent biological replicates were carried out for each sample and expression levels were normalized using *Eukaryotic Initiation Factor 2 (eIF2)* mRNA levels. The relative expressions of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study are listed in Table S1.

Results and discussion

Read assembly, functional annotation and classification of unigenes

Three cDNA libraries were generated with the mRNA from C, M2 and M4 bark tissues of rubber trees and sequenced with the Illumina HiSeq 4000 platform. After preprocessing and quality trimming, a total of 44.92, 44.82 and 45.12 Mb of clean reads were obtained in the C, M2 and M4 sample, respectively. The clean reads are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number of SRP093864. The reads were quantitatively mapped on the rubber tree genome (or against the rubber tree genome) (Tang et al. 2016) with the Tophat software. The 80% sequences of the transcriptome could be aligned to the rubber genome, whereas the percentage of unique-mapped reads were more than 70%, showing that higher-quality transcriptome was obtained (Table S2).

Differential expression analysis of MeJA-responsive genes

To detect genes with differential expression under two MeJA treatments, we compared the transcriptome profiles of M2 and M4 with that of C, respectively. The results showed 1575 up-regulated genes and 1375 down-regulated genes existed in M2 compared with C, and 1301 up-regulated genes and 1549 down-regulated genes were obtained in M4 compared with C. The total number of differentially expressed genes

(DEGs) (2950) between M2 and C is larger than that (2850) of M4 and C. To represent DEGs under MeJA treatments, we generated a heatmap through hierarchical clustering. The heat map reveals a similar pattern of up-regulated gene distribution in M2 and M4 compared with C (Fig. 1). The top 20 most up- and down-regulated genes between C and M2, and C and M4 are shown in Table S3.

Identification of DEGs involved in IPP and rubber biosynthesis

MeJA has been reported to be as an effective elicitor for enhancement of the production of some secondary metabolites in plants (Gundlach et al. 1992; Singh et al. 1998; van der Fits and Memelink 2000; Kim et al. 2004; Zhao et al. 2004; Thanh et al. 2005; Zhao et al. 2010). In rubber tree, jasmonic acid has been suggested to play a role in laticifer differentiation (Hao and Wu 2000; Shi and Tian 2012; Tian et al. 2003). But very limited information about the effect of JAs on rubber biosynthesis is available. Our study showed that the expression of 3-hydroxy-3-

methylglutaryl coenzyme A reductase (HMGR) in MVA pathway was down-regulated by MeJA treatment, but the expression of farnesyl diphosphate synthase (FPS) and cis-prenyltransferase (CPT, or rubber transferase) in rubber biosynthesis pathway were up-regulated by MeJA treatment (Fig. 2). In addition, a systemic up-regulation of key genes by exogenous MeJA in MEP pathway, including genes encoding 1-deoxy-d-xylulose 5-phosphate (DXP) synthase (DXS), DXP reductoisomerase (DXR), 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol (CDP-ME) synthase (CMS), 2C-methyl-d-erythritol 2,4-cyclodiphosphate (MEcPP) synthase (MCS), 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) synthase (HDS) and HMBPP reductase (HDR), was observed (Fig. 2). In fact, main pathways of primary metabolism were also induced by MeJA application. Notably, some genes encoding ribulose biphosphate carboxylase small chain 1A (RBCS1A), phosphofructokinase (PFK), pyruvate kinase (PK) and triose-phosphate isomerase (TPI or TIM), in carbon fixation (Calvin cycle) and glycolysis pathway (the upstream long-range biosynthesis pathway of natural rubber) were also up-regulated by MeJA treatment (Table S4).

HMGR has been reported to play only limiting role in plant IPP biosynthesis of MVA pathway (Rodríguez-Concepción et al. 2013; Vranová et al. 2012). Although HMGR expression could be up-regulated by treatment with MeJA in different species (Schmidt and Baldwin 2006; Wang et al. 2007; Dai et al. 2011; Wang et al. 2014; Spyropoulou et al. 2014; Shi et al. 2015; Sharma et al. 2015), transcriptional suppression or dose-dependent regulation of HMGR expression by MeJA treatment has also been reported (Burnett et al. 1993; Maldonado-Mendoza et al. 1994; Choi et al. 1994). In rubber tree, exogenous applications of JAs could increase latex production in a small-scale field experiment (Duan et al. 2004). But it has been shown that the transcriptional regulation of HMGR seemed not to be important for enhancing latex productivity and the down-regulation of HMGR by MeJA was reminiscent of the case of ethephon stimulation (Zhu and Zhang 2009; Adiwilaga and Kush 1996; Wang et al. 2016; Liu et al. 2016; Liu 2016). However, in a recent study, the expression of HMGR was enhanced with jasmonic acid (JA), linolenic acid (LA) (Loh et al. 2016). The exact reason for this variation is unclear to us, but the different sampling strategies could partly explain the difference. In their study, bark samples were harvested from the young rubber plants (3 layer stage) after 2 months of the treatments with JA and LA. In present study, bark tissues were collected from the mature rubber trees (22-year-old rubber trees with 14 years of tapping) 24 h after MeJA treatments.

Up-regulated genes in MEP pathway included genes encoding the major rate-determining enzymes (DXS and HDR, the first and the last enzyme of the MEP pathway)

Fig. 1 Heatmap of DEGs between M2 and M4, C and M2, and C and M4 based on RNA-seq results. Red and blue colors indicate the up- and down-regulated genes, respectively (color figure online)

Fig. 2 DEGs involved in IPP and rubber biosynthesis in rubber tree bark treated with MeJA. *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *MVA* mevalonate, *MVP* 5-phosphomevalonate, *MVPP* 5-diphosphomevalonate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *FPP* farnesyl diphosphate, *G3P* glyceraldehyde 3-phosphate, *DXP* 1-deoxy-d-xylulose 5-phosphate, *MEP* 2-C-methyl-d-erythritol 4-phosphate, *CDP-ME* 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol, *CDP-MEP* 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol phosphate, *MEcPP* 2C-methyl-d-erythritol 2,4-cyclodiphosphate, *HMBPP* 4-hydroxy-3-methylbut-2-enyl

diphosphate. Enzymes include *AACT* acetoacetyl-CoA thiolase, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MVK* MVA kinase, *PMVK* MVP kinase, *DPMD* MVPP decarboxylase, *IDI* IPP isomerase, *FPS* FPP synthase, *CPT* rubber transferase or cis-prenyltransferase, *DXS* DXP synthase, *DXR* DXP reductoisomerase, *CMS* CDP-ME synthase, *CMK* CDP-ME kinase, *MCS* MEcPP synthase, *HDS* HMBPP synthase; *HDR* HMBPP reductase. Pale pink and pale green cells represent up- and down-regulated DEGs respectively (color figure online)

and other two potential control points (*DXR* and *HDS*) of the metabolic flux to plastidial isoprenoids (Vranová et al. 2012). Additionally, our result is consistent with the increase of transcript abundance of genes such as *DXS*, *DXR*, *MCS* and *HDS* in MEP pathway following elicitation of MeJA (Sun et al. 2013).

The increased expression levels of *FPS* and *CPT* in rubber biosynthesis pathway possibly resulted in an increased latex production in rubber tree treated with MeJA.

Contrary to our results, it has been documented that genes for primary metabolism such as glycolysis and ribulose biphosphate carboxylase/oxygenase (*Rubisco*) in carbon fixation were repressed with MeJA treatment (Schmidt and Baldwin 2006; Jung et al. 2007). Even in barley (*Hordeum vulgare* L.) leaf, a decrease in content and activity of *Rubisco* treated with JA or MeJA were reported (Popova and Vaklinova 1988; Weidhase et al. 1987). Whether the up-regulated expression of the genes with exogenous application of MeJA can cause the enhancement of photosynthetic capacity and glycolytic flux in rubber tree, need to be investigated.

DEGs involved in JA biosynthesis

Two critical genes encoding lipoxygenase 2 (*LOX2*), first enzyme of JA biosynthesis, and 12-oxophytodienoate reductase 3 (*OPR3*), first enzyme of peroxisomal JA biosynthesis, were up-regulated by MeJA treatment (Fig. 3). In addition, the transcript of JA-amino acid synthetase (*JAR1*), the enzyme which catalyses the final step in the production of the bioactive JA compound, were found to be accumulated after MeJA treatment (Fig. 3). The increase of transcript abundance of genes in JA biosynthesis induced by exogenous MeJA is consistent with the self-activation of JA biosynthesis reported in the literature (Sun et al. 2013; Yan et al. 2013; Wasternack 2007; Pauwels et al. 2008; Men et al. 2013). The positive feedback loop is one of important regulations of JA biosynthesis and can be explained by SCF^{COI1}-JAZ regulatory module (Wasternack and Hause 2013).

Fig. 3 DEGs involved in JA biosynthesis pathway in rubber tree bark treated with MeJA. α -LeA a-linolenic acid, 13-HPOT (13S)-hydroperoxyoctadecatrienoic acid, 12,13-EOT 12,13-epoxyoctadecatrienoic acid, OPDA 12-oxophytodienoic acid, OPC-8 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid, JAs jasmonates. The enzymes are LOX2 lipoxygenase 2, AOC allene oxide cyclase, OPR3 12-oxophytodienoate reductase 3, JAR1 JA-amino acid synthetase. Pale pink and pale green cells represent up- and down-regulated DEGs respectively (color figure online)

DEGs of hormone signaling components and transcription factors

After MeJA application, not only the genes encoding JA signaling components such as JASMONATE ZIM DOMAIN (JAZ) and MYC2 were differentially expressed, but also the genes encoding signaling components of other major plant hormones such as auxin, cytokinin (CK), ethylene (ET), abscisic acid (ABA), brassinosteroid (BR), salicylic acid (SA) and gibberellin (GA) were up- or down-regulated (Table S5). Notably, MYC2, the most prominent JAZ-interacting transcription factor and a master switch in JA signaling which regulates the majority of JA responses (Wasternack and Hause 2013; Kazan and Manners 2013), were shown to be up-regulated. Some genes encoding the components involved in the cross-talk between JA and other hormones were obviously up-regulated, such as ETHYLENE RESPONSE FACTOR 1 (ERF1) and ETHYLENE INSENSITIVE 3 (EIN3), which are required for the both JA-ET synergistic and antagonistic interaction (Zhu 2014; Zhu et al. 2011; Zhang et al. 2012; Song et al. 2014a, b; Zhang et al. 2014), were found to be up-regulated. NONEXPRESSOR OF PR GENES1 (NPR1), the

central regulator in SA signaling and an essential factor which mediates the antagonistic interaction between JA and SA (Spoel et al. 2003; Dong 2004; Leon-Reyes et al. 2009), was observed to be up-regulated in M2 but down-regulated in M4 compared with C. The transcripts of ABA receptor PYR/PYL proteins which are linked to ABA-JA cross-talk (Lackman et al. 2011; Aleman et al. 2016), and ARF and IAA proteins which are related to JA-auxin (Nagpal et al. 2005; Tabata et al. 2010; Reeves et al. 2012), showed enhancement after MeJA treatment. It seems that the output of JA signaling depends on the complex cross-talk between JAs and other hormones, and ultimately mediates transcription regulation of secondary metabolic pathways (Pauwels et al. 2009).

JAs regulate a wide variety of biological processes in plants via triggering a transcription cascade (Pauwels et al. 2009). In our study, a large number of DEGs of putative TFs were identified (Table S6). We found that gene expression of many TFs of different families shown to be as regulators of the biosynthesis of different groups of secondary metabolites such as AP2/ERF (APETALA2/Ethylene-Response Factors), bHLH (basic Helix-Loop-Helix), MYB, and WRKY were induced by MeJA treatment, consistent with the previous studies (De Geyter et al. 2012; Zhou and Memelink 2016; Chezem and Clay 2016). However, their detailed regulatory role and mode in IPP and rubber biosynthesis remained to be solved. The real time quantitative PCR (qRT-PCR) results validated the authenticity of the expression patterns obtained in this transcriptome analysis (Fig. 4).

Conclusions

Our results suggested that activation of the expression of rubber biosynthesis genes such as FPS and CPT possibly leads to increase in latex production in rubber tree with MeJA application. MeJA induced up-regulation of key genes in glycolysis and carbon fixation (Calvin cycle) pathway might also play an important role in the stimulation of latex production. Elevation of transcript levels of critical genes in JA biosynthesis revealed that a JA autoregulatory loop existed. Identification of MeJA-responsive components in cross-talk between JA and other hormone signaling, and MeJA-responsive TFs demonstrated a complex control of MeJA-mediated downstream processes. Overall, our transcriptome data provides a valuable start point to identifying key JA-responsive TFs and enzymes that control the metabolic flow through the biosynthetic pathway of IPP and rubber, and can serve as a resource for unraveling the molecular basis of MeJA regulation of other cellular processes. For example, besides injuries inflicted by plant-eating organisms and typhoon,

Fig. 4 qRT-PCR validation of six DEGs involved in *H. brasiliensis* bark tissues treated with MeJA compared with the control. The axis indicates treatments; the y-axis indicates relative expression level. **a** *JAZ1* **b** *MYC2*; **c** *OPR3*; **d** *HDR*; **e** *ERF1*; **f** *AUX2*

tapping represents a routine injury of rubber tree. JA, a wound hormone, mediate systemic wound responses which depend on large-scale changes in gene expression (Koo and Howe 2009).

Acknowledgements We would like to thank Prof. Tang and Dr. Fang (Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences) for their support with the genome sequencing data of rubber tree. This work was funded by the National Natural Science Foundation of China (No. 31560573) and the Hainan Province Major Science and Technology Project (ZDZX2013023).

Authors contributions J.P.L. supervised the experiments and wrote the manuscript. J. H. conducted the bioinformatics studies and carried out qRT-PCR validation. L.S.Z. supervised the he bioinformatics studies and revised the paper. Y.H.L. participated in the bioinformatics studies. C.P.Y. and Y.F.Z. participated in the qRT-PCR validation. X.L.G. and Y.J.L. participated in collection of plant materials. All the authors read and approved the manuscript for publication.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

Ethical approval This study was conducted according to compliance with ethical standards. This study does not involve the use of any human, animal and endangered or protected plant species as materials.

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