Jasmonate-regulated ERF109-MYB51-MYC3 ternary complexes control indolic glucosinolates biosynthesis

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One-sentence summary: The JA-responsive ERF109-MYB51-MYC3 ternary complex controls JAs-regulated GLSs biosynthesis.

AUTHOR CONTRIBUTIONS

M.Z. conceived and supervised the research. K.Z., Y.M., J.L., M.D., Q.L. and X.L. conducted the experiments. Y.M., J.L. and M.K. analyzed the data. K.Z.,Y.M., J.L. M.K. and M.Z. wrote the paper.

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Summary:

Jasmonates (JAs) are plant hormones which regulate biosynthesis of many secondary metabolites, such as glucosinolates (GLSs), through JAs-responsive transcription factors (TFs). The JAs-responsive *CYP83B1* gene, has been shown to catalyze the conversion of indole-3-acetaldoxime (IAOx) to indolic glucosinolates (IGLSs). However, little is known about the regulatory mechanism of *CYP83B1* gene expression by JAs. In yeast one-hybrid screens using the *CYP83B1* promoter as bait we isolated two JAs-responsive TFs ERF109 and MYB51 that are involved in JAs-regulated IGLS biosynthesis. Furthermore, using a yeast two-hybrid assay, we identified ERF109 as an interacting partner of MYB51, and Jasmonate ZIM-domain (JAZ) proteins as interactors of MYB51, and BTB/POZ-MATH (BPM) proteins as interactors of ERF109. Both JAZ and BPM proteins are necessary for the full repression of the ERF109-MYB51-MYC3 ternary complex activity on *CYP83B1* gene expression and JA-regulated IGLS biosynthesis. Biochemical analysis showed that the 26S proteasome-mediated degradation of ERF109 protein is mediated by a $CRL3^{BPM}$ E3 ligase independently of JA signaling. Genetic and physiological evidence shows that MYB51 acts as an adaptor and activator to bridge the interaction with the co-activators MYC3 and ERF109, for synergistically activating the *CYP83B1* gene expression, and all three factors are essential and exert a coordinated control in JAs-induced IGLS biosynthesis. Overall, this study provides insights into the molecular mechanisms of JAs-responsive ERF109-MYB51-MYC3 ternary complexes in controlling JAs-regulated GLSs biosynthesis, which provides a better understanding of plant secondary metabolism.

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INTRODUCTION

Plants produce an enormous diversity of secondary metabolites. Not only These compounds mediate the interaction with biotic and abiotic environmental factors but also determine the food quality of crop plants. Glucosinolates (GLSs) are a group of plant secondary metabolites recognized for their distinctive benefits to human nutrition and plant defense. Unlike major classes of plant secondary metabolites, GLSs are largely limited to species of the order Brassicales, including important crops (e.g., broccoli, oilseed rape and cabbage) as well as the model plant *Arabidopsis thaliana* (Kliebenstein et al., 2005). In the GLS-defining core structure (the glucone), there has extensive side-chain modification and amino acid elongation which are responsible for the variation of its chemical structures (Grubb and Abel, 2006). Myrosinases, the enzymes responsible for GLS breakdown, co-exist with GLSs, are attacked by chewing herbivores, GLSs are hydrolysed by myrosinases into different unstable intermediates, leading to the rapid generation of the toxic compounds like thiocyanates, isothiocyanates, or nitriles, which play important roles in defense responses against pathogens and generalist herbivores, and in controlling soil-borne pathogens (Barth and Jander, 2006; Martínez-Ballesta et al., 2013). In addition, these final metabolites, such as isothiocyanates, influence the potential utility of plants,

by accounting for the distinctive flavors and exhibiting anticarcinogenic and antitumoral properties (Keck and Finley, 2004; Dinkova-Kostova and Kostov, 2012). Therefore, to rationally improve crop quality by engineering of GLS content, it is necessary to explore the molecular mechanism of GLS metabolism and regulatory network.

The biosynthesis of GLS has three different stages: 1) side-chain elongation of amino acids, 2) formation of the core GLS structure, and 3) secondary side-chain modifications (Sotelo et al., 2016). Based on the nature of the source amino acid, there are three different types of GLSs, aliphatic, indole and aromatic GLSs (Sotelo et al., 2016). During the chain elongation step, aliphatic undergo four successive reactions catalyzed by branched-chain amino acid aminotransferase (BCATs), methylthioalkylmalate synthase (MAMs), isopropylmalate isomerase (IPMIs) and isopropylmalate dehydrogenase (IPMDHs) (Ishida et al., 2014; Robin et al., 2016). Five cytochrome P450 monooxygenases 79 (CYP79), CYP79A2, CYP79B2 and CYP79B3, CYP79F1 and CYP79F2 have been shown to form the core GLS structure by catalyzing the conversion of amino acids chain-elongated methionines into aldoximes. These are further oxidized by CYP83A1 and CYP83B1 to form *S*-alkylthiohydroximates, and then cleaved by carbon–sulfur lyase (SUR1) which is further catalyzed into GLS structure by glutathione *S*-glucosyltransferases (GSTF), uridine diphosphate glycosyltransferase 74 (UGT74) and sulfotransferases (SOT) (Ishida et al., 2014; Robin et al., 2016). In last step of side chain modifications, three types of GLSs are oxygenated by three different enzymes. *S*-oxygenation of aliphatic GLSs (AGLSs) is carried out by a flavin monooxygenase (FMOGS-OXs) and the oxidation of indole GLSs (IGLSs) is catalyzed by CYP81F (Sønderby et al., 2010). Modified IGLSs originate from indole-3-ylmethyl-GLS (I3M), then the end products 4-methoxy-I3M (4MO-I3M) and 1-methoxy-I3M (1MO-I3M) are synthesized *via* hydroxylation and methylation of I3M (Pfalz et al., 2009, 2011).

During the last few years, many efforts have been made to discover the transcription factors (TFs) that regulate GLSs biosynthesis in Arabidopsis. Many reports show that TFs representing the subgroup 12 of R2R3-MYBs are mainly involved in the transcriptional regulation of GLS biosynthesis (Sánchez-Pujante et al., 2017). Furthermore, it has been reported that MYB28, MYB29, and MYB76 TFs are involved in the transcriptional regulation of AGLSs (Sønderby et al., 2007, 2010; Malitsky et al., 2008), while MYB34, MYB51, and MYB122 TFs are involved in the transcriptional regulation of IGLSs (Gigolashvili et al., 2009, Frerigmann and Gigolashvili, 2014). Then again, other types of TFs, such as IQD1 and Dof1.1, are known to regulate the biosynthesis of both AGLSs and IGLSs (Mikkelsen et al., 2003; Mewis et al., 2005; Kliebenstein et al., 2002; Brader et al., 2001; Skirycz et al., 2006). In general, the production of many kinds of secondary metabolites is induced by the plant stress hormones, jasmonates (JAs). The regulation of JA response occurs largely at the level of gene expression and protein accumulation (Zhou and Memelink, 2016). Several studies on different plant species indicated that JA induces the production of IGLSs and AGLSs, but not aromatic GLSs (Bodnaryk et al., 1994; Doughty et al., 1995; Brader et al., 2001; Mikkelsen et al., 2003; Sasaki-Sekimoto et al., 2005; Liu et al., 2010). It has been shown that MYB34, MYB122 and MYB29 plays key role in JA-mediated responses by inducing a set of genes involved in both AGLS and IGLS biosynthetic pathway (Hirai et al., 2007; Frerigmann and Gigolashvili, 2014). Interestingly, three JA-responsive basic helix-loop-helix (bHLH) TFs MYC2, MYC3 (bHLH05), and MYC4, not only directly activate the expression of GLS biosynthesis genes, but also as co-activators interact directly with GLS-related MYB TFs

(Schweizer et al., 2013; Frerigmann et al., 2014), indicating that JA signaling module is essential for GLSs biosynthesis.

Combinatorial interactions among TFs are necessary to control gene expression in response to a variety of environmental signals, which will benefit the plant without being too costly. Indole-3-acetaldoxime (IAOx) is a key branching point between IGLSs and other metabolites (such as Auxin and camalexin) in Arabidopsis. Both CYP83B1 and CYP83A1CYP83A1, the first key enzymes have been shown to catalyze the conversion of IAOx to IGLSs (Bak et al., 2001). MeJA treatment induces the expression of *CYP83B1* gene, but not *CYP83A11* (Mikkelsen et al., 2003) and triggers GLS accumulation (Liu et al., 2010). However, TFs regulating the *CYP83B1* gene expression is largely unknown. Scanning of promoter sequence motif suggests that the *CYP83B1* genes is regulated by one or more upstream TFs. This study was designed to search for such TFs. Using the fragments of the *CYP83B1* promoter as bait in a yeast one-hybrid (Y1H) screening, two TFs AP2/ETHYLENE RESPONSE FACTOR 109 (ERF109) and GLS-related MYB51 turned out to be interacting with the promoter, and both of them JA-responsive TFs. Furthermore, protein-protein interaction assays indicated that ERF109 interacts directly with MYB51. This specific ERF-MYB interaction is coordinately regulated under JA signaling pathway, and plays a crucial role in the transcriptional regulation of *CYP83B1* gene and GLSs biosynthesis.

RESULTS

ERF109 and MYB51 directly activate the *CYP83B1* promoter

To check whether *CYP83B1* and *CYP83A1* respond differentially to the upstream signaling molecule MeJA, we first analysed the expression of these two genes in wild-type (WT) Arabidopsis plants treated with MeJA. As shown in **Supplemental Fig. S1**, MeJA caused a significant induction of the expression of *CYP83B1*, but not of *CYP83A1*, which is consistent with a previous report (Mikkelsen et al., 2003). To identify more factors binding to the *CYP83B1* promoter, a yeast one-hybrid (Y1H) assay was performed using the various *CYP83B1* promoter fragments (**Supplemental Fig. S2**) as bait to screen a JA-treated Arabidopsis cDNA library (Li et al., 2018). Screening of the library with the fragment I (-19 bp to -214 bp) of the *CYP83B1* promoter gives positive colonies with yielding clones i.e. pACT/I-3 (At4g34410, ERF109) and pACT/I-6 (At1g18570, MYB51). However, the fragment II, III and IV gives no positive colony. To confirm the direct interaction between either ERF109 or MYB51 and the *CYP83B1* promoter, Electrophoresis mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP) quantitative PCR were performed. As shown in **Fig. 1a**, ERF109, MYB51 were able to bind to *CYP83B1* promoter fragment I. Binding of ERF109 and MYB51 were completely abolished when the ERF109 binding site GTCCACCT at positions -163 to -170 and the MYB51 binding site ACCAACC at the position -95 to -101 were mutated to GGGGGGGG and CCCCCCC, respectively. Furthermore, a ChIP quantitative PCR assay showed that ERF109-HA and MYB51-HA fusion proteins binds to the *CYP83B1* promoter (**Fig. 1b and c**). These results confirmed that ERF109 and MYB51 bind directly to the promoter of the *CYP83B1* gene.

To study the trans-activation of the *CYP83B1* promoter by ERF109 and MYB51, Arabidopsis protoplasts were co-transformed with the reporter plasmid consisting of the *CYP83B1* promoter (-19 to -889) fused to GUS (*CYP83B1FLpro*-GUS), and the effector plasmids carrying either of the *ERF109* and *MYB51* genes. ERF109 and MYB51 were found to activate the

CYP83B1FLpro-GUS reporter gene 5 or 3 folds respectively, whereas the IGLS-associated MYB TFs MYB34 and MYB122 (Frerigmann and Gigolashvili, 2014), as a control reporter construct of CYP83A1 promoter (-9 to -1019) was used which had no effect (**Fig. 1d** and **Supplemental Fig. S3**). These results indicates that ERF109 and MYB51 interacts and activates the *CYP83B1* promoter.

ERF109 interacts directly with MYB51, but not MYC2/3/4

The IGLS-associated TF MYB51 was previously shown to interact with MYC3, and the related bHLH TFs MYC2, MYC4 and bHLH28 (Schweizer et al., 2013; Frerigmann et al., 2014). Since ERF109, like MYB51, acts as a transcriptional activator in JA-responsive *CYP83B1* gene expression, we decided to perform a yeast two-hybrid (Y2H) assay to test whether MYB51 protein could interact with ERF109. MYC2/3/4 were also tested as positive controls. As shown in **Fig. 2a**, MYB51 interacts with MYC2/3/4 in consistence with previous reports (Schweizer et al., 2013; Frerigmann et al., 2014). Also, we saw that the MYB51–MYC3 interaction is stronger than MYB51–MYC2/4 interaction. Interestingly, MYB51, but not MYB34, interacts with ERF109 (**Fig. 2a**), and this interaction depends on the C-terminus of MYB51 (MYB51CT) (**Supplemental Fig. S4**). However, ERF109 did not interact with MYC2/3/4 (**Supplemental Fig. S5**). To verify the interactions between MYB51 and ERF109 *in planta*, we performed bimolecular fluorescence complementation (BiFC) assays in Arabidopsis protoplasts. As shown in **Fig. 2b**, strong YFP signals were observed in Arabidopsis protoplasts upon co-expression of MYB51-cYFP and nYFP-ERF109. A similar result was observed upon co-expression of MYB51-cYFP with nYFP-MYC3 as a positive control. None or only background YFP fluorescence was detected upon co-expression of ERF109-cYFP with nYFP-MYC3 and in negative controls (nYFP-ERF109 or nYFP-MYC3 co-expressed with cYFP, and nYFP co-expressed with MYB51-cYFP). These results indicated that the ERF109 interacts with MYB51, but not MYC3. To verify the protein–protein interactions *in vitro*, we performed pull-down assays using HA- and Strep-tagged proteins (**Fig. 2c**). As shown in **Fig. 2c**, Strep-MYB51 interacts with either HA-ERF109 and HA-MYC3. These data are consistent with the results from yeast two-hybrid and BiFC assays.

Combinatorial ERF109-MYB51-MYC3 interactions synergistically activate *CYP83B1* gene expression

Binding of MYB51 to MYC2/3/4 on the one hand, and to ERF109 on the other hand, supported an adaptor role for MYB51. To show that the proteins can form a multimeric complex *in vivo*, we set up a yeast three-hybrid bridge (Y3H) assay, where MYC2/3/4, fused to the GAL4 AD, was co-transformed with ERF109 fused to the GAL4 BD and unfused MYB51 or MYB34. The results from the colony-lift filter β-galactosidase assay, show that MYB51, but not MYB34, was able to function as an adaptor bridging the interaction between ERF109 and MYC2/3/4 (**Fig. 3a and Supplemental Fig. S6**).

To functionally test the interaction between ERF109, MYB51 and MYC3, protoplasts trans-activation assays were performed. Arabidopsis protoplasts were co-transformed with the identical amounts of over expression effector plasmids carrying ERF109, MYB51 or MYC3 and *CYP83B1FLpro*-GUS reporter construct. The results show that ERF109, MYB51 and MYC3 act synergistically instead of additively (**Fig. 3b**), indicating that they act dependently *via* interaction with the different target sites. MYC3 plasmid had no positive effect on ERF109 activity, an

additional positive effect was observed in the presence of MYB51 (**Fig. 3b**), suggesting that a ternary complex between ERF109, MYB51 and MYC3 is necessary for full activation. Co-expression of a C-terminal deletion derivative of MYB51 (MYB51NT, 1-117AA) did not lead to an additional positive effect on ERF109 activity, consistent with its inability to interact with both MYC3 (Schweizer et al., 2013) and ERF109 proteins in yeast, suggesting that MYC3-ERF109 exerts its additive effect *via* interaction with MYB51. To study whether MYB51 acts *via* the binding site ACCAACC at positons -95 to -101, we mutated it generating the *mMBSCYP83B1FLpro*-GUS derivative. This mutation abolished GUS activity conferred by MYB51 (**Fig. 3c**), indicating that ACCAACC is the key site interacting with MYB51. Taken together, our experiments show that MYB51 acts as an adaptor and activator, bridge the interaction with the co-activators MYC3 and ERF109 for synergistically activating the *CYP83B1* gene expression.

The ERF109-MYB51-MYC3 factors are involved in jasmonate-induced IGLS biosynthesis

It was previously shown that the MYB51 and MYC3 are involved in JAs-induced IGLS accumulation (Schweizer et al., 2013; Frerigmann et al., 2014). To study further the role of *ERF109* gene in the biosynthesis of IGLS, homozygous loss-of-function alleles in *ERF109* (SALK_150614, *erf109*) and over-expression lines of *ERF109* gene (CS2102255, OE^{ERF109}) were analyzed. Compared with the WT plants, the *erf109* and OE^{ERF109} lines showed no visible effects in plant morphology under the given growth conditions (Bahieldin et al., 2018). The level of IGLSs (I3M, 4MO-I3M, and 1MO-I3M) in the OE^{ERF109} lines was higher compared to corresponding WT lines, under continuous light, without JA treatment (**Fig. 4a**). Interestingly, the much lower levels of all IGLSs in the *erf109* mutant, treated with MeJA under continuous light, together with the much higher levels of all IGLSs in OE^{ERF109} plants, compared with the WT (**Fig. 4a**), suggest a positive role of ERF109 in IGLSs regulation following environmental challenge.

To analyze whether the *ERF109*, *MYB51* and *MYC3* expression correlates with the level of IGLS biosynthesis and to reveal the contribution of each of the three TFs in IGLs biosynthesis, IGLS contents were analyzed in *erf109*, *myb51* (GK-228B12), *myc3* (GK445B11), *erf109myb51*, *myb51myc3*, *erf109myc3*, and *erf109myb51myc3* mutants. These mutants were analyzed for the accumulation of IGLS (**Fig. 4b-d**). As shown in **Fig. 4b-d**, IGLS biosynthesis were not affected in either of the single mutants and neither the *erf109myb51* and *erf109myc3* double mutant lines compared with the WT without JA treatment. Consistent with a previous report (Frerigmann et al., 2014), the concentration of IGLSs in the double *myb51myc3* mutant was substantially lower than that of single mutants and WT (**Fig. 4b-d**). Moreover, the IGLSs levels in the triple *erf109myb51myc3* mutant seedlings were significantly lower than that of the double *myb51myc3* mutant (**Fig. 4b-d**), especially upon JA treatment, suggesting the importance of ERF109 in the combinatorial regulation of IGLSs together with MYB51 and MYC3.

Subsequently, qRT-PCR was performed to investigate the expression patterns of six IGLSs biosynthesis genes (*CYP79B3*, *CYP83B1*, *CYP83A1*, *GSTF10*, *SUR1* and *UGT74B1*) in all lines with and without MeJA treatment. As expected, expression levels of these five IGLS genes (except *CYP83A1*) were very similar in the all single mutant, the double *erf109myb51* and *erf109myc3* mutant lines as compared to WT, contrasting with their significantly reduced expression in the double *myb51myc3* mutant and the triple *erf109myb51myc3* mutant seedlings under the non-JA treatment (**Fig. 4e and Supplemental Fig. S7**). However, lower expression levels of these five

IGLS genes in all mutant lines, especially in triple mutant, upon MeJA treatment compared with the non-MeJA treatment (**Fig. 4e and Supplemental Fig. S7**), indicated that the ERF109-MYB51-MYC3 complex has a role in the JA-mediated induction of IGLSs.

MYB51 interacts with the JA repressor JAZ10

Most JAZ proteins were shown to interact with many TFs with a variety of different roles in regulating JAs-associated gene expression (Zhou and Memelink, 2016). These TFs include the bHLH TFs MYC2, MYC3 and MYC4 (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011), and R2R3-MYB TFs MYB21 and MYB24 (Song et al., 2011). Since MYB51 acts as a transcriptional activator in JAs-responsive *CYP83B1* gene expression, we performed Y2H assay to test whether MYB51 protein could interact with JAZ proteins or not. We used MYC3 as a positive control and MYB34 as a negative control. As shown in **Fig. 5a**, MYB34 cannot interact with any JAZ protein, but MYC3 interacted with all JAZ proteins except JAZ4 and JAZ7. This was consistent with previous reports (Chini et al., 2009; Fernández-Calvo et al., 2011). Interestingly, MYB51 only interacted with JAZ10. To investigate which domain of MYB51 is responsible for the interaction with JAZ10, we constructed plasmids with N-terminal parts (MYB51NT) containing the R2R3 domain and the C-terminal parts (MYB51CT), the JAZ10 N-terminal part (JAZ10NT) and C-terminal part (JAZ10CT). As shown in **Supplemental Fig. S8**, the N-terminal parts of the MYB51 protein was responsible for the interactions with JAZ10CT in yeast.

To verify the interactions between MYB51 and JAZ10 *in planta*, we performed BiFC assays in Arabidopsis protoplasts. As shown in **Fig. 5b**, strong YFP signals were observed upon co-expression of MYB51-cYFP with nYFP-JAZ10, MYB51NT-cYFP with nYFP-JAZ10, and MYB51-cYFP with nYFP-JAZ10CT, respectively. A similar result was observed upon co-expression of nYFP-MYC3 with JAZ10-cYFP as a positive control. None or only background YFP fluorescence was detected in negative controls (MYB51-cYFP co-expressed with nYFP or cYFP co-expressed with nYFP-JAZ10). To verify the protein–protein interaction *in vitro*, we performed pull-down assays using HA- and Strep-tagged proteins (**Fig. 5c**). As shown in **Fig. 5c**, Strep-MYB51 interacted with HA-JAZ10 but not HA-JAZ10NT. These results indicated that the MYB51 protein interacts with JAZ10 protein *in vitro* and *in vivo*.

ERF109 interacts with BPM family proteins, is degraded by the 26S proteasome and is stabilized under JA treatment

To identify proteins that interact with ERF109, Y2H screenings of an Arabidopsis seedling cDNA library were performed. Expression of full-length ERF109 fused to the GAL4 BD showed weak auto-activation that could be suppressed by the addition of 15 mM 3-AT in the medium. This resulted in the isolation 9 colonies that were able to grow on minimal medium lacking histidine. From these candidate ERF109 interactors, only one, cDNA sequence (9# clone), was in frame with the GAL4 AD. This plasmid contained a partial cDNA encoding the protein named BPM1 (At5g19000), lacking the last 263 amino acids (**Fig. 6a**), demonstrating that this region is not essential for assembly with ERF109 protein. BPM1 consists of 442 amino acids and contains a BTB domain (203-347AA) in the middle of the protein and a MATH domain (38-150 AA) in the N-terminal region. It has been reported that BPM1 belongs to the BTB/POZ family, that includes six members, BPM1 to BPM6 (Weber et al., 2005). This suggested that ERF109 could interact with other BPM proteins. We could indeed confirm a ERF109-BPM3 interaction by Y2H assays

(**Supplemental Fig. S9**). Furthermore, the partial BPM1 (1-179 AA) in the Y2H screens demonstrated that the BTB domain is not involved in the interaction with ERF109. To further confirm that a full-length MATH domain is sufficient for binding to ERF109, we generated a new truncated BPM1 version of 120 amino acids (35-154 AA, BPM1-MATH) comprised of complete MATH domain. As shown in **Supplemental Fig. S9**, BPM1-MATH is capable of binding to ERF109, making it highly probable that only the MATH domain is required for BPM1-ERF109 interaction. Likewise, we sought to determine which ERF109 region mediates the interaction with BPM1 protein. As shown in **Supplemental Fig. S10**, the N-terminal region of ERF109 was responsible for the interactions with BPM1. To further confirm the interaction of ERF109 with BPM1, BiFC assays were performed in Arabidopsis protoplasts. Interaction between ERF109 and BPM1 but also ERF109 and BPM1-MATH was visible in the nucleus (**Fig. 6b**). These results indicated that *in planta* ERF109 interacts with BPM1 in the nucleus.

It has been previously shown that the BTB / POZ family proteins could interact with CUL3 proteins, which act as scaffolding subunits of multimeric E3-ligases that can target their substrates for degradation *via* the 26S proteasome (Weber et al., 2005). Since ERF109 acts as an interactor of BPM1 and is responsive to JA on gene expression level, we investigated its stability in protoplasts was associated with the 26S proteasome in JA dependent manner. A GFP-tagged ERF109 protein or just GFP was expressed in Arabidopsis protoplasts treated with JA, MG132, or the solvent DMSO. As a result, the C-terminal fusion of ERF109 with GFP was found in the nucleus of Arabidopsis protoplasts, and fluorescence microscopy showed that MG132 and JA, but not DMSO treatment, increased the abundance of ERF109-GFP in protoplasts (**Fig. 6c**). As shown in **Fig. 6d**, immunoblot analysis with anti-GFP antibodies of the total cellular protein revealed that JA or MG132 treatment drastically increased the amount of ERF109-GFP protein, while the amount of GFP as a negative control was not affected. In addition, the accumulated ERF109-GFP protein was observed in the leaf protoplasts of *bpm1bpm3* double mutant, but not in the single mutants and WT (**Fig. 6e**). These results indicated that ERF109 protein is subjected to 26S proteasome-mediated degradation through binding with BPM proteins and JA, stabilizes ERF109 protein post-translationally.

BPM and JAZ repress the activity of ERF109-MYB51-MYC3 Ternary Complexes

In order to elucidate the functional significance of the interaction between BPM proteins and ERF109, JAZ proteins and MYB51, trans-activation assays were performed. Co-transformation of Arabidopsis protoplasts with a *CYP83B1FLpro*-GUS reporter construct and effector plasmids CaMV35S-ERF109, CaMV35S-MYB51 or CaMV35S-MYC3 resulted in strong activation of around 5-fold, 3-fold and 3-fold, respectively (**Fig. 3b** and **Supplemental Fig. S11a**). Addition of the JAZ10 effector plasmid resulted in the repression of MYB51 activity, and did not have a significant effect on the activity of ERF109, whereas the known MYC3 repressor JAZ10 had a strong repressive effect. In contrast to the effect of JAZ10, BPM1 significantly repressed the activity of ERF109 only, and did not affect either MYB51 or MYC3 activity (**Supplemental Fig. S12a**). These results are line with the above protein-protein interaction assays where JAZ10 was found to interact with MYB51 and MYC3, and BPM1 with ERF109. Furthermore, co-expression of JAZ10 or BPM1 with *CYP83B1FLpro*-GUS, ERF109, MYB51, and MYC3 dramatically reduced the GUS activity (**Supplemental Fig. S12a**), demonstrating that JAZ10 and BPM1 represses the transcriptional function of ERF109-MYB51-MYC3 **ternary complexes**. However,

the higher amounts of JAZ10 or BPM1 plasmid had negative effect on *CYP83B1FLpro*-GUS activity (**Supplemental Fig. S12**). To investigate further, whether accumulation of endogenous MYB51 or ERF109 proteins could mediate the repression of JAZ10 or BPM1 on endogenous *CYP83B1* gene expression, we examined the expression of *CYP83B1* in the Arabidopsis *myb51*, *erf109*, *erf109myb51myc3* mutant and WT leaf protoplasts with over-expression of JAZ10 or BPM1. As shown in **Supplemental Fig. S12b**, the negative effect of JAZ10 or BPM1 over-expression on *CYP83B1* gene expression was not observed in the *myb51* or *erf109* mutant, respectively, as compared to the WT, indicating that the negative effect of JAZ10 or BPM1 alone was mediated by endogenous MYB51 or ERF109 proteins serving as adaptors. In addition, over-expression of *JAZ10* or *BPM1* gene in Arabidopsis plants significantly decreased IGLS accumulation (**Supplemental Fig. S13**). Taken together, both JAZ10 and BPM1 are necessary for full repression of ERF109-MYB51-MYC3 **ternary complexes** at *CYP83B1* gene expression and IGLS biosynthesis level.

DISCUSSION

JAs, as the key signaling molecules, induce the expression of genes that are responsible for the biosynthesis of various secondary metabolites, such as GLS (Zhou and Memelink, 2016). JAs signaling results in the activation of TFs which regulate gene expression through binding to *cis*-acting elements of their target genes. Until now, several types of JAs-induced TFs that participate in the regulation of GLS biosynthesis have been characterized. These belong to the DOF, bHLH and MYB families (Skirycz et al., 2006; Sønderby et al., 2007, 2010; Malitsky et al., 2008; Gigolashvili et al., 2009, Frerigmann and Gigolashvili, 2014). However, our understanding about the molecular mechanisms of these TFs complexes in the regulation of GLSs biosynthesis is still lacking. In this study, we identified ERF109, an AP2/ERF (APETALA2/Ethylene-Response Factors), also called RRTF1 (REDOX RESPONSIVE TRANSCRIPTION FACTOR1), as an important regulator for IGLS biosynthesis. *ERF109* is highly responsive to MeJA and induces reactive oxygen species (ROS) accumulation (Cai et al., 2014; Matsuo et al., 2015). *CYP83B1* is the key enzyme which catalyzes the conversion of IAOx to IGLSs, importantly, this gene is strongly induced upon JAs signaling (Bak et al., 2001; Mikkelsen et al., 2003). Using the *CYP83B1* promoter as bait in a Y1H screening, two JAs-responsive TFs, ERF109 and MYB51, were identified (Schweizer et al., 2013; Cai et al., 2014). Further in a Y2H assay, we identified two BPMs as interacting partners of ERF109, and JAZ10 as an interacting partner of MYB51. Previous report shows that MYC2/3/4 are interacting partners of MYB34/51, and mediate the transcriptional regulation of IGLS biosynthesis (Schweizer et al., 2013; Frerigmann et al., 2014). In addition, there is high redundancy between MYC2/3/4 and MYB34/MYB51 in the regulation of IGLSs (Schweizer et al., 2013; Frerigmann et al., 2014). Thus, these specific JAs-responsive ERF-MYB-bHLH interactions play an essential role in the transcriptional activation of the *CYP83B1* gene and the production of IGLSs in plants.

Gene expression analysis of the triple *erf109myb51myc3* mutant revealed that IGLS biosynthetic genes are down-regulated, and thus the production of IGLSs impaired (**Fig. 4b-d**), indicating the important roles of ERF109, MYB51, and MYC3 in the regulation of IGLS biosynthesis. In contrast with the IGLS profile of the triple *erf109myb51myc3* mutant, the single mutants and *erf109myb51*, *erf109myc3* double mutants show similar IGLS levels compared to WT plants, indicating functional redundancy of ERF109. However, unlike the *erf109myb51* and *erf109myc3*

double mutants, the production of IGLSs was significantly affected in double *myb51myc3* mutant (**Fig. 4b-d**), pointing to an essential role of MYB51 and MYC3 in the production of IGLSs in the absence of JA. Based on this observation, it can be assumed that mainly MYC2/3/4 and MYB51 and secondarily ERF109 are important in JA inducible *CYP83B1* gene regulation.

The expression of a gene is determined by the *cis*-acting DNA elements located in the vicinity of the gene and the trans-acting TFs that interact with them. Until now, several *cis*-acting elements in various gene promoters that mediate JA responsiveness have been identified. The most common *cis*-acting elements in JAs-responsive promoter sequences are the G-box (CACGTG) or G-box-like sequences (e.g. CACCTG) (Zhou and Memelink, 2016). In this study, the promoter motif analysis of *CYP83B1* gene showed that a G-box-like sequence CACCTG is present at the position -120 to -125. It has been previously reported that AC-rich sequences, such as ACC[A/T]A[A/C][T/C] and ACC[A/T][A/C/T][A/C/T], are typical motifs important for binding R2R3-MYB TFs (Prouse and Campbell, 2012; Chezem and Clay, 2016). Here, we showed that MYB51 directly binds to the AC-rich sequence ACCAACC at the position -95 to -101, similarly to the way that ZmMYB31 binds the maize lignin gene promoters containing the ACC(T/A)ACC consensus sequence (Fornalé et al., 2010). The AP2/ERF family is a large group of TFs that contain AP2/ERF-type DNA binding domains, and can bind different *cis*-elements such as a GCC-box and a DRE element (Mizoi et al., 2012). Here, we show that ERF109 binds the GTCCACCT element at the position -163 to -170, this element includes a CCAC motif which is very similar to the coupling-element 1 (CE1)-like sequence recognized by the AP2/ERF TF ABI4 (ABA-INSENSITIVE 4) (Mizoi et al., 2012). In the *CYP83B1* gene promoter, three binding motifs were located in the 200 nucleotides upstream of the start codon ATG, which may allow complex regulation of gene expression in response to multiple changes in external and internal environments.

Gene regulation of plant's secondary metabolism typically involves formation multiprotein complexes through combinatorial interactions between different families or subfamilies of TF protein. For example, the interactions between MYB, bHLH and WD40 domain proteins form the MYB-bHLH-WD40 (MBW) complexes that can activate a set of biosynthetic genes in anthocyanin biosynthesis (Quattrocchio et al., 2006; Zhou and Memelink, 2016). In this ternary complex, the MYB protein recognizes its *cis*-regulatory element and typically provides regulatory specificity for a given target pathway in plant secondary metabolism, the bHLH proteins act as cofactors to regulate the activity of MYB factors and is considered redundant, while the conserved WD40 domain proteins are essential for the integrity of the MBW complexes (Feller et al., 2011). Recently, interactions between proteins of different subfamilies such as, MYB and bHLH family have been shown to regulate different gene modules in GLSs biosynthesis (Schweizer et al., 2013; Frerigmann et al.,2014; Li and Sack, 2014). In this study, we demonstrated that a key GLS biosynthetic pathway gene is not only under transcriptional control of MYBs and bHLHs as was previously shown (Schweizer et al., 2013; Frerigmann et al., 2014), but is also regulated by AP2/ERFs. In addition, ERF109, MYB51 and MYC2/3/4 proteins form the ERF109-MYB51-MYCs (EMM) complexes using MYB51 as an adaptor. This conclusion was made from the results which showed. ERF109 binds directly to the CE element and activates the *CYP83B1* gene expression. The amount of IGLSs in the triple *erf109myb51myc3* mutant is strongly impaired, and combinatorial gain of function of ERF109, MYB51 and MYC3 proteins has an additive effect on the *CYP83B1* gene expression. Finally, MYB51 not only binds to the

AC-rich sequence, but also could act as an adaptor to bridge the interactions with MYC2/3/4 and ERF109 in our Y3H assays. These results point to indispensable roles to the transcriptional control of GLSs genes for AP2/ERF, MYB and bHLH proteins.

The Skp-Cullin-F-box protein complex containing COI1 (SCF^{COI1}) facilitates the degradation of JAZ proteins (JAsmonate ZIM domain), key repressors within JA signaling cascades through the ubiquitin-26S proteasome pathway. The JAZs repress a variety of TFs that have different roles in regulating JAs-responsive gene expression (Zhou and Memelink, 2016). In the JAs-regulated anthocyanin biosynthesis, JAZ proteins directly interact with bHLH and MYB factors of MBW complexes, impair their transcriptional activity, repressing anthocyanin biosynthesis (Qi et al., 2011). Previous reports showed that JAZ proteins interact with MYC3, and that mutating the JAZ Interaction Domain (JID) of MYC3 inhibited the interaction with JAZ proteins, thereby increasing the MYC3 activity resulting in elevated **GLS levels** (Frerigmann et al., 2014). Here, we demonstrated that JAZ10 directly interacts with MYB51 and MYC3, and attenuated their transcriptional function. Over-expression of *JAZ10* in plants significantly decreased the accumulations of both anthocyanin and IGLSs (**Supplemental Fig. S13**), indicating that MYB51, like MYC3, is regulated by JAZ repressors.

Several JA-responsive members of the AP2/ERF family function as key regulators in plant secondary metabolite biosynthesis, including monoterpenoid indole alkaloid, nicotine and artemisinin biosynthesis (Zhou and Memelink, 2016). It has been reported that ERF109 is a JAs-responsive factor and mediates the cross-talk JA and auxin (Cai et al., 2014). Recently, ERF109 has also been shown to directly activate a set of biosynthetic enzyme genes and promote tryptophan biosynthesis and tryptophan metabolism (Bahieldin et al., 2018). Here, we show that ERF109 is directly involved in the regulation of IGLS biosynthesis. In this study, JAs control ERF109 not only at the transcriptional level, but also on the *de novo* synthesized protein. Our western blot results showed that JA directly affects ERF109 protein activity by inducing stabilization, and this established ERF109 as a component of JA signal transduction. In addition, ERF109 was also stabilized by MG132, indicating that it's degraded via 26S proteasome. It has been widely accepted that E3 ubiquitin (UBQ) ligase binds to the UBQ conjugating enzyme E2 and a substrate protein to facilitate transfer of the UBQ moiety, and thereby the ubiquitylated protein is marked for degradation via the 26S proteasome (Hua and Vierstra, 2011). CULLIN3 (CUL3)-based Really Interesting New Gene (RING) E3 ligases (CRL3) are composed of the scaffolding subunit CUL3 protein, the RING-finger protein RING-Box protein 1 (RBX1) which binds to the C-terminal region of CUL3, while the N-terminal part of CUL3 is recognized by proteins containing a BTB/POZ fold (Hua and Vierstra, 2011). BTB/POZ-MATH (BPM) family contains a MATH domain located in their N-terminal region and a BTB/POZ fold in their C-terminal region (Weber and Hellmann, 2009). It has been shown that the MATH domain of BPM proteins is used to assemble with members of the AP2/ERF TFs, such as RELATED TO APETALA2.4 (RAP2.4) and WRINKLED1 (WRI1) (Weber and Hellmann, 2009; Chen et al., 2013). In addition, it has been demonstrated that the degradation of WRI1 by 26S proteasome depends on the $CRL3^{BPM}$ E3 ligase in the fatty acid metabolism (Chen et al., 2013). In this study, we confirmed the interactions between ERF109 and the MATH domain of BPM proteins, and the stability of ERF109 is controlled by $CRL3^{BPM}$ E3 ligase, which is in agreement with the instability of WRI1, mediated by a $CRL3^{BPM}$ E3 ligase (Chen et al., 2013). Overall, it is likely that BPM proteins, as potential regulators, affect the transcriptional activity and protein stability of AP2/ERF

TFs in higher plant.

In conclusion, we proposed a 'relief of repression' model for the function of the ERF109-MYB51-MYC3 **ternary complexes** in controlling the key enzyme *CYP83B1* gene expression and JA-regulated GLS biosynthesis (**Fig. 7**). This model proposes that JAs promote GLS biosynthesis *via* the degradation of JAZ proteins which interact with MYB51 and MYC3 that control *CYP83B1* gene transcription, and the stability of ERF109 which is a target by $CRL3^{BPM}$ E3 ligase. It is fascinating that both BPM and JAZ proteins interact with ERF109-MYB51-MYC3 which function as repressors of JAs responsive ERF-MYB-bHLH activity. Further comparative investigation of the regulatory mechanisms of ERF-MYB interaction will provide a better understanding of plant secondary metabolism.

MATERIALS AND METHODS

Yeast one hybrid assays

The promoter fragments of *CYP83B1proI* (-214 to -1), *CYP83B1proII* (-430 to -193), *CYP83B1proIII* (-648 to -410) and *CYP83B1proIV* (-889 to -628) were PCR-amplified from genomic DNA of *A. thaliana* digested with NotI and SmaI and fused to a TATA box-*His*3 gene in plasmid pHIS3NX, respectively. Through homologous recombination, the *His3* gene constructs were integrated in the yeast (strain Y187) genome (Zhou et al., 2015; Li et al., 2018). After transformation of the JA-treated Arabidopsis cDNA libraries in the different *CYP83B1pro* yeast strains, cells were grown on synthetic dextrose minimal medium lacking Leu and His (SD/-LH) supplemented with increasing 3 amino-1,2,4-triazole (3-AT; Sigma) concentrations ranging from 0 to 15 mM, respectively. The positive colonies were confirmed by re-transformation and sequencing. All primer sequences are mentioned in **Supplemental Table S1**.

Yeast two/three hybrid assays

The open reading frames (ORFs) of ERF109, MYB34, MYB51, MYC2/3/4, BPM1-5, JAZ1-12 and their deletion derivatives, ERF109NT, ERF109CT, MYB51NT, MYB51CT, BPM1-MATH, JAZ10NT and JAZ10CT were cloned into pACT2 or pAS2.1 vectors (Zhou et al., 2015). pAS2.1-ERF109 was used as bait for yeast two-hybrid screening. Co-transformation of pAS2.1-ERF109 and Arabidopsis cDNA libraries, or any bait and prey plasmids, was performed into yeast strain PJ64-4A according to the protocol as previously described (Zhou et al., 2015). For the three-hybrid bridge assay, the *MYB51* and MYB34 was cloned in pYPGE15 (Brunelli and Pall, 1993) and p423GPD (Mumberg et al., 1995) for Ura synthesis, respectively. The positive transformants could grow on SD/-LWH and SD/-LWHU selection media, and the colony-lift filter β-galactosidase assay was performed as described in the Yeast Protocols Handbook (Clontech). **Supplemental Table S1** shows sequence of all the primers used in this study, all constructed plasmids were sequenced.

EMSA

To produce His-tagged proteins, ERF109 and MYB51 were PCR amplified and cloned in pASK-IBA45plus. These constructs were transformed into *Escherichia coli* strain BL21 (DE3) pLysS for protein extraction according to the protocol as previously described (Zhou et al., 2017; Li et al., 2018). EMSAs were performed using biotin-labeled double-stranded probes (*CYP83B1* promoter fragment I and its mutants) and the Light Shift Chemiluminescent EMSA Kit according

to the manufacturer's instructions (Thermo Fisher). All primer sequences are mentioned in **Supplemental Table S1**.

ChIP-qPCR and qRT-PCR Assay

The plasmid pRT101-ERF109-HA and pRT101-MYB51-HA (5 µg) were transformed into *Arabidopsis* cell suspension protoplasts. After 18 h incubation at 28°C under dark condition, protoplasts were harvested and washed with PBS buffer (1% formaldehyde, pH 7.4) to crosslink the proteins to the DNA at 4°C (Li et al., 2018). HA antibody (Roche, Mannheim, Germany) was used for immunoprecipitation of chromatin-bound proteins. qPCR was performed using primers designed on the flanking sequence of MYB51 and ERF109-binding sites in *CYP83B1* promoter. The coding region of *CYP83B1* gene and the reference gene *UBQ10* promoter were used as a negative control. ChIP assays were performed as previously described (Zhou et al., 2017; Li et al., 2018). Total RNA extraction and reverse transcription (Revert Aid first-strand cDNA synthesis kit; Fermentas) were performed according to the manufacturer's instructions and qRT-PCR was performed as previously described (Zhou et al., 2017; Li et al., 2018). The qPCR primers for six IGLSs biosynthesis genes (*CYP79B3*, *CYP83B1*, *CYP83A1*, *GSTF10*, *SUR1* and *UGT74B1*) were used as previously described (Hirai et al. 2007; Frerigmann et al., 2014; Frerigmann and Gigolashvili, 2014). All primer sequences are mentioned in **Supplemental Table S1**.

Arabidopsis protoplast trans-activation assays

The full-length *ERF109*, *MYB34*, *MYB122*, *MYB51*, *MYB51NT*, *MYB51CT*, *MYC3*, *JAZ10*, *JAZ10NT*, *JAZ10CT* and *BPM1* were PCR amplified cloned into effector plasmid pRT101 under the CaMV 35S promoter (Töpfer et al., 1987). The promoter fragments of *CYP83B1* pro-fused GUS were cloned in reporter plasmid GusXX (Töpfer et al., 1987). Arabidopsis cell suspension or leaf protoplasts were co-transformed with effector plasmids together with reporter plasmid as previously described (Schirawski et al., 2000; He et al., 2007). GUS activity assays were performed as previously described (van der Fits and Memelink, 1997). All primer sequences are mentioned in **Supplemental Table S1**.

BiFC

The N-terminal (YN) or C-terminal (YC) fragments of the yellow fluorescent protein (nYFP or cYFP) were fused either N-terminally or C-terminally with MYB51, MYB51NT, ERF109, MYC3, BPM1, BPM1-MATH, JAZ10 and JAZ10CT and cloned in pRTL2-YNEE (nYFP-) or pRTL2-HAYC (-cYFP). The all possible combinations of nYFP and cYFP fusion protein constructs were transiently co-expressed in Arabidopsis cell suspension as previously described (Schirawski et al., 2000). Microscopy images were acquired with a Leica DM IRBE confocal laser scanning microscope and analyzed using the ImageJ software (Abràmoff et al., 2004; Zhou et al., 2017). All primer sequences are mentioned in **Supplemental Table S1**.

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the wild-type plant. The knockout T-DNA insertion lines *erf109* (SALK_150614, namely KO^{ERF109}) and over-expression lines of *erf109* gene (CS2102255, namely OE^{ERF109}) of locus *AT4G34410* were provided by the SALK Institute, Genomic Analysis Laboratory (SIGnAL) (http://signal.salk.edu/tdnaprimers.2.html). The

T-DNA insertion lines *myb51* and *myc3* (GK445B11) had been described previously (Gigolashviliet al., 2007; Frerigmann et al., 2014). Pollen from homozygous *myb51* plants were used to pollinate emasculated homozygous *erf109* and *myc3* flowers to generate *erf109myb51* and *myb51myc3* double homozygous plants. The triple mutant *erf109myb51myc3* plants were generated from a cross between *myb51myc3* and *erf109* plants. For JA treatments, two-week-old 20–25 seedlings of each genotype under continuous light were treated for different time periods with 50 µM JA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO, 0.05% final concentration). As a control, seedlings were treated with 0.05% DMSO.

Immunoblot analysis

The full-length *ERF109* and *JAZ10* were PCR amplified cloned into pTH2 under the CaMV 35S promoter to generate ERF109-GFP and JAZ10-GFP proteins constructs. Transformed Arabidopsis protoplasts were ground in 50 µL of cold protein extraction buffer (50 mM HEPES-KOH pH 7.2, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 mM DTT and 1 mM phenylmethylsulfonylfluoride (PMSF)). After centrifugation at 12000 rpm for 15 min at 4°C, supernatants were separated on SDS-PAA gels and transferred to protran nitrocellulose by semidry blotting. The Western blots with GFP peroxidase antibodies (1:2000; Roche) were performed as previously described (Zhou et al., 2015). All primer sequences are mentioned in **Supplemental Table S1**.

In Vitro Pull-Down Assay

To produce HA-tagged proteins, *MYC3*, *ERF109*, *JAZ10* and *JAZ10NT* were PCR amplified and cloned in pASK-IBA45plus. To produce Strep-tagged proteins, *MYB51* was amplified and cloned in pASK-IBA45plus. These constructs were transformed into *E. coli* strain BL21 (DE3) pLysS for protein expression. The soluble Strep- and HA-fusion proteins were extracted and immobilized onto Strep-tactin Sepharose (IBA) and anti-HA agarose beads (Thermo Scientific), respectively. Pull-down assays were performed as described previously (Zhou et al., 2016). All primer sequences are presented in **Supplemental Table S1**.

Measurement of IGLSs

The isolation and quantification of the concentration of I3M, 4MO-I3M, and 1MO-I3M were performed using the desulpho-GS method on a UPLC (Waters, Eschborn) as described recently (Gigolashvili et al., 2012; Frerigmann et al., 2014; Frerigmann and Gigolashvili, 2014).

Statistical analysis

All data were analyzed using Student's t-test and one-way ANOVA. Values of $P<0.05$ were considered to be significant.

Accession numbers

Sequence data for the genes described in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ERF109 (At4g34410), MYB51 (At1g18570), MYC2 (At1g32640), MYC3 (At5g46760), MYC4 (At4g17880), MYB34 (At5g60890), MYB122 (At1g74080), JAZ1 (At1g19180), JAZ2

(At1g74950), JAZ3 (At3g17860), JAZ4 (At1g48500), JAZ5 (At), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 (At1g70700), JAZ10 (At5g13220), JAZ11 (At3g43440), JAZ12 (At5g20900), BPM1 (At5g19000), BMP2 (At3g06190), BPM3 (At2g39760), BPM4 (At3g03740), BPM5 (At5g21010), BPM6 (At3g43700), CYP79B3 (At2g22330), CYP83B1 (At4g31500), CYP83A1 (At4g13770), GSTF10 (At2g30870), SUR1 (At2g20610), UGT74B1 (At1g24100), and UBQ10 (At4g05320).

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AUTHOR CONTRIBUTIONS

M.Z. conceived and supervised the research. K.Z., Y.M., J.L., M.D., Q.L. and X.L. conducted the experiments. Y.M., J.L. and M.K. analyzed the data. K.Z.,Y.M., J.L. M.K. and M.Z. wrote the paper.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression pattern of *CYP83B1* and *CYP83A1* in 2-week-old plant seedlings treated with 50 μM MeJA for various times (in h) as indicated by qRT–PCR.

Supplemental Figure S2. Different fragments of *CYP83B1* gene promoter sequence.

Supplemental Figure S3. Trans-activation assays of *CYP83B1* and *CYP83A1* promoter by MYB51, MYB34 and MYB122.

Supplemental Figure S4. ERF109 interacts directly with MYB51 C-terminal in quantitative Y2H assays.

Supplemental Figure S5. ERF109 did not interact with MYC2/3/4 in quantitative Y2H assays.

Supplemental Figure S6. MYB51 can bridge the interaction between ERF109 and MYC2/4.

Supplemental Figure S7. Expression levels of *CYP79B3*, *CYP83B1*, *CYP83A1*, *GSTF10*, *SUR1* and *UGT74B1* in 2-week-old different genotypes seedlings treated with 50 μM MeJA for various times (in h) as indicated by qRT–PCR.

Supplemental Figure S8. MYB51 N-terminal interacts directly with JAZ10 C-terminal in quantitative Y2H assays.

Supplemental Figure S9. ERF109 interacts directly with BPM family proteins in quantitative Y2H assays.

Supplemental Figure S10. BPM1 interacts directly with ERF109 N-terminal in quantitative Y2H assays.

Supplemental Figure S11. BPM and JAZ repress the activity of ERF109-MYB51-MYC3 Ternary Complexes.

Supplemental Figure S12. Trans-activation assays of *CYP83B1* promoter by different amounts of JAZ10 and BPM1.

Supplemental Figure S13. Over-expressed *JAZ10* and *BPM1* represses the accumulation of IGLS.

Supplemental Table S1. Primers used in this study.

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Figure Legends:

Figure 1. ERF109 and MYB51 binds and activate *CYP83B1* promoter. a, EMSA of a wild type or mutated *CYP83B1* promoter fragment I with His-MYB51 and His-ERF109. The arrow indicates the protein probe complex. **b and c**, ChIP assays show MYB51 (**b**) and ERF109 (**c**) bind to the promoter of *CYP83B1*. ChIP assays were conducted by real-time PCR after normalizing with the input DNA. The reference gene UBQ10 promoter and the fragment of *CYP83B1* coding sequence were used as a negative control. **d**, ERF109 and MYB51 trans-activate the *CYP83B1* promoter. Trans-activation assays of *CYP83B1* and *CYP83A1* promoter by ERF109 and MYB51. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying 2 μg *CYP83B1FLpro-GUS* or *CYP83A1pro-GUS* and 2 μg overexpression vectors containing 35S::ERF109 or 35S::MYB51, as indicated. Asterisks denotes one-way analysis of variance t test significance **P \0.01 and *P \0.05 and ns (not significant different). Values represent mean standard error (SE) of triplicate experiments and are expressed relative to the control.

Figure 2. ERF109 interacts with MYB51 *in vitro* and *in vivo*. a, ERF109 interacts with MYB51 in quantitative Y2H assays. Yeast cells expressing ERF109, MYC2 or MYC3 proteins fused to the GAL4 AD and MYB34 or MYB51 fused to the GAL4 BD were spotted on SD/-LW medium to select for the plasmids and on SD/-LWH medium containing 10 mM 3-amino-1,2,4-triazole (3AT) to select for transcriptional activation of the *His-3* and β-galactosidase gene. A liquid culture β-galactosidase assay was performed on the transformed yeasts after 5 d growth. The activity of β-galactosidase was measured in arbitrary units. Values are means ±SD of three biological repeats. Asterisks denotes one-way analysis of variance (ANOVA) test significance **P \0.01 and *P \0.05

and ns (not significant different). **b**, Bimolecular fluorescence complementation (BiFC) assays in planta. YFP fluorescence images alone or merged with bright-field images of Arabidopsis cell suspension protoplasts co-transformed with constructs encoding the indicated fusion proteins with YFP at the C terminus or the N terminus. Bar = 10 μm. **c,** In vitro interaction between HA-MYC3 or HA-ERF109 and Strep-MYB51. HA-MYC3 or HA-ERF109 protein was incubated with immobilized Strep-MYB51. The immuno-precipitated fractions were detected with anti-HA antibody. Input was detected with anti-Strep antibody. Asterisks denote bands from HA antibody.

Figure 3. Combinatorial ERF109-MYB51-MYC3 interactions synergistically activate *CYP83B1* gene expression. a, MYB51 can bridge the interaction between ERF109 and MYC3. Yeast cells expressing MYC3 fused to GAL4AD, ERF109 fused to GAL4BD and MYB51 protein or corresponding control plasmids were spotted on minimal (-3 = -LWH) or selective SD medium (-4 = -LWHA), growth and the activity of β-galactosidase assay was performed on the transformed yeast cells after 5 d growth. Yeast cells transformed with the empty plasmids were used as control. **b** and **c,** Trans-activation assays of *CYP83B1* and *mMBSCYP83B1* promoter by ERF109, MYB51 and MYC3. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying 2 μg *CYP83B1FLpro-GUS* or mMBS*CYP83B1FLpro-GUS* and 2 μg overexpression vectors containing 35S::ERF109, 35S::MYB51, 35S::MYB51NT or 35S::MYC3, as indicated. Values are means ±SE of three biological repeats. Asterisks denotes one-way analysis of variance (ANOVA) test significance *P\0.05.

Figure 4. The ERF109-MYB51-MYC3 factors are involved in jasmonate-induced IGLS biosynthesis. a, MeJA induced the accumulation of IGLS in wild-type (Col-0), OE^{ERF109} (OE) and *erf109* (KO) Arabidopsis plants. The three major IGLS I3M, 4MO-I3M and 1MO-I3M content of two-week-old plants grown on MS plates and treated with 50 μM MeJA for 16h and 32h. **b-d,** MeJA-induced accumulation of I3M **(b)**, 4MO-I3M **(c)** and 1MO-I3M **(d)** in *myb51myc3* and *erf109myb51myc3* loss-of-function mutants. The three major IGLS I3M, 4MO-I3M and 1MO-I3M content of two-week-old Col-0, *erf109*, *myb51*, *myc3*, *erf109myb51*, *myb51myc3*, *erf109myc3*, *erf109myb51myc3* plants grown on MS plates and treated with 50 μM MeJA for 32h. Data are presented as means ± SE from three independent experiments with four biological replicates.

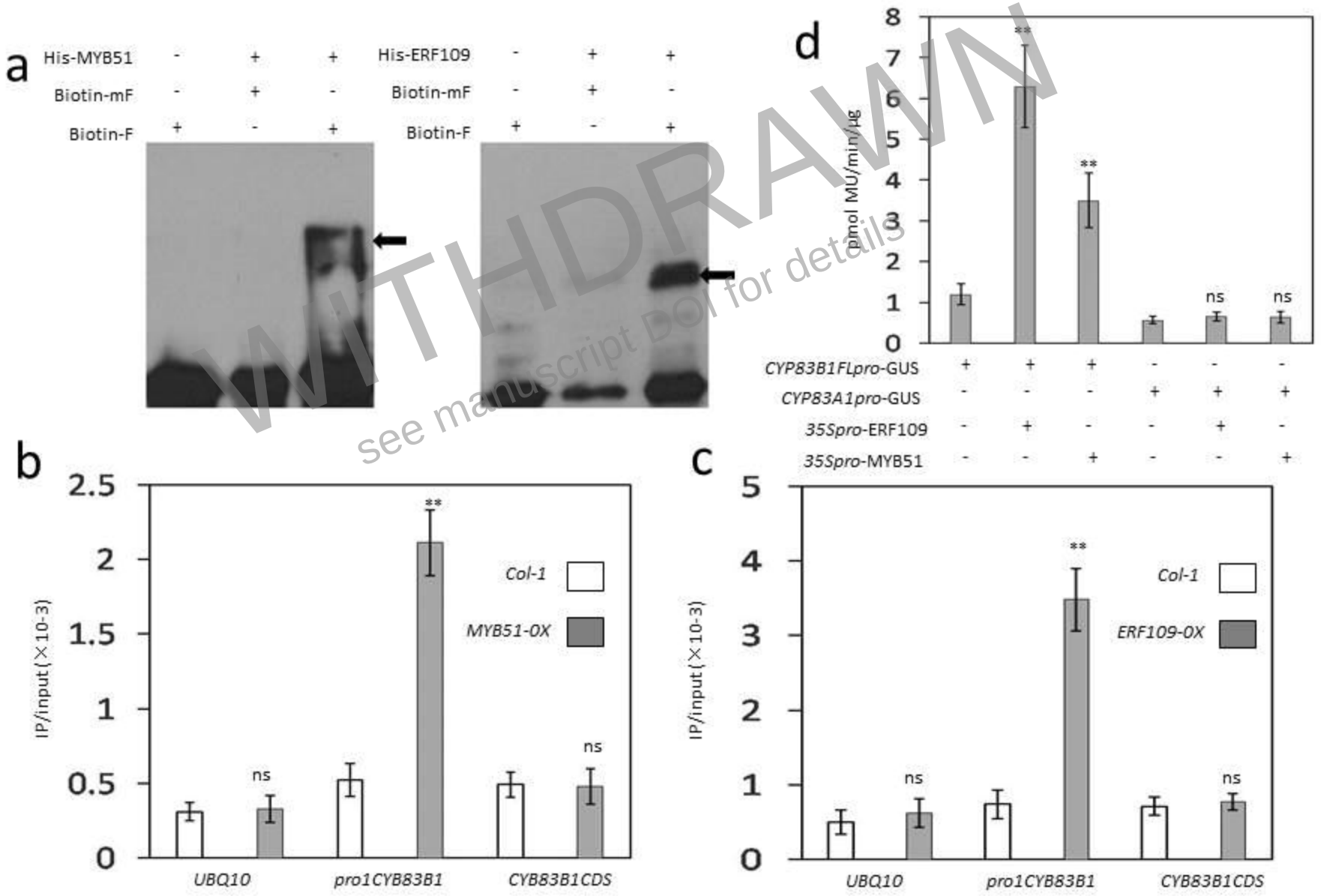
Significant differences between values are indicated with different letters (P<0.05, one-way ANOVA) . **e,** Expression pattern of *CYP79B3*, *CYP83B1*, *CYP83A1*, *GSTF10*, *SUR1* and *UGT74B1* in 2-week-old different genotypes seedlings treated with 50 µM MeJA for various times (in h) as indicated by qRT–PCR. *UBQ10* was used as an internal control. Mock denotes JA treatment for 0 h. Heat maps were generated by Microsoft excel based on expression levels. Different shades indicate higher or lower expression as indicated by expression bar.

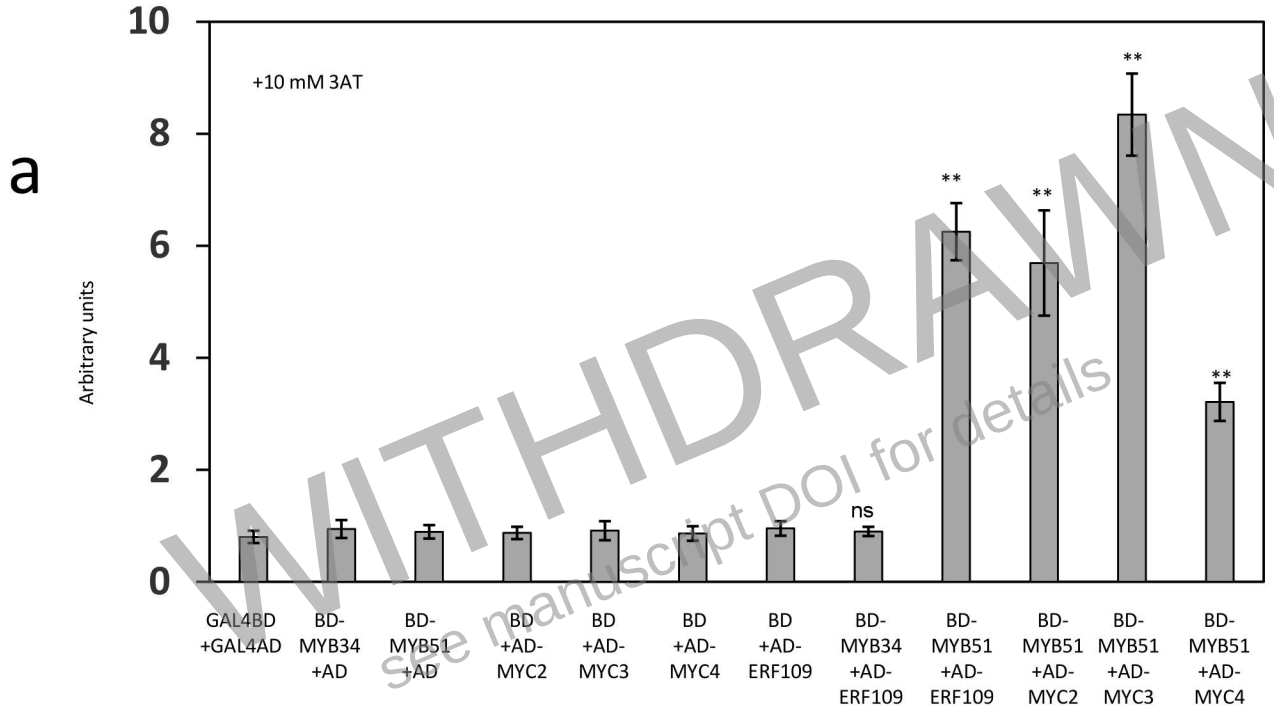
Figure 5. MYB51 interacts with JAZ10 *in vitro* and *in vivo*. a, MYB51 interacts with JAZ10 in quantitative Y2H assays. Yeast cells expressing MYB51, MYB34 or MYC3 proteins fused to the GAL4 AD and JAZ1-12 fused to the GAL4 BD. A liquid culture β-galactosidase assay was performed on the transformed yeast cells after 5 d growth. Values are means ±SD of three biological repeats. Asterisks denotes one-way analysis of variance (ANOVA) test significance **P \0.01 and *P \0.05 and ns (not significant different). +++, ++ and + represent very strong, strong and weak interaction, respectively. **b,** BiFC assays in planta. YFP fluorescence images alone or merged with bright-field images of Arabidopsis cell suspension protoplasts co-transformed with constructs encoding the indicated fusion proteins with YFP at the C terminus or the N terminus. Bar = 10 µm. **c,** In vitro interaction between Strep-MYB51 and HA-JAZ10 or HA-JAZ10NT. HA-JAZ10 or HA-JAZ10NT protein was incubated with immobilized Strep-MYB51. The immuno-precipitated fractions were detected with anti-HA antibody. Input was detected with anti-Strep antibody. Asterisks denote bands from HA antibody.

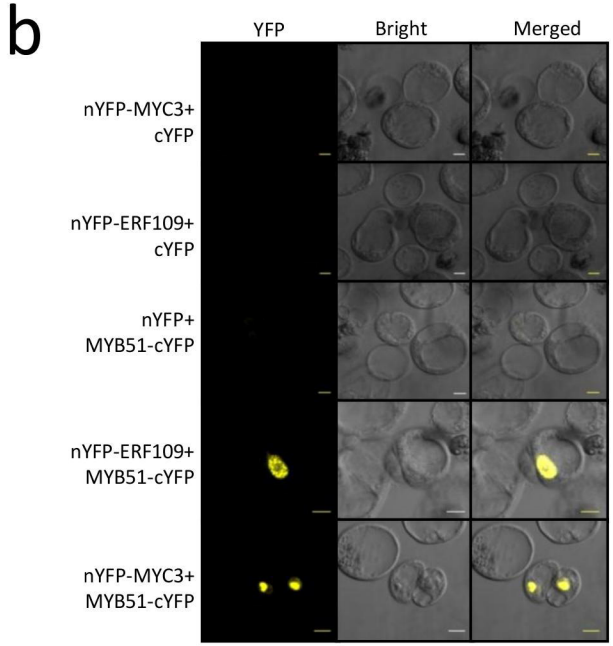
Figure 6. ERF109 interacts with BPM family proteins resulting into its degradation by 26S proteasome pathway. a, ERF109 interacts with BPM1 lacking the last 263 amino acids in Y2H screening. Schematic representation of BPM1 protein. **b,** BiFC assays of the interaction between ERF109 and BPM1 or BPM1-MATH domain in planta. YFP fluorescence images alone or merged with bright-field images of Arabidopsis cell suspension protoplasts co-transformed with constructs encoding the indicated fusion proteins with YFP at the C terminus or the N terminus. Bar = 10 µm. **c** and **d,** ERF109 is degraded by the 26S proteasome and stabilized under JA treatment. **c,** Confocal laser scanning microscopy images of Arabidopsis protoplasts expressing ERF109-GFP or GFP. Eighteen hours after transformation, protoplasts were treated for 2 and 4 h with 50 µM

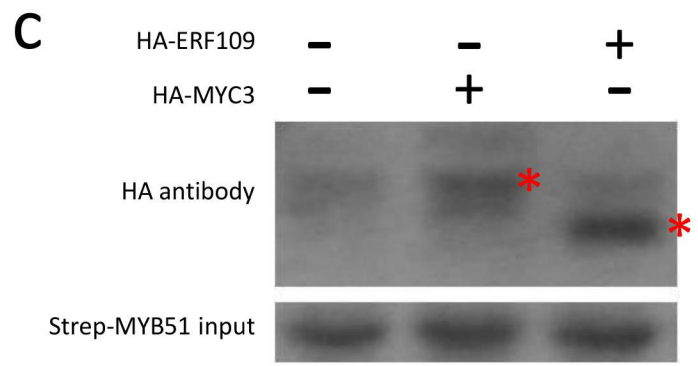
MG132, 50 μM MeJA or 0.1 % DMSO. Bar = 20 μm. **d** and **e,** Transiently expressed ERF109-GFP and GFP proteins in Col-0, *bpm1*, *bpm3*, *bpm1bpm3* Arabidopsis protoplasts. The proteins were extracted 18 h after transformation of protoplasts and detected with anti-GFP antibodies.

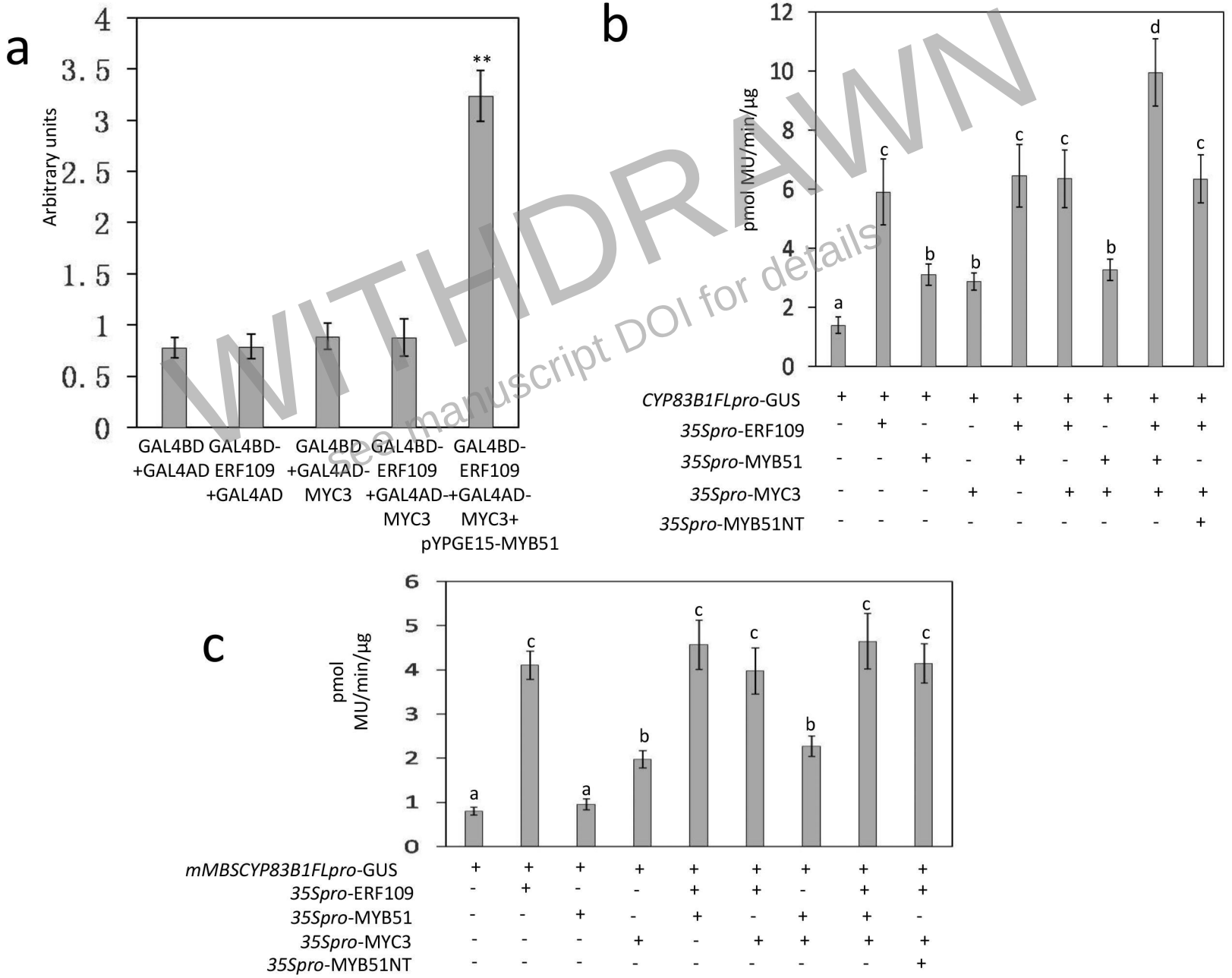
Figure 7. The 'relief of repression' model for the function of ERF109-MYB51-MYC3 ternary complexes in controlling the key enzyme *CYP83B1* gene expression and JAs-regulated GLSs biosynthesis. All three TFs are recruited to the promoters of *CYP83B1* gene to activate its expression; MYB51 binds to AC-rich sequence, MYC2/3/4 bind to G-box-like sequence, whereas ERF109 binds to CE element. Without JAs, BPM proteins interact with ERFs, function as substrate adaptors to CUL3-based E3-ligases $CUL3^{BPM}$, docking of the BPM–ERF complex to the $CUL3^{BPM}$ E3-ligase results in ubiquitination and subsequent degradation of ERFs; stabilized JAZ proteins interact with MYBs and MYCs, thereby inhibit the activity of MYBs and MYCs. JAs induce degradation of JAZs via the SCF^{COI1} complex and the 26S proteasome to release MYBs and MYCs that in turn activates the expression of *CYP83B1*, in addition, JA stabilized ERFs not only at the transcriptional induction, but also on the de novo synthesized protein. JA promotes the formation of a ERF-MYB-bHLH ternary complexes, thus activating the expression of GLSs biosynthesis genes.

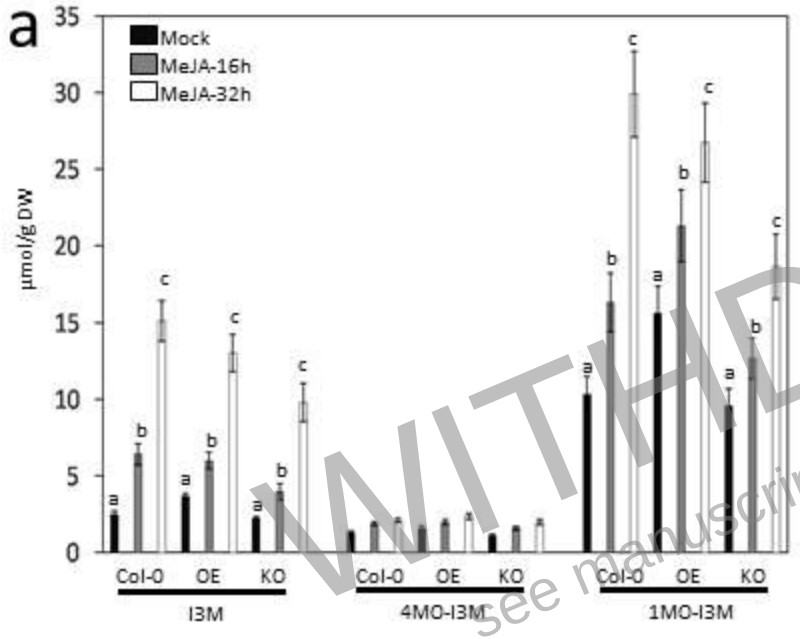


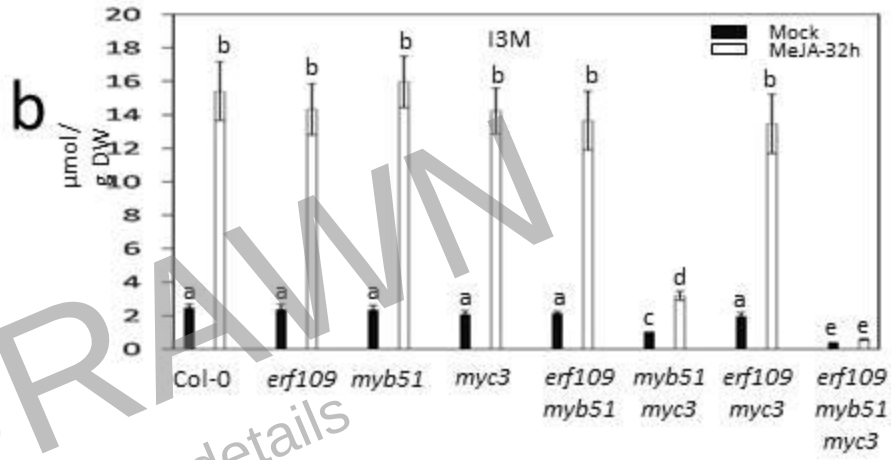


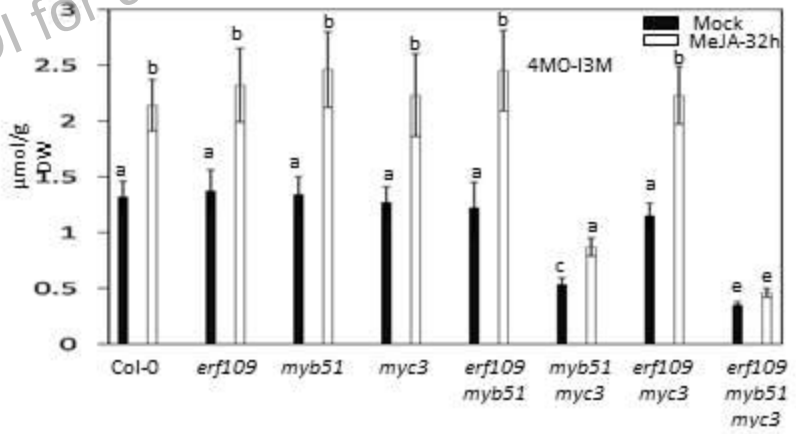


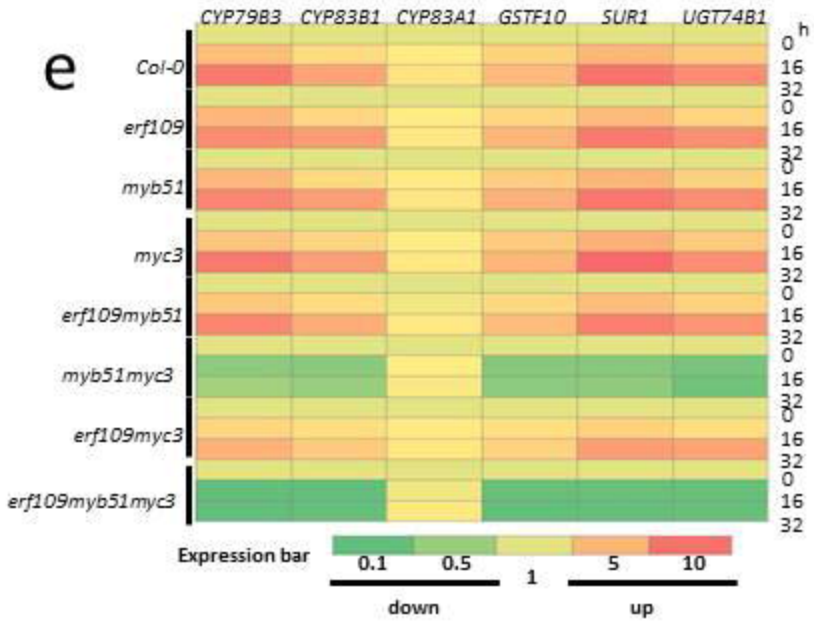


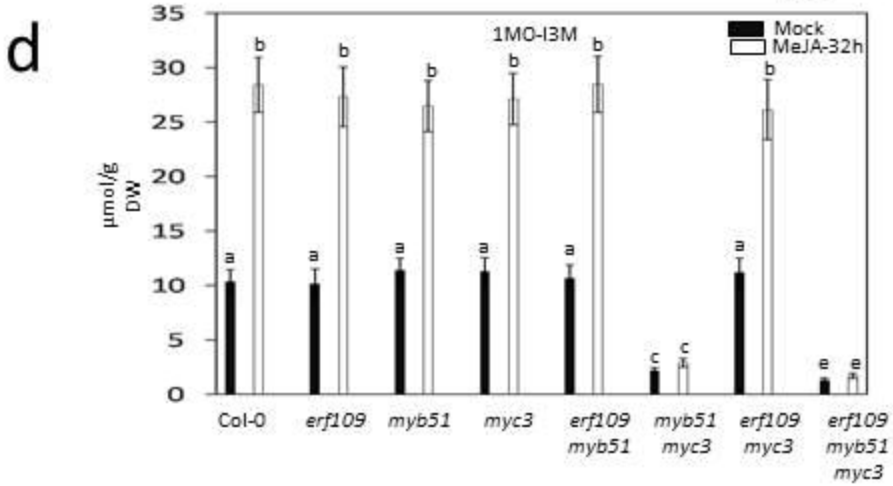








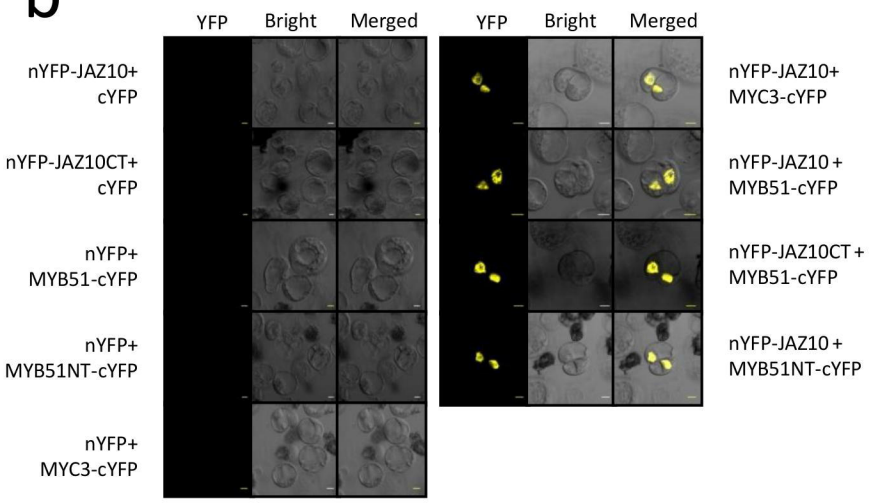




a

+10 mM 3AT	GAL4AD	GAL4AD-MYC3	GAL4AD-MYB51	GAL4AD-MYB34
GAL4BD	0.821	1.014	1.258	0.962
GAL4BD-JAZ1	0.937	5.425 (+++)	1.269	0.997
GAL4BD-JAZ2	0.875	4.278 (+++)	0.998	1.034
GAL4BD-JAZ3	0.926	3.514 (++)	0.975	1.031
GAL4BD-JAZ4	0.976	1.029	1.058	1.011
GAL4BD-JAZ5	0.895	2.985 (+)	1.243	0.963
GAL4BD-JAZ6	0.836	3.698 (++)	1.115	1.103
GAL4BD-JAZ7	0.824	1.112	0.963	0.998
GAL4BD-JAZ8	0.892	4.367 (+++)	0.994	0.994
GAL4BD-JAZ9	0.912	5.697 (+++)	1.065	1.014
GAL4BD-JAZ10	0.876	3.998 (++)	**4.652 (+++)**	1.002
GAL4BD-JAZ11	0.813	4.567 (+++)	1.265	0.987
GAL4BD-JAZ12	0.875	3.925 (++)	1.127	0.996

b



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