

# Jasmonate regulates the FAMA/mediator complex subunit 8-THIOGLUCOSIDE GLUCOHYDROLASE 1 cascade and myrosinase activity

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## Abstract

Myrosinases are  $\beta$ -thioglucoside glucosidases that are unique to the Brassicales order. These enzymes hydrolyze glucosinolates to produce compounds that have direct antibiotic effects or that function as signaling molecules in the plant immune system, protecting plants from pathogens and insect pests. However, the effects of jasmonic acid (JA), a plant hormone that is crucial for plant disease resistance, on myrosinase activity remain unclear. Here, we systematically studied the effects of JA on myrosinase activity and explored the associated internal transcriptional regulation mechanisms. Exogenous application of JA significantly increased myrosinase activity, while the inhibition of endogenous JA biosynthesis and signaling reduced myrosinase activity. In addition, some myrosinase genes in *Arabidopsis* (*Arabidopsis thaliana*) were upregulated by JA. Further genetic and biochemical evidence showed that transcription factor FAMA interacted with a series of JASMONATE ZIM-DOMAIN proteins and affected JA-mediated myrosinase activity. However, among the JA-upregulated myrosinase genes, only *THIOGLUCOSIDE GLUCOHYDROLASE 1* (*TGG1*) was positively regulated by FAMA. Further biochemical analysis showed that FAMA bound to the *TGG1* promoter to directly mediate *TGG1* expression in conjunction with Mediator complex subunit 8 (MED8). Together, our results provide evidence that JA acts as an important signal upstream of the FAMA/MED8–*TGG1* pathway to positively regulate myrosinase activity in *Arabidopsis*.

## Introduction

Glucosinolates are secondary metabolites that are found only in plants in the Brassicales order (Canistro et al., 2004; Wang et al., 2011; Fernandez-Calvo et al., 2020). Although

these compounds themselves do not have biological activity, they can be decomposed via myrosinase catalysis into many types of metabolites that are deterrent or toxic to insects and pathogens (Andersson et al., 2009). Glucosinolates and myrosinases are normally physically separated at the tissue

level or at the single-cell level in plants. However, these components can be brought together as a result of tissue damage caused by insects or pathogens. Glucosinolate–myrosinase interactions release large amounts of biologically active hydrolysis products (Sugiyama and Hirai, 2019; Bhat et al., 2020; Kayum et al., 2020; Mocniak et al., 2020).

In *Arabidopsis* (*Arabidopsis thaliana*), myrosinases are divided into two categories based on conserved amino acid residues: the QE- and the EE-type myrosinases. The QE-type myrosinases contain Gln and Glu residues in the catalytic site. In *Arabidopsis*, there are six QE-type myrosinases, which are encoded by the thioglucoside glucohydrolase (*TGG*) genes (*TGG1–6*). *TGG1* and *TGG2* are expressed in the aboveground parts of the plant, and their encoded enzymes decompose aliphatic glucosinolates and indole glucosinolates (Barth and Jander, 2006); *TGG3* and *TGG6* are pseudogenes in *Arabidopsis* Col-0 (Xue et al., 1995); and *TGG4* and *TGG5* are only expressed in plant roots and are associated with auxin synthesis (Xue et al., 1995; Fu et al., 2016). A previous study showed that, despite the replacement of the Gln residue in the PENETRATION2 (PEN2) protein by Glu, PEN2 still exerted myrosinase activity against indol-3-ylmethyl glucosinolate (I3G) and its 4-methoxy analog (Bednarek et al., 2009). Similarly, PYK10 (BGLU23) myrosinase activity was maintained despite the substitution of the Gln residue by Glu (Nakano et al., 2017). Proteins containing the two glutamic acid residues conserved in PEN2 and PYK10 are designated EE-type myrosinases (Sugiyama and Hirai, 2019). There are 16 EE-type myrosinase genes in *Arabidopsis*: *BGLU18–BGLU33*. Some of these genes are involved in specific stresses. *BGLU18*, one of the primary plant proteins inducing ER bodies, can be upregulated by jasmonic acid (JA) or wounding (Lee et al., 2006; Ogasawara et al., 2009; Yamada et al., 2011; Nakazaki et al., 2019). *BGLU19* is induced by high salt stress, and *BGLU19* mutants are tolerant to salt stress (Cao et al., 2017). PEN2 plays an important role in plant resistance to pathogens (Bednarek et al., 2009). Both *BGLU28* and *BGLU30* are induced by sulfur depletion (Hirai et al., 2003; Matsushima et al., 2003; Hirai and Saito, 2004); *BGLU30* is also induced by extended darkness or senescence (Fujiki et al., 2001; Lee et al., 2007). *BGLU33* mutants are more sensitive to salt stress than wild-type (Xu et al., 2012). In addition, these proteins have myrosinase activities with substrate selectivity. In vitro hydrolysis of ABA glucose ester (ABA-GE) showed that *BGLU18* and *BGLU33* exhibit ABA-GE hydrolyzing activity in vitro (Lee et al., 2006; Xu et al., 2012). The hydrolysis of 4-methoxyindol-3-ylmethyl glucosinolate (4MI3G) was remarkably retarded in the *bglu18 pyk10* mutant compared with that in wild-type, indicating that 4MI3G is hydrolyzed by *BGLU18* and/or PYK10 (Nakazaki et al., 2019). In vitro glucosinolate hydrolysis experiments showed that PYK10 (also known as *BGLU23*) and its closest homologs (*BGLU21* and *BGLU22*) specifically hydrolyzed scopolin and some substrates whose aglycone moiety is similar to scopolin (e.g. esculin and 4-MU-glucoside; Ahn et al., 2010). However, they did not

hydrolyze sinigrin (Ahn et al., 2010). In addition to coumarin glucosides, in vitro activity testing of recombinant PYK10 protein toward I3G showed that PYK10 exhibited a high hydrolysis activity toward I3G (Nakano et al., 2017). Recent research on glucosinolate hydrolysis experiments with *pyk10 bglu21* double mutant showed PYK10 and/or *BGLU21* constitute the main source of myrosinase activity against aliphatic and indolic glucosinolates in young *Arabidopsis* seedlings (Yamada et al., 2020). Heterologous expression of a form of PEN2 lacking 64 residues from the C-terminal region could hydrolyze I3G and 4MI3G in vitro (Bednarek et al., 2009).

JA is an important signal molecule that regulates many plant physiological processes. In *Arabidopsis*, JA directly regulates root growth, plant fertility, anthocyanin accumulation, senescence, and stress resistance (Wasternack and Hause, 2013; Wasternack and Feussner, 2018). Previous studies have thoroughly characterized the JA signal transduction pathway. First, JA-Ile, the active form of JA, is recognized by the F-box protein COI1 in the nucleus (Fonseca et al., 2009; Sheard et al., 2010). COI1 interacts with the Jas domain of JASMONATE ZIM-DOMAIN (JAZ) proteins and degrades JAZ proteins through the 26S proteasome (Chini et al., 2007; Thines et al., 2007). JAZ proteins also directly interact with a series of specific transcription factors to inhibit their transcriptional activities, thus inhibiting specific physiological functions of JA (Fernandez-Calvo et al., 2011; Hu et al., 2013; Zhai et al., 2015; Chini et al., 2016; Howe et al., 2018). When JAZ proteins are degraded, transcription factors are released, reactivating JAZ transcription and triggering the JA signal. For example, the basic helix-loop-helix (bHLH)-class transcription factors MYC2, MYC3, and MYC4, as well as transcription factors TARGET OF EAT1 (TOE1) and TOE2, regulate plant root growth, resistance responses, and flowering by directly interacting with JAZ proteins (Fernandez-Calvo et al., 2011; Zhai et al., 2015). This suggests that JA regulates plant-specific responses via direct physical interaction with JAZ proteins and specific transcription factors that influence the expression of downstream genes.

FAMA is a bHLH transcription factor that was first shown to play an important role in stomatal development (Ohashi-Ito and Bergmann, 2006; Serna, 2007). FAMA and two other bHLH transcription factors, SPCH and MUTE, regulate three different stages of stomatal development (Lampard and Bergmann, 2007). In *fama* mutants, stomatal development is abnormal, and the plant is sterile (Ohashi-Ito and Bergmann, 2006). FAMA has more recently been shown to participate in the development of myrosin cells. The expression levels of *TGG1* and *TGG2* in *fama* mutants were substantially lower than expression levels in controls (Li and Sack, 2014; Shirakawa et al., 2014). Recently, we showed that, in conjunction with MED8, FAMA regulated plant resistance to *Botrytis cinerea* (Li et al., 2018). Therefore, FAMA may have multiple key functions in plants.

Although studies have shown that JA induces the expression of myrosinase genes (e.g. *BGLU18*, PYK10, and *TGG1*;

Capella et al., 2001; Matsushima et al., 2002; Ogasawara et al., 2009), there is no evidence that JA-signaling specifically regulates myrosinase activity. Here, we systematically investigate the effect of JA on myrosinase activity using molecular and genetic methods. We found that treatment with exogenous methyl jasmonate (MeJA) significantly increased myrosinase activity, but that myrosinase activity was reduced in JA-synthesis and JA-signaling mutants. Our mechanistic study showed that JAZ proteins interacted with FAMA and inhibited the transcription function of FAMA to regulate *TGG1* expression. MED8 was also involved in this process. Our results provide evidence that JA affects myrosinase activity by regulating the FAMA/MED8–*TGG1* pathway.

## Results

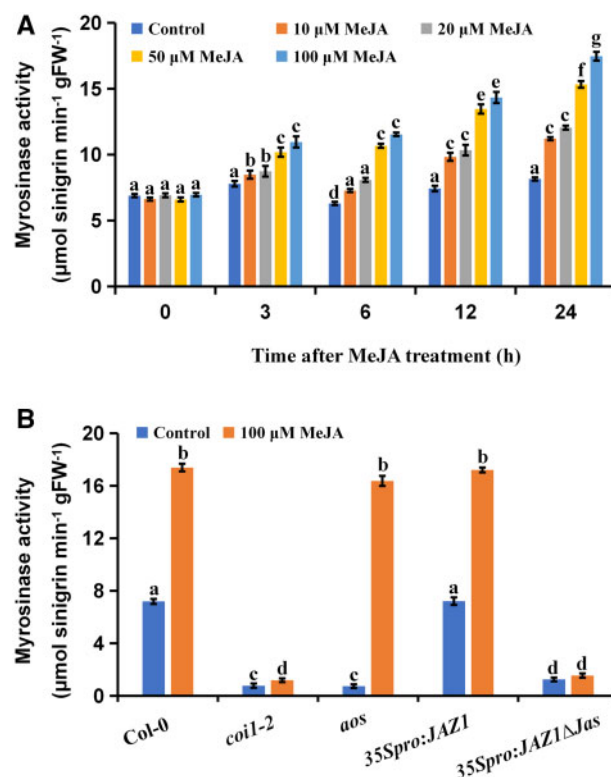
### The JA signal positively regulates myrosinase activity

To verify the role of the plant hormone JA in glucosinolate hydrolysis, 21-d-old *Arabidopsis* Col-0 seedlings were treated with MeJA, and myrosinase activity was detected after treatment. We found that, as treatment time increased, plants treated with MeJA exhibited higher levels of myrosinase activity than the untreated controls, and that, as MeJA concentration increased from 0 to 100  $\mu$ M, myrosinase activity increased concomitantly (Figure 1A). This suggested that MeJA increased myrosinase activity, in a time- and concentration-dependent manner.

To verify the role of JA in glucosinolate hydrolysis, we performed glucosinolate hydrolysis experiments using *Arabidopsis* treated with various concentrations of MeJA for 72 h. We found that the glucosinolate contents of plants treated with MeJA decreased faster, and the rate of glucosinolate decrease was proportional to the concentration of MeJA (Supplemental Figure S1, A and B). That is, plants treated with higher concentrations of MeJA exhibited higher rates of glucosinolate hydrolysis (Supplemental Figure S1, A and B). This further indicated that JA promoted glucosinolate hydrolysis.

We investigated the role played by JA biosynthesis and signaling in the regulation of myrosinase activity. As allene oxide synthase (AOS) is an important enzyme in JA synthesis (Park et al., 2002), we tested whether AOS levels affected myrosinase activity. We found that myrosinase activity in *aos* mutants was low, but that *aos* mutants treated with MeJA exhibited similar levels of myrosinase activity to MeJA-treated wild-type *Arabidopsis* seedlings (Figure 1B). This suggested that AOS affected myrosinase activity, and that JA played an important role in the regulation of myrosinase activity.

The F-box protein COI1 is the JA receptor and a key positive regulator in the JA signaling pathway (Xie et al., 1998). Previous studies have shown that myrosinase activity in *coi1* mutants is decreased (Capella et al., 2001). Consistent with this, we found that myrosinase activity decreased by 88.9% in the *coi1-2* mutant compared with the wild-type (Figure 1B). To determine whether other molecular elements in the



**Figure 1** The JA signal positively regulates myrosinase activity. A and B, Myrosinase activity of 21-d-old seedlings at different times after treatment with different concentrations of MeJA (A) and of 21-d-old seedlings of different genetic materials (for JA synthesis and signal molecules) after treating with 100  $\mu$ M of MeJA for 24 h (B). Values are means  $\pm$  SEM of 8–16 plants. The experiments were repeated at least 3 times with similar results. Different letters represent significant differences ( $P < 0.05$ , Student's *t* test).

JA pathway are also involved in the regulation of myrosinase activity, we examined the effects of JAZ1, which acts as a transcriptional repressor of JA-responsive genes (Chini et al., 2007; Thines et al., 2007), on myrosinase activity. For this purpose, we detected myrosinase activity in 35S<sub>pro</sub>::JAZ1 and 35S<sub>pro</sub>::JAZ1 $\Delta$  JAS plants, which ectopically express the JAZ1 cDNA and a Jas-domain deletion version of JAZ1 cDNA, respectively (Zhai et al., 2015). Myrosinase activity was significantly reduced in 35S<sub>pro</sub>::JAZ1 $\Delta$  JAS plants, but was similar to that of the wild-type in 35S<sub>pro</sub>::JAZ1 plants (Figure 1B). These results suggested that the inhibition of the JA pathway led to the inhibition of myrosinase activity. Along with our glucosinolate hydrolysis experiment, this experiment provides further evidence that JA biosynthesis and signaling are important for glucosinolate hydrolysis (Supplemental Figure S1, C and D).

### Effects of JA on myrosinase gene expression

We then tested whether JA affected myrosinase gene expression. A total of 22 myrosinase genes have been reported in *Arabidopsis* (Sugiyama and Hirai, 2019), but two of these (*TGG3* and *TGG6*) have been identified as pseudogenes in *Arabidopsis* Col-0 (Xue et al., 1995). Thus, we quantified

changes in the relative expression levels of the remaining 20 myrosinase genes after MeJA treatment. We found that *TGG1*, *BGLU18*, *BGLU21*, *BGLU22*, *PYK10*, *BGLU24*, *BGLU27*, *BGLU28*, *BGLU30*, and *BGLU32* were significantly upregulated after JA treatment in the wild-type Arabidopsis (Figure 2). Surprisingly, *TGG2* and *PEN2* were significantly downregulated by JA (Supplemental Figure S2), suggesting that JA might negatively regulate *TGG2* and *PEN2* expression levels. To confirm the regulatory effects of JA on myrosinase genes, we also quantified myrosinase gene expression in the *coi1-2* mutants. We found that the JA-driven upregulation of the myrosinase genes was reduced in the JA-treated *coi1-2* mutants as compared to the JA-treated wild-type plants (Figure 2). Conversely, *TGG2* and *PEN2* were upregulated in the JA-treated *coi1-2* mutants as compared to the JA-treated wild-type plants (Supplemental Figure S2). Thus, these results suggest that JA positively regulates the expression of selected myrosinase genes in Arabidopsis.

### A series of JAZ proteins interact with the FAMA transcription factor

It has previously been shown that certain plant-specific responses are regulated via the direct physical interaction of JAZ proteins with specific transcription factors (Fernandez-Calvo et al., 2011; Zhai et al., 2015; Howe et al., 2018). Thus, we screened the proteins interacting with JAZ1 using a yeast two-hybrid (Y2H) system to find these potential transcription factors involved in the process of JA-regulated myrosinase activity. Among the identified JAZ1-interacting proteins, we focused on a bHLH transcription factor, FAMA, which was previously reported to regulate the expression of *TGG1* (Li and Sack, 2014; Shirakawa et al., 2014). FAMA and two other transcription factors (MUTE and SPCH) are critically involved in stomatal development (Lampard and Bergmann, 2007; Ortega et al., 2019). Therefore, we used the Y2H system to test whether JAZ1 specifically interacted with FAMA, MUTE, and SPCH. We verified that JAZ1 specifically interacted with FAMA (Figure 3A), but that JAZ1 did not interact with either MUTE or SPCH (Supplemental Figure S3). We then examined the interactions between other JAZ family proteins and FAMA. In the Y2H system, JAZ1, JAZ2, and JAZ9 interacted with the FAMA protein, while other members of the JAZ family did not (Figure 3A).

We verified the interaction between JAZ proteins and FAMA using a split-luciferase (Split-LUC) complementation assay. We inserted the *FAMA* gene into an nLUC vector, and inserted the *JAZ1* and *JAZ9* genes into a cLUC vector. Then, *JAZ1* and *JAZ9* were co-injected with *FAMA* into *Nicotiana benthamiana* for transient co-expression. LUC fluorescence was observed in *N. benthamiana* leaves co-injected with *FAMA* and *JAZ1* or *JAZ9*, but not leaves injected with *FAMA*, *JAZ1*, or *JAZ9* alone (Figure 3B). This indicated that *JAZ1* and *JAZ9* also interacted with *FAMA* in vivo.

The interaction between *JAZ1* and *FAMA* was further verified using co-immunoprecipitation (Co-IP) assays.

Transgenic Arabidopsis carrying the *FAMA-GFP* plasmid were hybridized with transgenic Arabidopsis carrying the *35S<sub>pro</sub>:JAZ1-GUS* plasmid, and total protein from the F1 offspring was extracted and co-precipitated with the GFP antibody. Subsequent immunoblotting with the GUS antibody showed that the *JAZ1-GUS* protein was expressed in the immunoprecipitated cells (Figure 3C). This indicated that *FAMA* interacted with *JAZ1* in plant cells (Figure 3C).

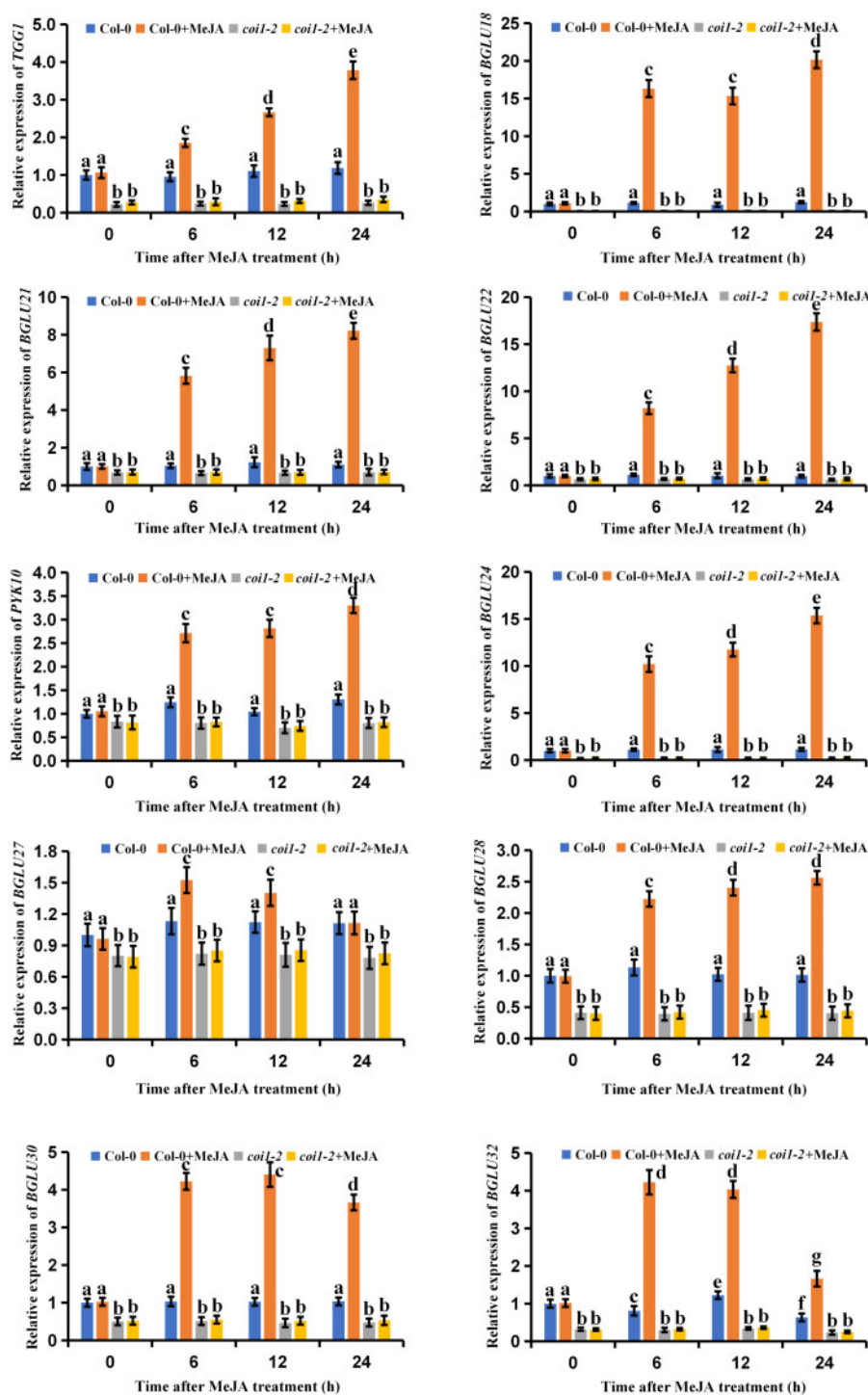
### The domains implicated in the JAZ1–FAMA interaction

The JAZ protein harbors three primary domains (Pauwels and Goossens, 2011). In general, the JAS domain interacts with COI1 and various transcription factors, the NT domain interacts with various other transcription factors, and the ZIM domain interacts with other JAZ proteins or with NINJA (Pauwels and Goossens, 2011). To verify the specific *JAZ1* domain(s) that interacted with *FAMA*, we truncated the *JAZ1* protein such that each truncated protein included only one primary domain: *JAZ1* Jas, *JAZ1* NT, or *JAZ1* ZIM. We then used these truncated proteins as bait in *FAMA* interaction analyses. Only the truncated protein containing the *JAZ1* Jas domain interacted with *FAMA* (Figure 4A), indicating that the JAS domain, but neither the NT domain nor the ZIM domain, interacted with *FAMA*.

To similarly identify which part of the *FAMA* protein interacted with *JAZ1*, we split the *FAMA* protein into an N-terminal fragment lacking the bHLH domain (*FAMA*-head) and a C-terminal fragment with the bHLH domain (*FAMA*-tail). We then used Y2H assays to test which of these fragments interacted with *JAZ1*-BD. We found that the *FAMA*-head fragment interacted with *JAZ1*, but the *FAMA*-tail fragment did not (Figure 4B), indicating that the domain of *JAZ1*–*FAMA* interaction was located at the N-terminal, before the bHLH domain.

### FAMA positively regulated JA-mediated myrosinase activity

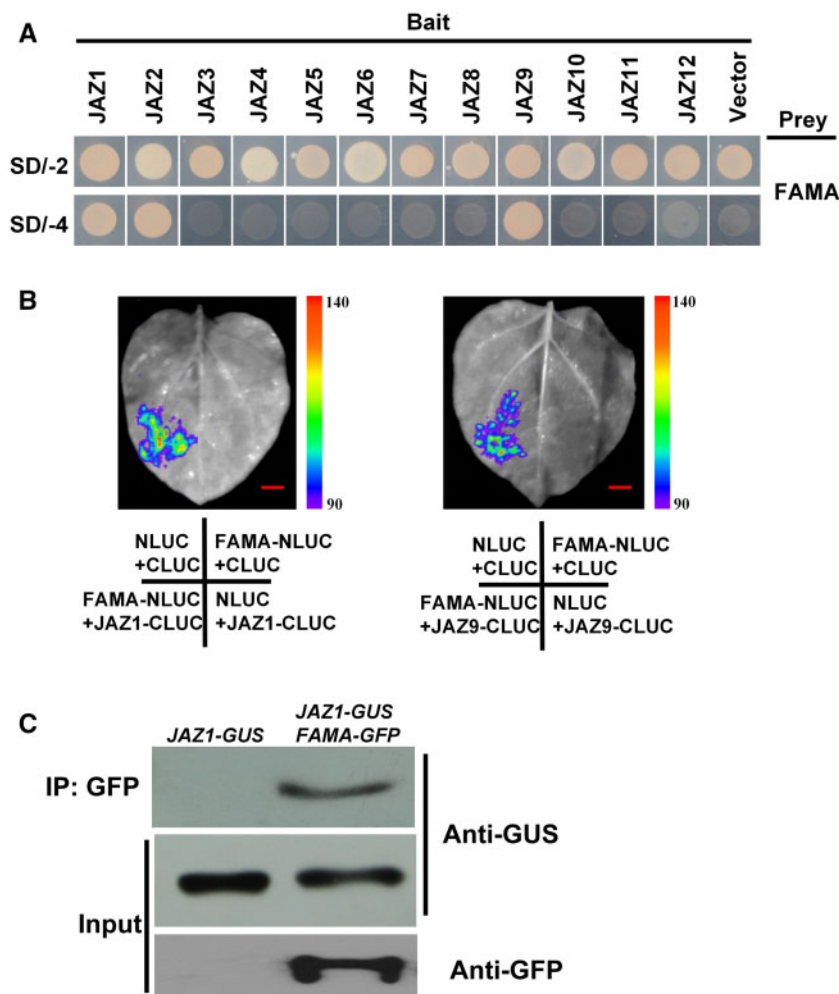
To understand the physiological significance of the interaction between *FAMA* and *JAZ1* with respect to JA-mediated myrosinase activity, we first quantified *FAMA* expression in Arabidopsis after treatment with 10–100  $\mu$ M MeJA. *FAMA* was upregulated with respect to the untreated control in all plants treated with MeJA (Figure 5A). We also quantified *FAMA* expression in the *coi1-2* mutants. We found that *FAMA* expression was reduced in the *coi1-2* mutants as compared to the wild-type plants, irrespective of MeJA treatment (Figure 5B). These results suggest that JA positively regulates *FAMA* expression. We then tested the effects of *FAMA* on myrosinase activity. We used four transgenic Arabidopsis lines previously designed in our laboratory: *fama-1* and *fama-2*, in which *FAMA* is inhibited, and *OE-3* and *OE-7*, in which *FAMA* is overexpressed (Li et al., 2018). As previously reported, *FAMA* was upregulated in 5-d-old *OE-3* and *OE-7* plants as compared to the wild-type controls, but downregulated in 21-d-old *OE-3* and *OE-7* plants



**Figure 2** Effects of JA on myrosinase gene expression. Twenty-one-day-old seedlings of wild-type (Col-0) and *coi1-2* were treated with 100  $\mu$ M of MeJA for the indicated time periods. The treated plants were harvested for total RNA extraction and RT-qPCR assays. Means  $\pm$  SEM are relative values obtained from three technical replicates. Different letters represent significant differences ( $P < 0.05$ , Student's  $t$  test).

compared to the wild-type controls (Supplemental Figure S4, A and B; Li et al., 2018). In addition, in 21-d-old *OE-3* and *OE-7* plants, myrosinase activity and *TGG1* expression were also reduced compared with controls, irrespective of MeJA treatment (Supplemental Figure S4, C and D). Conversely, in 5-d-old *OE-3* and *OE-7* plants, myrosinase activity and *TGG1* expression were increased compared with

controls, irrespective of MeJA treatment (Figures 5, B and 6). The 5-d-old *fama-1* and *fama-2* mutants exhibited inverse responses to MeJA treatment as compared to the 5-d-old *OE-3* and *OE-7* mutants (Figures 5, B and 6; Supplemental Figure S5). Similar trends were observed in hydrolysis experiments using aliphatic glucosinolates and indole glucosinolates (Supplemental Figure S5). Thus, the regulation of



**Figure 3** A subset of JAZs interact with FAMA. Y2H assay to detect interactions of FAMA with the 12 JAZ proteins. Yeast cells cotransformed with pGADT7-FAMA, or pGADT7-AP2 (preys) and pGBKT7-JAZ1-12 (bait) were grown on yeast synthetic dropout lacking Leu and Trp (SD/-2) as transformation control or on selective media lacking Ade, His, Leu, and Trp (SD/-4) to test protein interactions. pGADT7-FAMA, and pGADT7-AP2 cotransformed with pGBDT7 vector were included as a control. Split-LUC assays showing that JAZ1 and JAZ9 can interact with FAMA in *N. benthamiana* leaves. Representative images of *N. benthamiana* leaves 72 h after infiltration are shown. The images were digitally extracted for comparison. The right part of pseudocolor bar shows the range of luminescence intensity in each image. The bottom part indicates the infiltrated constructs. Bars = 10 mm. Three biological replicates were performed, and similar results were obtained. C, Co-IP assays to verify the interaction of JAZ1 with FAMA in vivo. Protein extracts from transgenic plants carrying both  $35S_{pro}$ :JAZ1-GUS and  $ProFAMA$ :FAMA-GFP (FAMA-GFP JAZ1-GUS) or from transgenic plants harboring  $35S_{pro}$ :JAZ1-GUS (JAZ-GUS) were immunoprecipitated with GFP antibody, and immunoprecipitated proteins were analyzed by immunoblotting using anti-GUS and anti-GFP antibodies. The experiments were repeated 3 times with similar results.

myrosinase activity by FAMA is a complex and precise process in plants.

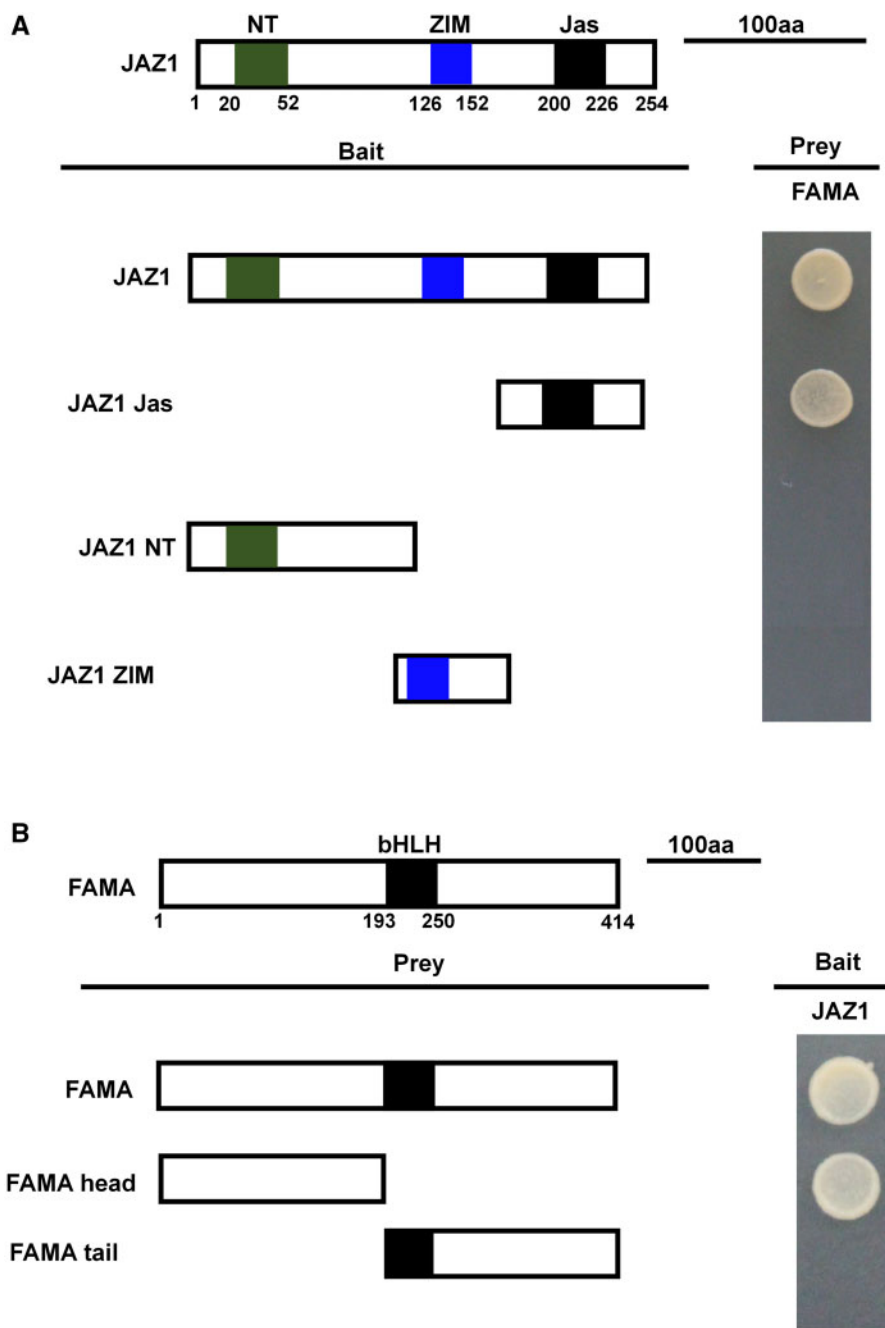
### FAMA alters the expression patterns of JA-upregulated myrosinase genes

We next investigated the effects of FAMA on the expression profiles of JA-upregulated myrosinase genes, including *TGG1*, *BGLU18*, *BGLU21*, *BGLU22*, *PYK10*, *BGLU24*, *BGLU27*, *BGLU28*, *BGLU30*, and *BGLU32*. The results showed that *TGG1* was significantly upregulated in the FAMA-overexpressing plants, while *BGLU18*, *PYK10*, *BGLU28*, and *BGLU30* were significantly downregulated; and in the *fama-1* and *fama-2* mutants, the expression patterns showed opposite trend compared with that in the FAMA-overexpressing

plants (Figure 6). The expression levels of the genes *BGLU21*, *BGLU22*, *BGLU24*, *BGLU27*, and *BGLU32* were not affected by FAMA expression or MeJA treatment (Figure 6). Together, these results indicated that FAMA had gene-specific effects on the expression patterns of JA-upregulated myrosinase genes.

### FAMA binds to the *TGG1* promoter

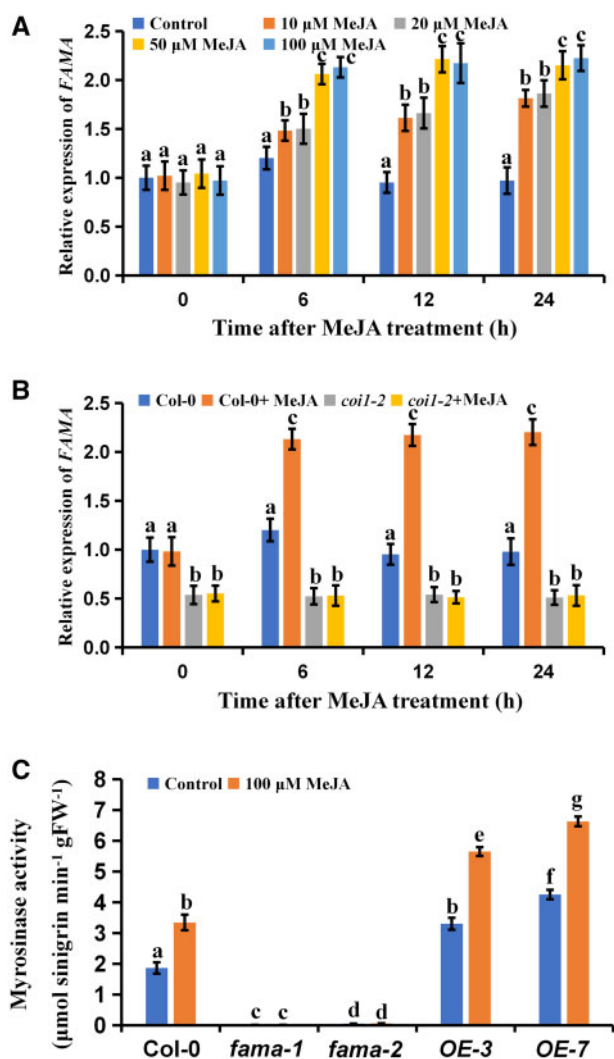
To verify that FAMA induced *TGG1* expression by binding to its promoter, we performed chromatin immunoprecipitation (ChIP) using previously developed transgenic Arabidopsis lines carrying the *ProFAMA*:FAMA-GFP plasmid (Li et al., 2018). We identified a G-box-like sequence (GCACTTGC) in the *TGG1* promoter region (Figure 7A). We



**Figure 4** Mapping of the protein domains involved in the interaction of FAMA with JAZ1, using Y2H assays. A, Based on the schematic protein structure of JAZ1, full-length JAZ1, or its derivatives (pGBKT7-JAZ1 or pGBKT7-JAZ1 derivatives) were tested for interactions with FAMA (pGADT7-FAMA). Yeast cells cotransformed with pGBKT7-JAZ1 or pGBKT7-JAZ1 derivatives (baits) and pGADT7-FAMA (prey) were grown on yeast synthetic dropout lacking Leu and Trp (SD/-2) as transformation control, or on selective media lacking Ade, His, Leu, and Trp (SD/-4) to test protein interactions. The different truncations of JAZ1 are represented. B, Based on the schematic protein structure of FAMA, full-length FAMA, or its derivatives (pGADT7-FAMA or pGADT7-FAMA derivatives) were tested for interactions with JAZ1 (pGBKT7-JAZ1). Yeast cells cotransformed with pGADT7-FAMA or pGADT7-FAMA derivatives (prey) and pGBKT7-JAZ1 (bait) were grown on yeast synthetic dropout lacking Leu and Trp (SD/-2) as transformation control, or on selective media lacking Ade, His, Leu, and Trp (SD/-4) to test protein interactions. The different domains of FAMA are represented.

therefore selected this site, two other promoter regions, the coding region, and the 3'-UTR of *TGG1* for ChIP-PCR assays. The results of these assays showed that FAMA was

significantly enriched in the G-box-like region of the promoter, as well as in exon 9 (Figure 7B). Treatment with MeJA significantly increased FAMA accumulation on the



**Figure 5** FAMA positively regulates JA-mediated myrosinase activity. Expression levels of *FAMA* of 5-d-old seedlings at different times after treatment with different concentrations of MeJA. Means  $\pm$  SEM are relative values obtained from three technical replicates; different letters represent significant differences ( $P < 0.05$ , Student's *t* test). Five-day-old seedlings of wild-type (Col-0) and *coi1-2* were treated with 100  $\mu$ M of MeJA for the indicated time periods. The treated plants were harvested for total RNA extraction and RT-qPCR assays. Means  $\pm$  SEM are relative values obtained from three technical replicates. Different letters represent significant differences ( $P < 0.05$ , Student's *t* test). C, Myrosinase activity of five-day-old seedlings of the indicated genotypes after treating with 100  $\mu$ M of MeJA for 24 h. Values are means  $\pm$  SEM of 8–16 plants. The experiments were repeated at least 3 times with similar results. Different letters represent significant differences ( $P < 0.05$ , Student's *t* test).

*TGG1* promoter (Figure 7C). These results indicated that FAMA was able to bind to the *TGG1* promoter, which was also regulated by JA.

To verify the binding of FAMA to the *TGG1* promoter, we performed electrophoretic mobility shift assays (EMSAs)

using the G-box-like sequence of *TGG1* as a probe. We found that FAMA bound the *TGG1* G-box-like sequence in vitro, and that this binding was not affected by the presence of the JAZ1 protein (Figure 7D). This may be because other factors have synergistic effects on JAZ1 as it interacts with FAMA.

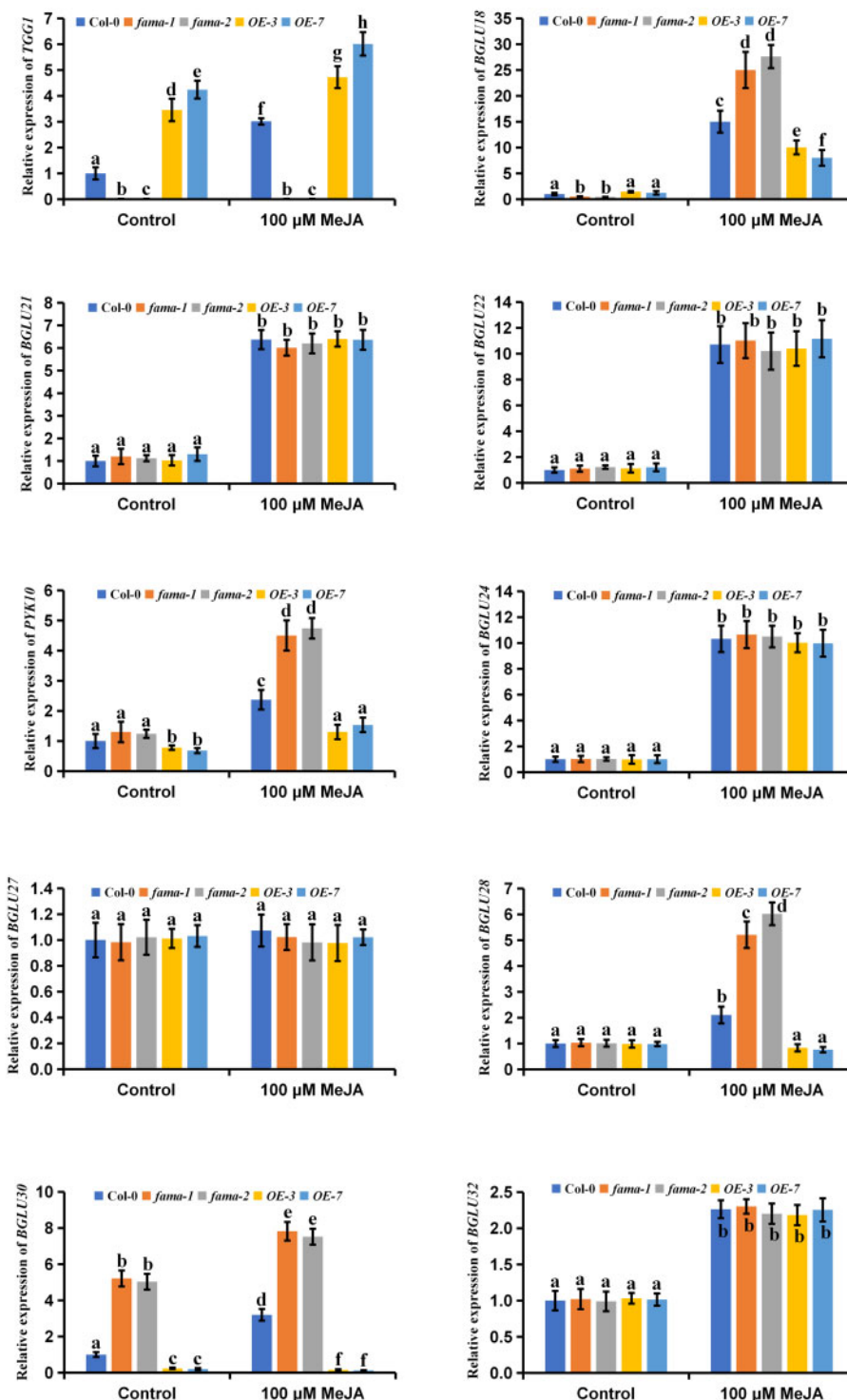
### JAZ1 inhibits the transcriptional regulation of FAMA

As transcriptional inhibitors, JAZ proteins downregulate transcription factors in the JA pathway (Chini et al., 2007; Thines et al., 2007). Therefore, we speculated that JAZ1 might also inhibit the transcriptional regulation of *FAMA*. To test this hypothesis, we examined the effects of JAZ1 on the transcription factor function of *FAMA* in *N. benthamiana*. We constructed the LUC reporter vector *ProTGG1:LUC*, and injected *ProTGG1:LUC* into *N. benthamiana* leaves for transient expression. We then measured the fluorescence intensity in *N. benthamiana* leaves. When *ProTGG1:LUC* and *35S<sub>pro</sub>:FAMA* were co-expressed in *N. benthamiana*, the fluorescence intensity of the co-expressed plants was significantly stronger than that of plants injected with *ProTGG1:LUC* alone (Figure 8, A and B). However, when *ProTGG1:LUC* was co-expressed with *35S<sub>pro</sub>:FAMA* and *35S<sub>pro</sub>:JAZ1*, the fluorescence intensity was the same as that of plants expressing *ProTGG1:LUC* alone, while the fluorescence intensity of plants expressing *35S<sub>pro</sub>:JAZ1*  $\Delta$ Jas was the same as those of plants co-expressing *ProTGG1:LUC* and *35S<sub>pro</sub>:FAMA* (Figure 8, A and B). These results indicated that FAMA regulates *TGG1* expression, and that JAZ1 inhibits the transcriptional regulation of *FAMA*.

### Transgenic FAMA expression reversed the reduced myrosinase activity of the *coi1-2* mutant

In conjunction with corroborating what is known to date about the molecular mechanisms underlying JA-mediated gene transcription, our results were also consistent with a system in which JAZ proteins accumulate in the *coi1-2* mutant and repress FAMA function, inhibiting *TGG1* expression. This downregulation of *TGG1* in the *coi1-2* mutant explains the reduced myrosinase activity observed in this mutant. We, therefore, speculated that FAMA overexpression might induce *TGG1* expression and rescue myrosinase activity in *coi1-2*. To test this, we crossed the FAMA overexpression line OE-7 with the *coi1-2* mutant to obtain OE-7/*coi1-2* plants. We then detected myrosinase activity and *TGG1* gene expression levels in the wild-type, *coi1-2*, OE-7, and OE-7/*coi1-2* plants. Consistent with expectations, myrosinase activity, and *TGG1* transcription levels were significantly lower in the *coi1-2* plants and significantly higher in the OE-7 plants, as compared to the wild-type (Figure 9, A and B). However, examination of myrosinase activity and *TGG1* transcription levels showed that, in contrast with *coi1-*

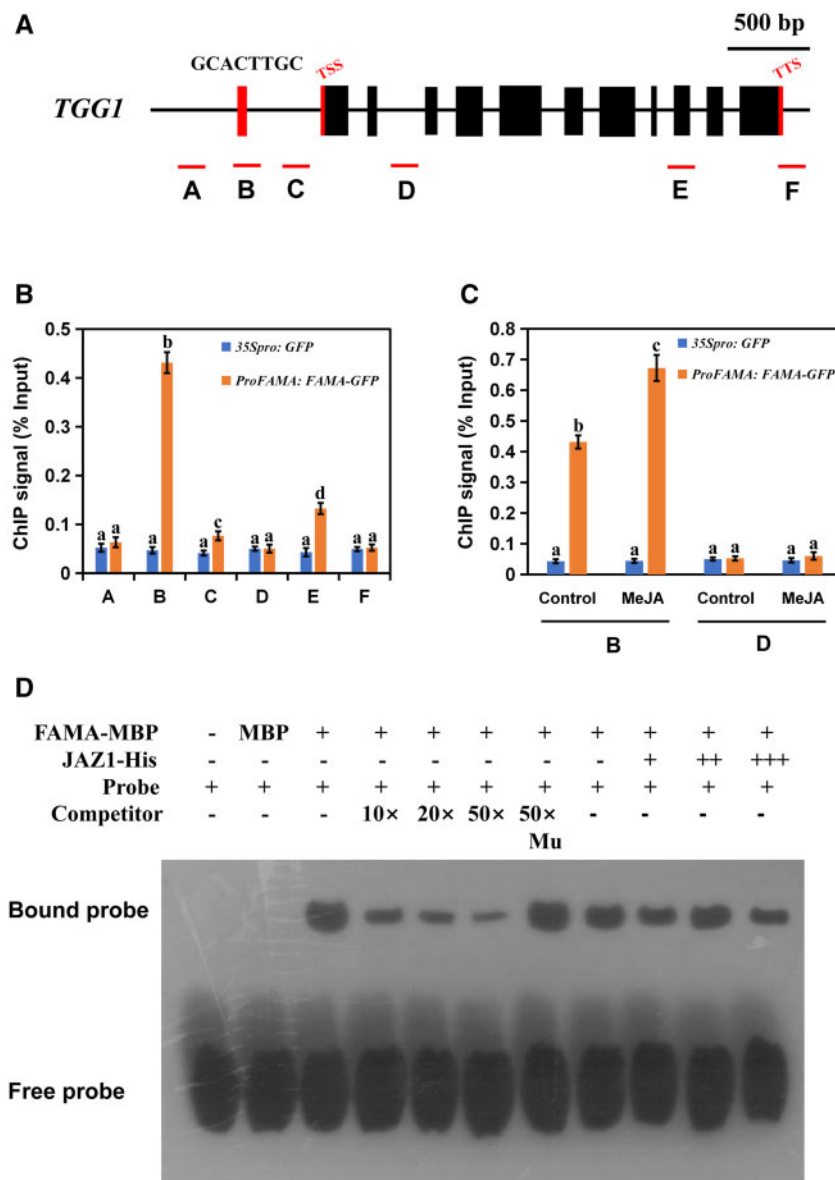




**Figure 6** Effects of FAMA on myrosinase gene expression. Five-day-old seedlings of the indicated genotypes after treating with 100 μM of MeJA for 24 h. The treated plants were harvested for total RNA extraction and RT-qPCR assays. Means ± SEM are relative values obtained from three technical replicates. Different letters represent significant differences ( $P < 0.05$ , Student's  $t$  test).

2 plants, myrosinase activity and *TGG1* transcription levels in the *OE-7/coi1-2* plants were much higher than in wild-type plants, irrespective of MeJA treatment (Figure 9, A and B). This indicated that the ectopic expression of FAMA

rescued the reduced enzyme activity associated with the *coi1-2* mutant. This possibility was supported by our tests of aliphatic- and indole-glucosinolates hydrolysis (Supplemental Figure S6, A and B).

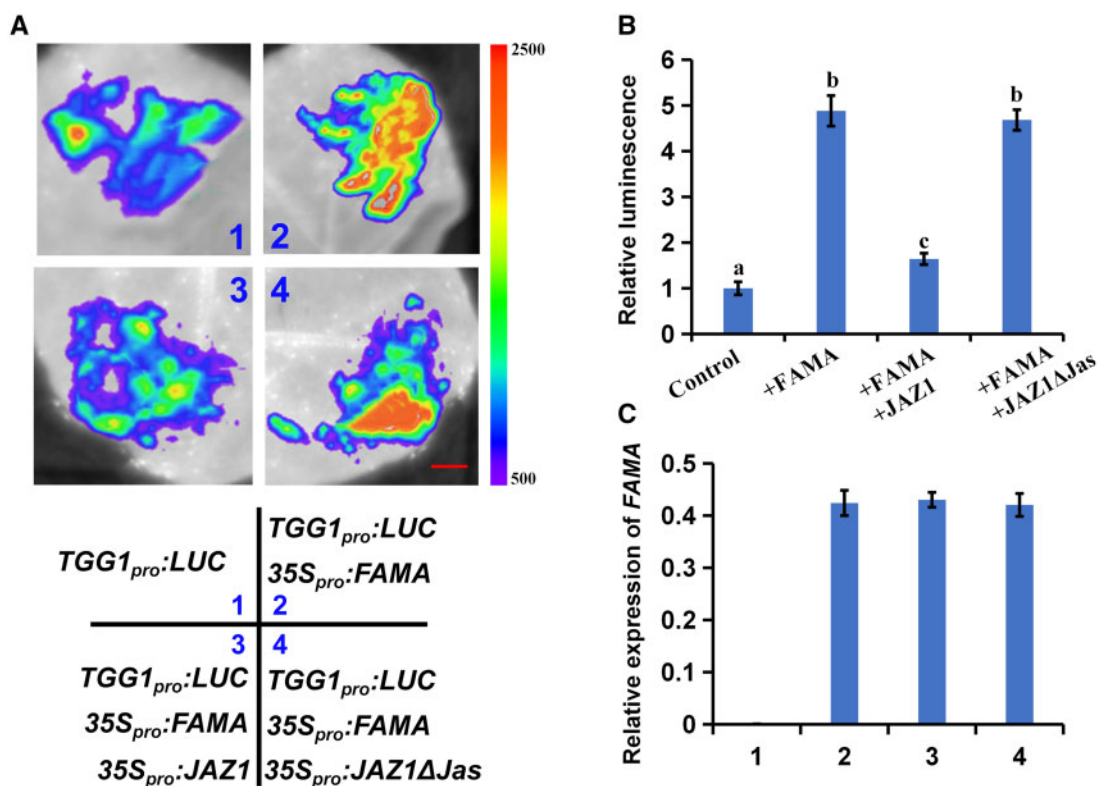


**Figure 7** FAMA could occupy the G-box-like region in the promoter of *TGG1*. Schematic diagram of *TGG1* indicating the amplicons and probe used for the ChIP-qPCR assay and EMSA. A–C are located in the promoter region of *TGG1*; D is located in the second intron region of *TGG1*; E contains the ninth exon and part of the intron region of *TGG1*; F is located in the 3′-UTR region of *TGG1*. Positions of the transcription start site and transcription termination site are indicated with thin red bars. B, ChIP-qPCR assays showing that FAMA associates with the *TGG1* locus. The chromatin of transgenic plants expressing *ProFAMA: FAMA-GFP* or *35S<sub>pro</sub>: GFP* was immunoprecipitated with an anti-GFP antibody, and *35S<sub>pro</sub>: GFP* plants served as control. Immunoprecipitated chromatin was analyzed by RT-qPCR using primers corresponding to the amplicons represented by the schematic diagram of *TGG1* (A). ChIP signal is displayed as the percentage of total input DNA. Means  $\pm$  SEM are relative values obtained from three technical replicates; different letters represent significant differences ( $P < 0.05$ , Student's *t* test). C, Dynamic recruitment of FAMA to the *TGG1* locus. ChIP assays were performed as in (B), except that *ProFAMA: FAMA-GFP* and *35S<sub>pro</sub>: GFP* plants were treated with 100  $\mu$ M of MeJA for 30 min before cross-linking. Means  $\pm$  SEM are relative values obtained from three technical replicates; different letters represent significant differences ( $P < 0.05$ , Student's *t* test). D, EMSA showing that the FAMA–maltose binding protein (MBP) fusion protein binds to the DNA probes of *TGG1* in vitro. Biotin-labeled probes were incubated with FAMA–MBP protein or FAMA–MBP and JAZ1-His proteins, and the free and bound DNAs were separated on an acrylamide gel. As indicated, the unlabeled probe and unlabeled mutant probe were used as competitors. Mu, mutated probe in which the 5′-GCACTTGC-3′ motif was replaced with 5′-TTTTTTTT-3′. A gradient concentration of JAZ1-His was applied (0.5 mg for +; 1.0 mg for ++; 1.5 mg for +++).

### MED8 is involved in the JA-mediated regulation of myrosinase activity

Previously, we showed that FAMA interacts with MED8 to regulate plant resistance to *B. cinerea* (Li et al., 2018). We

therefore also investigated whether MED8 affected myrosinase activity using the *med8* mutant. We found that myrosinase activity and *TGG1* gene expression levels in the *med8* mutant were significantly lower than those in the controls,



**Figure 8** JAZ1 represses the transcriptional function of FAMA in *N. benthamiana*. A, Transient expression assays showing that JAZ1 counteracts the function of FAMA in activating *TGG1* expression. Representative images of *N. benthamiana* leaves 72 h after infiltration are shown. The right part of pseudocolor bar shows the range of luminescence intensity in each image. The bottom part indicates the infiltrated constructs. Bars = 10 mm. B, Quantitative analysis of luminescence intensity in (A). Means  $\pm$  SEM are relative values obtained from three independent determinations; different letters represent significant differences ( $P < 0.05$ , Student's *t* test). C, RT-qPCR analysis of *FAMA* expression in the infiltrated leaf areas shown in (A). Total RNA was extracted from leaves of *N. benthamiana* coinfiltrated with the indicated constructs. Means  $\pm$  SEM are relative values obtained from three independent determinations.

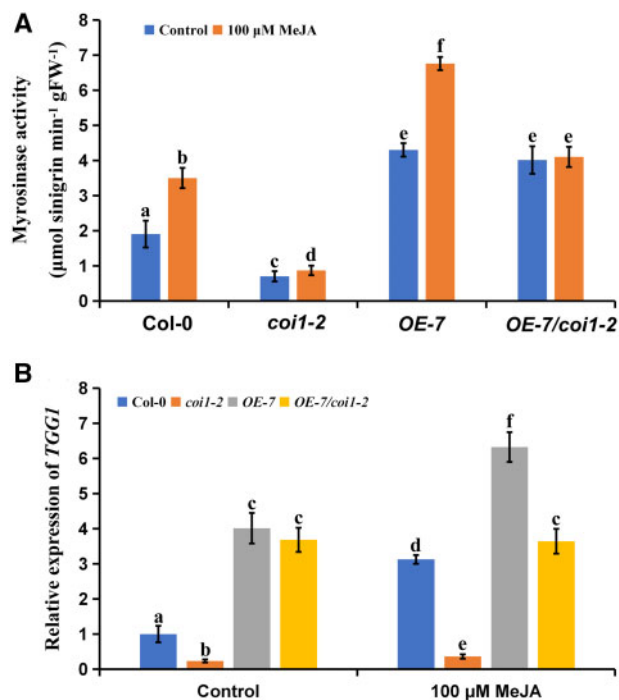
irrespective of MeJA treatment (Figure 10, A and B). Further glucosinolate hydrolysis experiments also indicated that MED8 played a positive role in the JA-mediated regulation of myrosinase activity (Supplemental Figure S7, A and B). We further tested whether MED8 could bind the *TGG1* promoter and found that, consistent with our FAMA results, MED8 bound to the G-box-like region of the *TGG1* promoter, and that MeJA treatment promoted this binding (Figure 10C). In addition, the binding of FAMA or MED8 to the *TGG1* promoter was significantly reduced in the *med8* or *fama-2* mutants (Figure 10, D and E). Therefore, our results suggested that both MED8 and FAMA participate in the JA-mediated regulation of myrosinase activity.

## Discussion

Glucosinolates play important roles in plant resistance to insect pests and pathogens (Friggmann and Gigolashvili, 2014; Vela-Corcia et al., 2019; Ting et al., 2020), and the plant hormone JA regulates glucosinolate metabolism (Mewis et al., 2005; Dombrecht et al., 2007; Liu et al., 2010). Although several previous studies have explored the regulatory effects of JA on glucosinolate synthesis (Liu et al., 2010; Mitreiter and Gigolashvili, 2021), the roles played by JA in the regulation

of glucosinolate hydrolysis remain unclear. Here, we elucidated the mechanisms underlying the JA-mediated regulation of glucosinolate hydrolysis with several different lines of evidence. First, exogenous MeJA treatment significantly increased myrosinase activity (Figure 1A). Second, the blockage of endogenous JA biosynthesis and signaling decreased myrosinase activity (Figure 1B). Third, exogenous MeJA treatment induced the expression of myrosinase genes (Figure 2). We, therefore, concluded that JA positively regulates myrosinase activity in Arabidopsis. Our FAMA binding results were consistent with this conclusion.

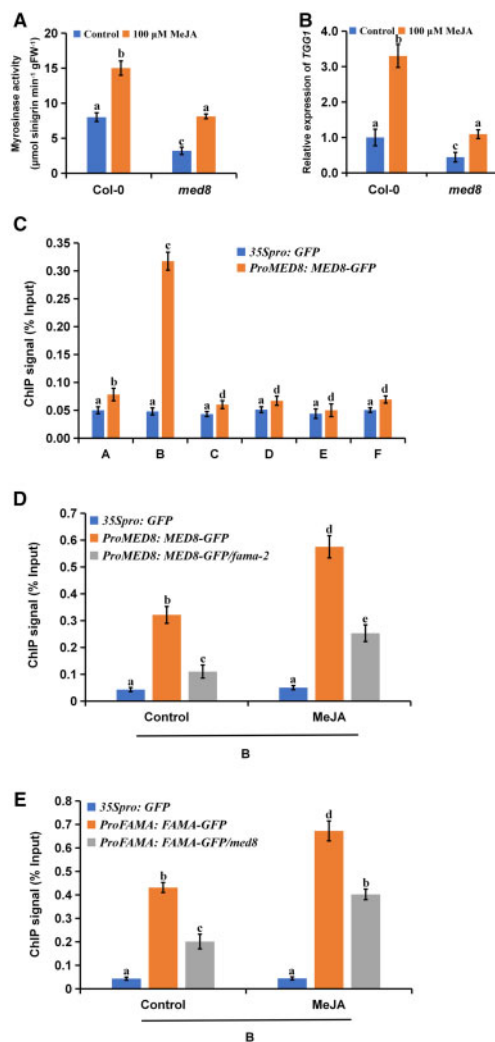
In Arabidopsis, *TGG1* and *TGG2* have redundant functions: these proteins contribute jointly to myrosinase activity in aerial plant parts (Barth and Jander, 2006). However, only *TGG1* was induced by JA (Figure 2); JA inhibited the expression of *TGG2* (Supplemental Figure S2). This indicated that *TGG1* and *TGG2* play different roles during the JA-mediated regulation of myrosinase activity. We also found that FAMA bound the promoters of both *TGG1* and *TGG2* (Figure 7; Supplemental Figure S8). MeJA treatment improved the ability of FAMA to bind to the *TGG1* promoter, but decreased the ability of FAMA to bind to *TGG2* promoter (Figure 7; Supplemental Figure S8). This suggested that JA might induce another regulator to compete with FAMA for the



**Figure 9** Transgenic expression of *FAMA* rescues the reduced enzyme activity of *coi1-2* mutant. A and B, Myrosinase activity (A) and expression levels of *TGG1* (B) of 5-d-old seedlings of the indicated genotypes after treating with 100  $\mu\text{M}$  of MeJA for 24 h. Values are means  $\pm$  SEM of 8–16 plants. The experiments were repeated at least 3 times with similar results. Different letters represent significant differences ( $P < 0.05$ , Student's *t* test).

binding site on the *TGG2* promoter, thus downregulating *TGG2*. To further clarify the mechanisms underlying the regulatory effects of JA on myrosinase activity, the factors that regulate *TGG2* expression should be thoroughly characterized in subsequent studies.

There are two types of myrosinases in Arabidopsis: the QE- and the EE-type myrosinases (Sugiyama and Hirai, 2019). In conjunction with previous studies, our results indicated that JA regulated the expression levels of both types of myrosinase genes (Figure 2). However, the relationship between the QE- and EE-type myrosinases has yet to be explored. Our results suggested that there might be antagonism between these two types of myrosinases whose activities are regulated by JA. For example, the transcription factor *FAMA* positively regulated JA-mediated *TGG1* gene expression, but negatively regulated the JA-mediated expression of the *BGLU18*, *PYK10*, *BGLU28*, and *BGLU30* genes (Figure 6). In addition, *FAMA* did not directly bind to the promoters of *BGLU18*, *PYK10*, or *BGLU28* (Supplemental Figure S9), indicating that *FAMA* may inhibit other factors that induce the expression of these three genes. Interestingly, we found that *FAMA* bound the promoter of *BGLU30* (Supplemental Figure S10), indicating that *FAMA* might compete with other factors to bind to the promoter of *BGLU30*, thus inhibiting the expression of *BGLU30*. In short, *FAMA* inhibited the expression of EE-type myrosinases



**Figure 10** MED8 positively regulates JA-mediated myrosinase activity. A and B, Myrosinase activity (A) and expression levels of *TGG1* (B) of 21-d-old seedlings of the indicated genotypes after treating with 100  $\mu\text{M}$  of MeJA for 24 h. Values are means  $\pm$  SEM of 8–16 plants. The experiments were repeated at least 3 times with similar results. Different letters represent significant differences ( $P < 0.05$ , Student's *t* test). C, ChIP-qPCR assays showing that MED8 associates with the *TGG1* locus. The chromatin of transgenic plants expressing *ProMED8: MED8-GFP* or *35S<sub>pro</sub>: GFP* was immunoprecipitated with an anti-GFP antibody, and *35S<sub>pro</sub>: GFP* plants served as control. Immunoprecipitated chromatin was analyzed by RT-qPCR using primers corresponding to the amplicons represented by the schematic diagram of *TGG1* (Figure 7A). ChIP signal was displayed as the percentage of total input DNA. Means  $\pm$  SEM are relative values obtained from three technical replicates; different letters represent significant differences ( $P < 0.05$ , Student's *t* test). D, *FAMA* affecting the recruitment of MED8 to the *TGG1* locus. ChIP assays were performed as in (C), except that *ProMED8: MED8-GFP*, *ProMED8: MED8-GFP/*fama-2**, and *35S<sub>pro</sub>: GFP* plants were treated with Control or 100  $\mu\text{M}$  of MeJA for 30 min before cross-linking. E, MED8 affecting the recruitment of *FAMA* to the *TGG1* locus. ChIP assays were performed as in (C), except that *ProFAMA: FAMA-GFP*, *ProFAMA: FAMA-GFP/*med8**, and *35S<sub>pro</sub>: GFP* plants were treated with Control or 100  $\mu\text{M}$  of MeJA for 30 min before cross-linking. D and E, Means  $\pm$  SEM are relative values obtained from three technical replicates; different letters represent significant differences ( $P < 0.05$ , Student's *t* test).

genes at a variety of levels. Thus, our results suggested that JA does not regulate the activity levels of the QE- and the EE-type myrosinases independently. Future studies should attempt to identify additional factors involved in the JA-mediated expression of *BGLU18*, *PYK10*, *BGLU28*, and *BGLU30*; the relationships between these as-yet unknown factors and FAMA are critical for the characterization of the JA-associated network through which myrosinase activity is regulated. Interestingly, we found that FAMA did not affect JA-mediated *BGLU21* or *BGLU22* gene expression (Figure 6), both of which are homologs of the *PRK10* gene (Nakano et al., 2017). This may be because *BGLU21* and *BGLU22* are expressed only in plant roots (Ahn et al., 2010), while the *FAMA* gene is expressed in aboveground parts (Ohashi-Ito and Bergmann, 2006).

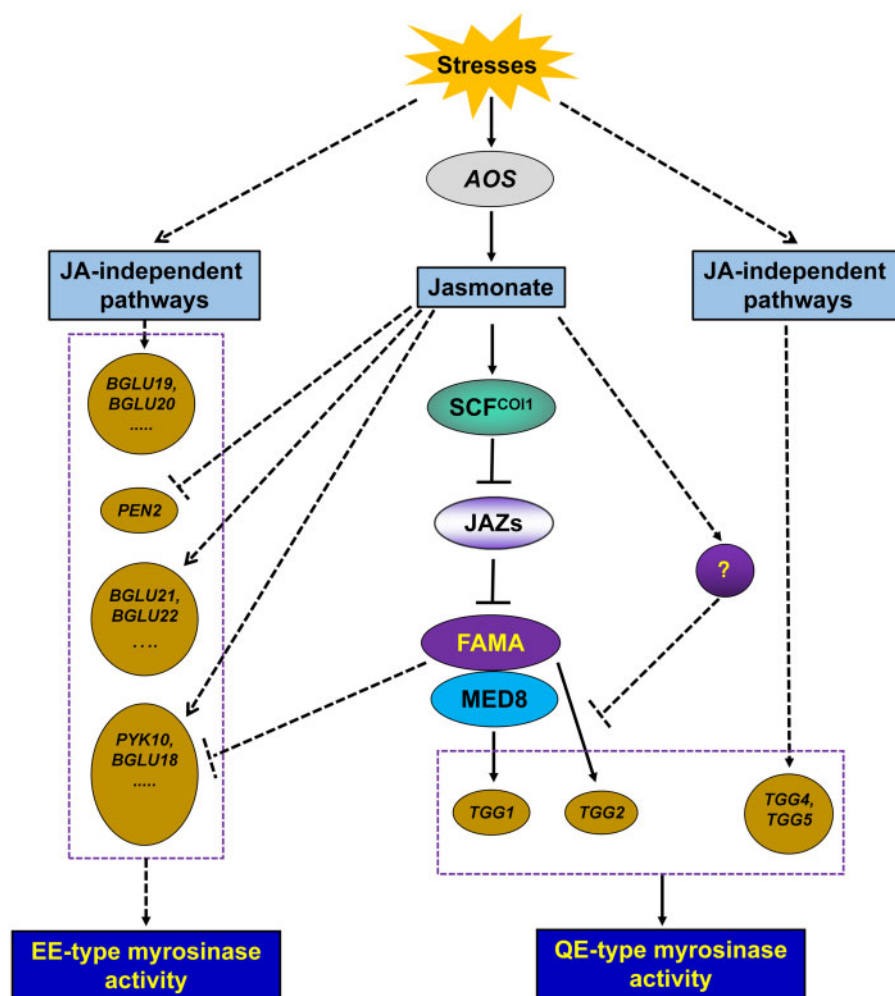
The molecular mechanisms underlying the regulation by JA of specific physiological processes can be clarified by identifying pairs of JAZ proteins and transcription factors. For example, previous studies have shown that JAZ proteins directly target several bHLH transcription factors (i.e. MYC2, MYC3, MYC4, ICE1, and ICE2), the WD-repeat/bHLH/MYB transcriptional complexes (e.g. GL3, EGL3, GL1, MYB21, and MYB24), ETHYLENE INSENSITIVE3, two APETALA2 transcription factors (TOE1 and TOE2), ROOT HAIR DEFECTIVE 6 (RHD6), and RHD6 LIKE1 to regulate various JA-mediated processes (Qi et al., 2011; Song et al., 2011; Hu et al., 2013; Zhai et al., 2015; Chini et al., 2016; Howe et al., 2018; Han et al., 2020). Here, we also found that FAMA interacted with JAZ proteins, and that JAZ proteins repressed the transcriptional effects of FAMA on *TGG1* (Figure 3; Figure 8). Moreover, the JAZ–FAMA pairing had important genetic and phenotypic effects on the mediation of myrosinase activity by JA. Thus, this study clarified the role played by JA in the regulation of myrosinase activity and elucidated the underlying molecular mechanisms.

FAMA, along with MUTE and SPCH, was initially described in relation to the regulation of various stages of stomatal development (Lampard and Bergmann, 2007). Recent studies have shown that JA is also involved in the regulation of stomatal development: JA inhibits stomatal development by regulating the expression of *FAMA*, *MUTE*, and *SPCH*, suggesting that FAMA negatively regulates JA-mediated stomatal development (Han et al., 2018). This was inconsistent with our results, which indicated that FAMA positively regulates JA-mediated myrosinase activity. Thus, FAMA may play a variety of different roles in various physiological processes involving JA. Interestingly, we found that JAZ proteins did not interact with MUTE or SPCH (Supplemental Figure S3), the other two important transcription factors in stomatal development (Torii et al., 2007), and that *TGG1* gene expression levels were similar among the wild-type, *mute*, and *spch* plants (Shirakawa et al., 2014). These results suggested that the interaction between JAZ proteins and FAMA might not be important for stomatal development, but instead might specifically help to regulate myrosinase activity.

Recent studies have shown that FAMA is also involved in the development of myrosin cells, which are idioblasts that store myrosinases (Li and Sack, 2014; Shirakawa et al., 2014). In Arabidopsis, myrosin cells play an important role in pest and disease resistance (Chhajed et al., 2020). However, there is as of yet no evidence that JA is associated with myrosin cells. Our results showed that JA induced the expression of *FAMA* (Figure 5A), and that, in the *coi1-2* mutant, *FAMA* expression was significantly reduced compared with the wild-type (Figure 5B). Previous studies have shown that FAMA overexpression increases the number of myrosin cells in hypocotyls and roots, and that myrosin cell numbers are substantially reduced in *fama* mutants (Li and Sack, 2014; Shirakawa et al., 2014). Our work, in conjunction with these previous results, indirectly indicates that JA may positively regulate the development of myrosin cells. Further genetic and cellular data are needed to more precisely define the role of JA in the development of myrosin cells.

The transcription mediator complex plays an important role in transcriptional regulation (Backstrom et al., 2007; Chen et al., 2012; Zhai and Li, 2019). For example, mediator complex subunit 25 (MED25), the most important transcription mediator complex subunit in the JA pathway, regulates JA-mediated root length inhibition and disease resistance (An et al., 2017; Wang et al., 2019; You et al., 2019; Wu et al., 2020). Previously, we demonstrated that MED8 regulates plant resistance to *B. cinerea* by interacting with FAMA (Li et al., 2018). Here, we found that MED8 was also involved in JA-mediated regulation of myrosinase activity (Figure 10). This suggested that MED8 and FAMA may work in concert to affect various plant physiological processes.

Based on our results, we propose a simple working model of the molecular mechanism underlying the JA-mediated FAMA/MED8–*TGG1* pathway in the regulation of myrosinase activity (Figure 11). Under normal growth conditions, JAZ repressors physically interact with the FAMA transcription factor, attenuating its transcriptional function, and thereby repressing the expression of the downstream *TGG1* gene. However, when plants are attacked by diseases or insect pests, endogenous JA is produced, which promotes glucosinolate synthesis. JA-Ile is recognized by the receptor COI1, leading to COI1 activation. Activated COI1 then degrades JAZ proteins, triggering FAMA transcriptional activity and activating downstream *TGG1* gene. This in turn promotes glucosinolate hydrolysis. However, many uncertainties remain, such as the role of *TGG2* in the JA-mediated regulation of myrosinase activity, the relationship between the two JA-mediated myrosinase activities (i.e. QE or EE), and the mechanisms underlying the coordination of JA-mediated stomatal development and myrosinase activity by FAMA. The answers to these questions will help us to understand the physiological processes mediated by JA in plants.



**Figure 11** A model of JA-dependent and JA-independent signaling pathways in regulating the enzyme activity of myrosinase. When plants are attacked by diseases and insect pests, stressed plants could induce the expression of myrosinase genes to resist the stresses. There are two signaling pathways to mediate the expression of myrosinase genes: One is the JA-independent signaling pathway which regulates the expression of *BGLU19*, *BGLU20*, *TGG4*, and so on; the other one is the JA-dependent signaling pathway. For the JA-dependent signaling pathway in regulating the expression of myrosinase genes, when plants are attacked by diseases and insect pests, endogenous JA will be produced through inducing AOS expression. JA-Ile can be recognized by the receptor COI1 ( $SCF^{COI1}$ ) and activate COI1. The activated COI1 can degrade the JAZ proteins, release the transcriptional activity of FAMA, and activate the downstream *TGG1* gene expression to promote the synthesis of myrosinase, to promote the hydrolysis of glucosinolate. JA also mediates myrosinase activity by inducing or inhibiting the expression of other myrosinase genes. The final outcome of jasmonate regulation is the enhancement of myrosinase activity. Purple boxes represent the two types of myrosinases; the solid lines represent the pathways that have been clarified in this study; dashed lines represent the pathways that need further study; the question mark bubble represents the important unknown element in the regulation of *TGG2* expression by JA, which needs further exploration.

## Conclusions

We demonstrated that JA mediates myrosinase activity by regulating *TGG1* expression in conjunction with the JAZ–FAMA/MED8–*TGG1* pathway: FAMA and MED8 bind the *TGG1* promoter and regulate *TGG1* expression, while JAZ proteins repress the transcriptional activity of FAMA. The results of this study help to extend and deepen our understanding of the roles played by JA in plants.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis* ecotype Col-0 was used as the wild-type. Some of the plant materials used in this study were previously

described: *coi1-2* (Xu et al., 2002);  $35S_{pro}::JAZ1$  and  $35S_{pro}::JAZ1\Delta Jas$  (Zhai et al., 2015); *aos* (Park et al., 2002);  $35S_{pro}::GFP$  (Li et al., 2011); *fama-1*, *fama-2*, *OE-3* (ProFAMA: FAMA-GFP 3#), *OE-7* (ProFAMA: FAMA-GFP 7#), *med8*, ProMED8: MED8-GFP, ProMED8: MED8-GFP/*fama-2*, and ProFAMA: FAMA-GFP/*med8* (Li et al., 2018). *OE-7/coi1-2* was generated by crossing the parental single homozygous lines.

Plants were grown initially on half-strength Murashige and Skoog (MS) agar plates for 10 d and then transferred to sterile soil in plastic trays at 22°C with a 16-h light/8-h dark photoperiod (light intensity 120  $\mu M$  photons  $m^{-2} s^{-1}$ ). *Nicotiana benthamiana* was grown in sterile soil under a 16-h light (28°C)/8-h dark (22°C) photoperiod.

### Measurement of total myrosinase activity

Total myrosinase activity was measured as described by Barth and Jander, with slight modifications (Barth and Jander, 2006). Plant tissues were collected and ground into a powder in dry ice. The powder was then added to 0.3 mL of pre-chilled sodium phosphate buffer (33 mM; pH 7.0) and vortexed well. The mixture was centrifuged at 10,000g at 4°C for 10 min. Then, 200 µL of the supernatant was added to a Sephadex G-50 (Sigma-Aldrich, St. Louis, MO, USA) column; Sephadex columns were filled with resin that was packed in 33 mM sodium phosphate buffer (pH 7.0) overnight at 4°C. After supernatant addition, columns were centrifuged at 720g at 4°C for 1 min. Then, 100 µL of filtrate was collected and diluted with water to a final volume of 200 µL. Next, 0.34 mM sinigrin and 0.3 mM ascorbic acid were separately diluted to 200 µL with the diluted filtrate mixture. The new mixtures were plated in 96-well plates, which were analyzed at 25°C for 15 min with a microplate reader at 227 nm; data were collected every 10 s. Myrosinase activity was calculated following Barth and Jander (2006).

### Measurement of glucosinolate breakdown

Glucosinolate breakdown experiments were performed as described by Barth and Jander, with minor modifications (Barth and Jander, 2006). Leaves were collected from the indicated plants (80 mg fresh weight). Samples collected 0 min after treatment were immediately snap-frozen in liquid nitrogen. Samples collected 1 min after treatment were ground with a plastic pestle in 400 µL of water for 0.25 min, then held an additional 0.75 min at room temperature before snap-freezing in liquid nitrogen. Samples collected at all other time points were ground with a plastic pestle in 400 µL of water for 0.25 min. Next, 60 µL of 100% (v/v) methanol was added to each sample and vortexed several times. Each mixture was immediately heated to 75°C for 15 min to inactivate myrosinase and to extract glucosinolates. Finally, 10 µL of 1.25 mM sinigrin was mixed with extracted glucosinolates as an internal standard. The desulfoglucosinolates in the mixtures were quantified using high-performance liquid chromatography following Barth and Jander (2006). Glucosinolate hydrolysis rate was calculated as (glucosinolate content before reaction – glucosinolate content after reaction)/reaction time.

### RNA analysis

Total RNA extraction was performed as previously described (Li et al., 2018). Reverse transcription quantitative PCR (RT-qPCR) was performed as previously described (Yu et al., 2020). All primers used for RT-qPCR are listed in Supplemental Table S1.

### Y2H assays

The full-length coding sequence of JAZ1 was amplified with the primers listed in Supplemental Table S1. The PCR products were enzyme-digested and cloned into the pGBKT7 vector. Y2H screening was performed using the pGADT7-

based Arabidopsis cDNA library described previously (Zhai et al., 2015). FAMA was identified by screening.

The interaction between JAZs and FAMA was verified following a previous study (Li et al., 2018). Briefly, 12 JAZs, including JAZ1, and the associated domain were cloned into separate pGBKT7 vectors. FAMA and its derivatives were cloned into the pGADT7 vector. Primers used for vector construction are listed in Supplemental Table S1. Interactions were observed after incubation with the yeast strain *Saccharomyces cerevisiae* AH109 for 3 d at 30°C.

### Transient expression assay in *N. benthamiana* leaves

The transient expression assays were performed in *N. benthamiana* leaves as previously described (Li et al., 2018). Briefly, for Split-LUC complementation assay, FAMA was cloned into vector pCAMBIA1300-nLUC, and JAZ1 or JAZ9 was cloned into vector pCAMBIA1300-cLUC. Primers are summarized in Supplemental Table S1. For transcriptional activation assays, the *TGG1* promoter was amplified using Gateway-compatible primers. The PCR products were cloned by pENTR Directional TOPO cloning kits (Invitrogen, Waltham, MA, USA) and recombined with the binary vector pGWB35 to generate the reporter construct *TGG1<sub>pro</sub>: LUC*. The FAMA effector construct was *35S<sub>pro</sub>: FAMA-GFP* (*35S<sub>pro</sub>: FAMA*). We used a low-light cooled CCD imaging apparatus (NightOWL II LB983 with indigo software) to capture the LUC image and to assess luminescence intensity. The leaves were sprayed with 0.5 mM luciferin and placed in darkness for 3 min prior to luminescence detection. The camera was cooled to –110°C and the images were taken with an exposure time of 2 min. Relative LUC activity was equivalent to luminescence intensity/0.2 mm<sup>2</sup> leaf area. Relative luminescence was the ratio of relative LUC activity between different experimental groups and control groups.

### Co-IP assays

To further investigate the interaction between JAZ1 and FAMA, Arabidopsis carrying the *35S<sub>pro</sub>: JAZ1-GUS* plasmid was crossed with plants carrying the *ProFAMA: FAMA-GFP* plasmid (Zhai et al., 2015; Li et al., 2018). Five-day-old F1 *ProFAMA: FAMA-GFP* (*JAZ1-GUS FAMA-GFP*) seedlings were used in Co-IP assays; 5-d-old seedlings carrying only *35S<sub>pro</sub>: JAZ1-GUS* were used as controls. All plants were harvested and ground in liquid nitrogen. The ground samples were added to extraction buffer containing 50 mM Tris-HCl (pH 7.5) 150 mM NaCl, 0.1% (v/v) Triton X-100, 0.2% (v/v) Nonidet P-40, 0.6 mM phenylmethylsulfonyl fluoride (PMSF), and 20 µM MG132, as well as 1× Roche protease inhibitor cocktail. After protein extraction, 20 µL protein G plus agarose (Santa Cruz Biotechnology, Dallas, TX, USA) was added to 3-mg protein extracts to reduce nonspecific immunoglobulin binding. After 1 h of incubation, the supernatant was transferred to a new tube. GFP antibody-bound agarose beads (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) were then added to each reaction and incubated for 1 h at 4°C. The precipitated samples were washed at least 3 times with lysis buffer. Samples were then eluted by adding

1 × SDS protein loading buffer and boiling for 5 min. Total and immunoprecipitated proteins were analyzed by immunoblotting using anti-GUS and anti-GFP antibodies.

### EMSA

The full-length coding sequences of FAMA and JAZ1 were amplified and cloned into pMAL-c2 and pET32a vectors, respectively. Primers are summarized in [Supplemental Table S1](#). The recombinant FAMA and JAZ1 proteins were expressed in *Escherichia coli*. Oligonucleotide probes were synthesized and labeled with biotin at the 3'-end (Invitrogen). EMSAs were performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific, Waltham, MA, USA) following a previous study (Zhai et al., 2015). Briefly, the synthesized probes were incubated with or without indicated proteins in incubation buffer (1 × binding buffer, 2.5% (v/v) glycerol, 50 mM KCl, 5 mM MgCl<sub>2</sub>, and 10 mM EDTA) at room temperature for 20–30 min. Unlabeled probes were added to the reactions as competition assays. The probe sequence was 5'-CCCCTTCC TCACATCTGCACTTGCTAAGCCAACCTTGCTTACTTATG ACCACC-3', and the mutated probe sequence was 5'-CCCCTTCCCTCACATCTTTTTTTTTTAAGCCAACCTTGGCT TACTTATGACCACC-3'.

### ChIP-PCR assays

Seedlings of several previously constructed transgenic lines (*ProFAMA*: FAMA-GFP, *ProMED8*: MED8-GFP, *ProMED8*: MED8-GFP/*fama-2*, *ProFAMA*: FAMA-GFP/*med8*, and *35S<sub>pro</sub>*: GFP; Li et al., 2018) were grown in MS medium. Five-day-old seedlings of each line were either treated with 50 μM of MeJA for 24 h or left untreated as controls. After 24 h, 1.5 g samples of treated and control plants were collected and cross-linked in 1% (v/v) formaldehyde. Following cross-linking, a GFP antibody (Abcam, Cambridge, UK) was used to immunoprecipitate the chromatin protein–DNA complex, and the precipitated DNA was purified using a PCR purification kit (Qiagen, Hilden, Germany) in preparation for real-time RT-qPCR analysis. All ChIP-PCR assays were performed 3 times. Chromatin precipitated from *35S<sub>pro</sub>*: GFP seedlings were considered the negative control, while chromatin isolated before precipitation was used as the input control. Primers used for this analysis are listed in [Supplemental Table S1](#).

### Accession numbers

The accession numbers of the genes mentioned in this article are given below and their sequence data can be found in Arabidopsis genome initiative ([www.arabidopsis.org](http://www.arabidopsis.org)): *COI1* (AT2G39940), *JAZ1* (AT1G19180), *JAZ2* (AT1G74950), *JAZ3* (AT3G17860), *JAZ4* (AT1G48500), *JAZ5* (AT1G17380), *JAZ6* (AT1G72450), *JAZ7* (AT2G34600), *JAZ8* (AT1G30135), *JAZ9* (AT1G70700), *JAZ10* (AT5G13220), *JAZ11* (AT3G43440), *JAZ12* (AT5G20900), *AOS* (AT5G42650), *FAMA* (AT3G24140), *SPCH* (AT5G53210), *MUTE* (AT3G06120), *TGG1* (AT5G26000), *TGG2* (AT5G25980), *TGG3* (AT5G48375), *TGG4* (AT1G47600), *TGG5* (AT1G51470), *TGG6* (AT1G

51490), *BGLU18* (AT1G52400), *BGLU19* (AT3G21370), *BGLU20* (AT1G75940), *PYK10* (AT3G09260), *BGLU21* (AT1G66270), *BGLU22* (AT1G66280), *BGLU24* (AT5G28510), *BGLU25* (AT3G03640), *PEN2* (AT2G44490), *BGLU27* (AT3G60120), *BGLU28* (AT2G44460), *BGLU29* (AT2G44470), *BGLU30* (AT3G60140), *BGLU31* (AT5G24540), *BGLU32* (AT5G24550), *BGLU33* (AT2G32860), and *MED8* (AT2G03070).

### Supplemental data

The following materials are available in the online version of this article.

**Supplemental Figure S1** The JA signal positively regulates the hydrolysis rate of glucosinolates.

**Supplemental Figure S2** Expression levels of JA-unaffecting and JA-downregulated myrosinase genes.

**Supplemental Figure S3** JAZ1 did not interact with MUTE or SPCH.

**Supplemental Figure S4** The phenotypes of 21-d-old FAMA mutants and overexpression plants in JA-regulated myrosinase activity.

**Supplemental Figure S5** FAMA positively regulates the hydrolysis rate of glucosinolates.

**Supplemental Figure S6** Transgenic expression of FAMA rescues the reduced hydrolysis rate of glucosinolates of *coi1-2* mutant.

**Supplemental Figure S7** MED8 positively regulates the hydrolysis rate of glucosinolates.

**Supplemental Figure S8** JA repressed the occupation of FAMA on the G-box-like region in the promoter of *TGG2*.

**Supplemental Figure S9** FAMA did not bind the promoters of *BGLU18*, *PYK10*, or *BGLU28*.

**Supplemental Figure S10** FAMA bound the promoters of *BGLU30*.

**Supplemental Table S1** List of primers used in this article.

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