The Jasmonate-ZIM-Domain Proteins Interact with the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin Accumulation and Trichome Initiation in Arabidopsis thaliana

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Jasmonates (JAs) mediate plant responses to insect attack, wounding, pathogen infection, stress, and UV damage and regulate plant fertility, anthocyanin accumulation, trichome formation, and many other plant developmental processes. Arabidopsis thaliana Jasmonate ZIM-domain (JAZ) proteins, substrates of the CORONATINE INSENSITIVE1 (CO1)-based SCFCOI1 complex, negatively regulate these plant responses. Little is known about the molecular mechanism for JA regulation of anthocyanin accumulation and trichome initiation. In this study, we revealed that JAZ proteins interact with bHLH (Transparent Testa8, Glabra3 [GL3], and Enhancer of Glabra3 [EGL3]) and R2R3 MYB transcription factors (MYB75 and Glabra1), essential components of WD-repeat/bHLH/MYB transcripational complexes, to repress JA-regulated anthocyanin accumulation and trichome initiation. Genetic and physiological evidence showed that JA regulates WD-repeat/bHLH/MYB complex-mediated anthocyanin accumulation and trichome initiation in a CO1-dependent manner. Overexpression of the MYB transcription factor MYB75 and bHLH factors (GL3 and EGL3) restored anthocyanin accumulation and trichome initiation in the coi1 mutant, respectively. We speculate that the JA-induced degradation of JAZ proteins abolishes the interactions of JAZ proteins with bHLH and MYB factors, allowing the transcriptional function of WD-repeat/bHLH/MYB complexes, which subsequently activate respective downstream signal cascades to modulate anthocyanin accumulation and trichome initiation.

INTRODUCTION

Anthocyanins, a class of flavonoids widely found in plants, are responsible for plant coloration (Springob et al., 2003; Lepiniec et al., 2006). They allure insects to pollinate flowers (Vogt et al., 1994), participate in pollen–pistil interactions (Mo et al., 1992), protect plants from UV radiation (Harborne and Williams, 2000; Solovchenko and Schmitz-Eiberger, 2003) and stress damage (Winkel-Shirley, 2002), and function as antimicrobial agents against insect attack and pathogen infection (Harborne and Williams, 2000; Winkel-Shirley, 2001; Gould, 2004).

Anthocyanin accumulation is stimulated by a series of endogenous developmental signals, such as Suc (Teng et al., 2005; Solfanelli et al., 2006) and plant hormones (Deikman and Hammer, 1995; Loreti et al., 2008; Shan et al., 2009), and by various environmental stresses, including UV irradiation, pathogen infection, insect attack, drought, wounding, and nutrient depletion (Harborne and Williams, 2000; Cominelli et al., 2008; Gonzalez et al., 2008; Lillo et al., 2008; Gonzalez, 2009).

The WD-repeat/bHLH/MYB complex acts as an important regulatory machinery to modulate anthocyanin accumulation in plant species (Payne et al., 2000; Broun, 2005; Ramsay and Glover, 2005). In Arabidopsis thaliana, the WD-repeat protein Transparent Testa Glabra1 (TTG1) (Walker et al., 1999) recruits basic-helix-loop-helix (bHLH) transcription factors (Toledo-Ortiz et al., 2003), such as Glabra3 (GL3) (Payne et al., 2000), Transparent Testa8 (TTB) (Nesi et al., 2000; Baudry et al., 2006), or Enhancer of Glabra3 (EGL3) (Zhang et al., 2003), and R2R3 MYB transcriptional factors (MYB75, MYB90, MYB113, or MYB114) (Borevitz et al., 2000; Stracke et al., 2001; Zimmermann et al., 2004; Stracke et al., 2007; Allan et al., 2008; Gonzalez et al., 2008; Rowan et al., 2009) to form the WD-repeat/bHLH/MYB complex, which mediates anthocyanin biosynthesis mainly by upregulating the expression of late anthocyanin biosynthetic genes, including NADPH-dependent dihydroflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), and UDP-Glc:flavonoid 3-O-glucosyltransferase (UFGT) (Dooner et al., 1991; Kubasek et al., 1992; Shirley et al., 1995; Gonzalez et al., 2008).
The WD-repeat/bHLH/MYB complex, consisting of TTG1 (Walker et al., 1999), the bHLH protein GL3 (Payne et al., 2000), or EGL3 (Bernhardt et al., 2003) and the R2R3 MYB protein GL1 or MYB23 (Oppenheimer et al., 1991; Kirk et al., 2005; Morohashi and Grotewold, 2009), also mediates trichome development (Payne et al., 2000; Zhao et al., 2008; Pesch and Hülskamp, 2009). Trichomes are fine structures differentiated from epidermal cells in the aerial part of plant (Ishida et al., 2008), which assist seed dispersal (Wagner et al., 2004) and function as barriers to protect plants against herbivores, insects, abiotic damage, UV irradiation, and excessive transpiration (Wagner et al., 2004; Ishida et al., 2008). Trichome formation is initiated by various environmental cues, such as wounding and insect attack (Larkin et al., 1996; Serna and Martin, 2006; Yoshida et al., 2009), and by different endogenous developmental signals, including phytohormones, such as gibberellin (Perazza et al., 1998) and jasmonate (JA; Traw and Bergelson, 2003; Li et al., 2004). Previous studies showed that JA can induce anthocyanin biosynthesis and trichome initiation (Chini et al., 2007; Thines et al., 2007; Fonseca et al., 2009a; Seo et al., 2011; Song et al., 2011). Upon perception of a JA signal, COI1 recruits MYC2 and its closely related members MYB90, MYB113, MYB114, and GL1 (Stracke et al., 2001), and a WD-repeat protein, TTG1, form WD-repeat/bHLH/MYB complexes to mediate diverse plant responses (Zimmermann et al., 2004; Gonzalez et al., 2008; Zhao et al., 2008). We used Y2H to investigate interactions of the 12 JAZ proteins with the major members of the WD-repeat/bHLH/MYB complexes, including three bHLH factors (TT8, GL3, and EGL3), two representative MYB factors (MYB75 and GL1), and the WD-repeat protein TTG1. Positive interactions of MYC2 (Lorenzo et al., 2004; Dombrecht et al., 2007) with 12 JAZ proteins (Figure 1) (Chini et al., 2009) and immunoblot analysis of the yeast strains (see Supplemental Figure 1 online) confirmed that all 12 JAZ fusion proteins were expressed in yeasts. As shown in Figure 1, the three bHLH transcription factors EGL3, GL3, and TT8 strongly interacted with eight JAZ proteins (JAZ1, JAZ2, JAZ5, JAZ6, JAZ8, JAZ9, JAZ10, and JAZ11) but not with the remaining four JAZ proteins (JAZ3, JAZ4, JAZ7, and JAZ12). The two MYB factors MYB75 and GL1 exhibited interactions with JAZ1, JAZ8, and JAZ11. GL1 exhibited additional interaction with JAZ10. However, the WD-repeat protein TTG1 did not interact with any JAZ protein in yeast. In conclusion, these results imply that several JAZ proteins may physically interact with the bHLH and MYB members but not with the WD-repeat protein from WD-repeat/bHLH/MYB complexes.

We next adopted bimolecular fluorescence complementation (BiFC) assays (Weinthal and Tzfira, 2009) to verify interactions of JAZ proteins with the bHLH (GL3, EGL3, and TT8) and MYB members (MYB75 and GL1) in planta. The N-terminal fragment of yellow fluorescent protein (nYFP) was ligated with JAZ1 and JAZ10 to produce JAZ1-nYFP and JAZ10-nYFP. The bHLH and MYB members were individually fused with the C-terminal fragment of YFP (cYFP). JAZ1-nYFP was transiently coexpressed with each bHLH or MYB member in Nicotiana benthamiana leaves. We found that coexpression of JAZ1-nYFP with cYFP-EGL3 resulted in strong YFP fluorescence in the nucleus of epidermal cells in N. benthamiana leaves (Figure 2A), whereas no YFP fluorescence was detected in negative controls (JAZ1-nYFP...
coexpressed with cYFP or nYFP coexpressed with cYFP-EGL3) (see Supplemental Figure 2 online). Similar results were observed for coexpression of JAZ1-nYFP with GL3, TT8, MYB75, GL1, TTG1, and MYC2 were fused with activation domain. MYC2 was used as a positive control (Chini et al., 2009). Protein–protein interactions were assessed on SD/Gal/Raf/X-gal (-Ura/-His/-Trp/-Leu) medium in 96-well plates.

We further applied pull-down assays to verify the JAZ interactions with these transcription factors in vitro. Maltose binding protein (MBP) and MBP-fused JAZ1 (MBP-JAZ1) were expressed in *Escherichia coli* and purified using amylose resin. Two transcription factors, GL3 and TT8, were fused with six myc tags (myc-GL3 and myc-TT8) and expressed in *N. benthamiana* leaves. The MBP-JAZ1 resin was incubated with the *N. benthamiana* leaves extracts expressing myc-GL3 (Figure 2B) or myc-TT8 (Figure 2C) and then separated on SDS-PAGE for immunoblotting with anti-c-myc antibody. As shown in Figure 2, the negative control (MBP resin) was unable to pull down myc-GL3 (Figure 2B, top panel) or myc-TT8 (Figure 2C, top panel), whereas the myc-TT8 and myc-GL3 were efficiently retained by the MBP-JAZ1 resin, suggesting that JAZ1 physically interacts with GL3 (Figure 2B, top panel) and TT8 (Figure 2C, top panel) in vitro.

Taken together, the Y2H assays, BiFC assays, and pull-down assays consistently demonstrate that the bHLH and MYB members in the WD-repeat/bHLH/MYB complex were responsible for interaction with JAZ proteins. The bHLH members (TT8 and EGL3) were divided into N-terminal fragments (TT8NT and EGL3NT) and C-terminal fragments (TT8CT and EGL3CT) containing the bHLH DNA binding domain (Figures 4A and 4C). The MYB members (MYB75 and GL1) were similarly divided into N-terminal fragments, containing the R2R3 DNA binding domain, and C-terminal fragments to produce MYB75NT, GL1NT, MYB75CT, and GL1CT, respectively (Figures 4E and 4G).

As shown in Figure 4, all the C-terminal fragments (TT8CT, EGL3CT, MYB75CT, and GL1CT), but no N-terminal fragments (TT8NT, EGL3NT, MYB75NT, and GL1NT) in these transcription factors, exhibited interactions with JAZ1 and JAZ8 in yeast. These results suggest that the C-terminal domains of EGL3, TT8, MYB75, and GL1 are responsible for interaction with JAZ proteins. This is different from the interaction of JAZ proteins with N-terminal domain of MYC2, which belongs to different bHLH subfamily (Toledo-Ortiz et al., 2003).

The C-Terminal Jas Domain in JAZ8 and JAZ11 Is Responsible for Interactions with TT8, GL3, EGL3, MYB75, and GL1

To investigate which domain of JAZ proteins was responsible for interactions with the bHLH and MYB members, we divided JAZ8 and JAZ11 into N-terminal (JAZ8NT and JAZ11NT) and C-terminal fragments (JAZ8CT and JAZ11CT). All the C-terminal fragments contained a Jas domain. JAZ8NT contained a ZIM domain, whereas JAZ11NT harbored two ZIM domains and one Jas domain (Figures 5A and 5B) (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008a). We found that GL3, EGL3, TT8, MYB75, and GL1 interacted with C-terminal fragments but not...
with N-terminal fragments of JAZ8 and JAZ11 in yeast (Figure 5). Consistent with previous data (Chini et al., 2007), these results indicate that the Jas domain in JAZ8 and JAZ11 is responsible for protein–protein interactions between JAZs and their down-stream transcription factors. Our results further indicate that, in JAZ11, the Jas domain nearer to the N terminus is not involved in the interactions with EGL3, GL3, TT8, MYB75, and GL1.

**JAZ1 Affects the Interactions between bHLH and MYB Proteins**

Having demonstrated the interaction of JAZ proteins with the bHLH and MYB transcription factors (Figures 1 to 5), we further examined the effect of JAZ proteins on the interaction of the bHLH proteins (GL3 or TT8) with the MYB proteins (GL1 or MYB75) in the BiFC assay.

When GL1-nYFP and cYFP-GL3 were transiently coexpressed in *N. benthamiana* leaves, we detected strong YFP fluorescence in the nucleus of epidermal cells in *N. benthamiana* leaves (Figures 6A and 6B), consistent with the previous observation that GL1 interacts with GL3 (Payne et al., 2000). When JAZ1 was coexpressed with GL1-nYFP and cYFP-GL3, the fluorescence signal was obviously reduced (Figures 6B to 6D), whereas coexpression of β-glucuronidase (GUS) with GL1-nYFP/cYFP-GL3

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did not reduce the fluorescence intensity (Figures 6B to 6D). These results indicate that JAZ1 can affect the interaction, through repression and/or structural modification, between the MYB GL1 and the bHLH GL3, essential components of the GL1/GL3-based WD-repeat/bHLH/MYB complex required for trichome formation.

Similar results were observed for coexpression of JAZ1 with MYB75-nYFP and cYFP-TT8 in *N. benthamiana* leaves (Figures 6E and 6F; see Supplemental Figure 3 online). The fluorescence signal was obviously reduced by JAZ1, but not by GUS, in BiFC assays (Figures 6E and 6F), which suggests that JAZ1 affects the interaction, through repression and/or structural modification, between MYB75 and TT8, essential components of the MYB75/TT8-based WD-repeat/bHLH/MYB complex required for anthocyanin accumulation.

Having shown that JAZ1 affected the interactions between the bHLH and MYB proteins in the BiFC assays (Figures 6B, 6C, and 6E), we investigated whether exogenous JA treatment could attenuate the JAZ1 repression of those interactions. Consistent with the data shown in Figures 6B and 6C, YFP fluorescence signal was obviously reduced when JAZ1 was coexpressed with GL1-nYFP and cYFP-GL3 (Figure 6G). Furthermore, we found...
that exogenous application of coronatine, a JA mimic molecule (Katsir et al., 2008b; Fonseca et al., 2009b; Sheard et al., 2010), restored the YFP fluorescence obviously (Figure 6G). Similarly, we observed that coronatine also rescued the JAZ1-repressed YFP fluorescence signal of MYB75-nYFP/cYFP-TT8 (Figure 6H). These results suggest that JA can reverse the JAZ1-mediated interference with the interaction between the bHLH and MYB proteins in these BiFC assays, probably by inducing the depletion of the transiently expressed Arabidopsis JAZ1 via the COI1 homolog and 26S proteasome of N. benthamiana.

JAZ1 Represses the Transcriptional Function of the bHLH Factor TT8 and MYB Member MYB75

DFR, the anthocyanin biosynthetic gene, is a direct target of the WD-repeat/bHLH/MYB complex (Nesi et al., 2000; Zhang et al., 2003; Gonzalez et al., 2008; Matsui et al., 2008). We further verified effect of JAZ1 on the transcriptional function of TT8 and MYB75 using the DFR promoter-driven luciferase reporter in a well-established transient expression assay.

When the promoter of DFR was fused with luciferase (LUC) reporter gene (ProDFR-LUC) and infiltrated into N. benthamiana leaves, we observed a certain amount of LUC activity that was reflected by luminescence intensity (Figure 7A), indicating that endogenous bHLH/MYB proteins of N. benthamiana may activate expression of ProDFR-LUC. Coexpression of ProDFR-LUC with TT8 and MYB75 generated much stronger luminescence intensity in the N. benthamiana leaves (Figures 7A to 7C). These results indicate that TT8 and MYB75 positively regulate the expression of DFR, consistent with previous research (Zimmermann et al., 2004; Matsui et al., 2008).

When ProDFR-LUC was coinfiltrated in N. benthamiana leaves with JAZ1 driven by the 35S cauliflower mosaic virus (CaMV35S) promoter, the LUC activity was obviously reduced in comparison with infiltration of ProDFR-LUC alone or coinfiltration of ProDFR-LUC and the GUS gene driven by the CaMV35S promoter (Figures 7A to 7D), indicating that JAZ1 may repress the transcriptional function of endogenous N. benthamiana bHLH/MYB proteins. Furthermore, coexpression of JAZ1 with ProDFR-LUC, TT8, and MYB75 dramatically reduced the luminescence intensity compared with the coexpression of ProDFR-LUC, TT8, and MYB75 without JAZ1 or with GUS (Figures 7A to 7D; see Supplemental Figure 4 online), demonstrating that JAZ1 represses the transcriptional function of TT8 and MYB75. In addition, we applied coronatine, the JA mimic molecule, onto the N. benthamiana leaf area coexpressing JAZ1/TT8/MYB75/ProDFR-LUC to investigate whether JA could attenuate the JAZ1 repression and restore the expression level of ProDFR-LUC. As shown in
Figure 6. *Arabidopsis* JAZ1 Affects the Interaction of GL3 with GL1 and the Interaction of TT8 with MYB75.

(A) BiFC assays to detect the interaction of GL3 (fused with cYFP) with GL1 (fused with nYFP). cYFP-GL3 and GL1-nYFP were coexpressed in leaves of *N. benthamiana*. YFP fluorescence was detected 50 h after infiltration. DAPI staining indicates the nuclei.

(B) BiFC assays showing that JAZ1 attenuates the interaction of GL3 with GL1. YFP fluorescence was detected 50 h after coexpression of GL1-nYFP/cYFP-GL3 (CK), JAZ1/GL1-nYFP/cYFP-GL3 (+JAZ1), or GUS/GL1-nYFP/cYFP-GL3 (+GUS). The identical amounts of *Agrobacterium tumefaciens* strains containing GL1-nYFP and cYFP-GL3 were used in these three combinations (CK, +JAZ1, and +GUS). The three combinations were infiltrated into the same leaf of *N. benthamiana* and detected under the microscope with identical gain settings (lasers, 514 nm, 8.0%; pinhole, 34 μm; master gain, 732). The experiment was repeated in more than 10 independent biological replicates with similar results. Bar = 100 μm.

(C) Quantitative analysis of YFP fluorescence intensity in (B). Fifty independent fluorescent spots were assessed for fluorescence intensity. Error bars represent SE.

(D) Real-time PCR analysis of JAZ1, GUS, cYFP-GL3 (GL3), and GL1-nYFP (GL1) expression in (B). Total RNAs were extracted from leaves of *N. benthamiana* coinfiltrated with the combinations of various constructs in (B). The primers specific for amplification of GUS, *Arabidopsis* JAZ1, cYFP-GL3 (GL3), and GL1-nYFP (GL1) are shown in Supplemental Table 1 online. *Nicotiana Actin* was used as the internal control. Expression levels of genes in the CK treatment were standardized as one unit in the real-time PCR system. Error bars represent SE.

(E) JAZ1 attenuates the interaction of TT8 with MYB75. YFP fluorescence was detected 50 h after coexpression of MYB75-nYFP/cYFP-TT8 (CK), JAZ1/MYB75-nYFP/cYFP-TT8 (+JAZ1), or GUS/MYB75-nYFP/cYFP-TT8 (+GUS) in the same leaf of *N. benthamiana*.
Figure 7E, treatment with coronatine (COR) obviously increased the luminescence intensity, suggesting that JA can reverse the JAZ1-mediated interference with the transcriptional function of TT8 and MYB75. Taken together, these results demonstrate that JAZ1 represses the transcriptional function of the bHLH factor TT8 and MYB member MYB75, thereby attenuating expression of ProDFR-LUC in the transient assay.

To examine further whether accumulation of endogenous JAZ proteins could repress the expression of endogenous DFR in planta, we detected the expression levels of DFR and other target genes of the TT8/MYB75-based complex in the Arabidopsis dominant-negative transgenic plant JAZ1Δ3A and the coi1-2 mutant, both of which highly accumulate JAZ proteins (Thines et al., 2007; Zhang and Turner, 2008; Chini et al., 2009; Chung and Howe, 2009). The results in Figure 7F showed that the JAZ1Δ3A transgenic plants and the coi1-2 mutant exhibited obvious repression of DFR, LDOX, and UFGT, the anthocyanin biosynthetic genes that are direct targets of the TT8/MYB75-based WD-repeat/bHLH/MYB complex (Zhang et al., 2003; Tohge et al., 2005; Gonzalez et al., 2008). In addition, we found that JAZ1Δ3A and coi1-2 also exhibited obvious repression of the trichome initiation factor GL2 (Figure 7F), a target gene of the GL1/GL3-based WD-repeat/bHLH/MYB complex (Zhang et al., 2003; Morohashi and Grotewold, 2009). These results suggest accumulation of endogenous JAZ proteins represses the respective target genes of MYB75/TT8- and GL1/GL3-based WD-repeat/bHLH/MYB complexes in planta.

The WD-Repeat/bHLH/MYB Complex Is Involved in JA-Regulated Anthocyanin Accumulation and Trichome Initiation

It has been reported that, in Arabidopsis, bHLH factors (GL3, EGL3, and TT8), MYB factors, including MYB75, and the WD-repeat protein TTG1 form the WD-repeat/bHLH/MYB complex to mediate anthocyanin accumulation (Zhang et al., 2003; Baudry et al., 2006). The bHLH factors (GL3 and EGL3) and TTG1 also form an alternative WD-repeat/bHLH/MYB complex with the MYB member GL1 to mediate trichome initiation (Payne et al., 2000; Bernhardt et al., 2003). We further investigated the role of these WD-repeat/bHLH/MYB complexes in JA-regulated anthocyanin accumulation and trichome initiation.

The Arabidopsis WD-repeat/bHLH/MYB mutants, the coi1-2 mutant (Xu et al., 2002), and the JAZ1Δ3A transgenic line (Thines et al., 2007) were used for measurement of JA-induced anthocyanin accumulation and trichome initiation. As shown in Figure 8, the anthocyanin content in wild-type seedlings was obviously upregulated when they were treated with JA. However, JA-induced anthocyanin accumulation decreased in the WD-repeat/bHLH/MYB mutants compared with that in the wild type (Figures 8A and 8B). When treated with methyl jasmonate (MeJA), the gl3 egl3 double mutant exhibited an obvious reduction in anthocyanin accumulation compared with the gl3 or egl3 single mutants (Figures 8A and 8B); the gl3 egl3 ttg1 triple mutant displayed much lower anthocyanin contents compared with the gl3 egl3 double mutant (Figures 8A and 8B), indicating that GL3, EGL3, and TT8 function redundantly in mediating JA-induced anthocyanin accumulation. In the ttg1 mutant, JA-induced anthocyanin accumulation was severely disrupted (Figures 8A and 8B), indicating that the single WD-repeat protein TTG1 is indispensable and essential for the integrity of the WD-repeat/bHLH/MYB complex (Zhao et al., 2008). These results suggest that the WD-repeat/bHLH/MYB complex is involved in JA-regulated anthocyanin accumulation.

We further measured total trichome number per leaf in ttg1, gl1, and the gl3 egl3 double mutant in response to JA induction. We found that JA-induced trichome formation was abolished in ttg1 (Figures 8D and 8E), whereas an obvious increase of trichome numbers was detected in JA-treated wild-type (Traw and Bergelson, 2003; Yoshida et al., 2009). Similar to previous observations (Yoshida et al., 2009), we also noticed that the JA-induced trichome initiation was disrupted in gl1 and the gl3 egl3 double mutant. These results suggest that the WD-repeat/bHLH/MYB complex is also involved in JA-regulated trichome initiation.

Consistent with our previous observation (Shan et al., 2009), we found that the coi1-2 mutant failed to accumulate anthocyanin in response to JA treatment (Figures 8A and 8B). Compared with the wild type, the trichome number in the coi1-2 mutant was also reduced obviously. In response to JA induction, trichome formation was dramatically attenuated in the coi1-2 mutant (Figures 8D and 8E) (Yoshida et al., 2009). These results indicate that JA regulates anthocyanin accumulation and trichome initiation in a COI1-dependent manner.

In the JAZ1Δ3A transgenic plants, JA-induced anthocyanin accumulation was largely decreased in comparison with that in the wild type (Figures 8A and 8B). Similarly, trichome formation, when treated with or without JA, was clearly reduced in the JAZ1Δ3A transgenic plants (Figures 8D and 8E). These results suggest that JAZ1Δ3A dominantly represses JA-regulated anthocyanin accumulation and trichome initiation. Consistent with the role for JAZ in repressing anthocyanin accumulation, expression of some JAZ genes, including JAZ1 and JAZ8, was detected in the leaves by quantitative real-time PCR (Figure 8C).

We also used sharp forceps, under a microscope, to harvest...
Figure 7. Arabidopsis JAZ1 Represses the Transcriptional Function of TT8 and MYB75.

(A) Transient expression assays show that JAZ1 represses the transcriptional function of TT8 and MYB75. Luminescence imaging of N. benthamiana leaves is shown 50 h after coinfiltration with the constructs indicated in the bottom circle.

(B) Quantitative analysis of luminescence intensity in (A). Five independent determinations were assessed. Error bars represent se.

(C) Real-time PCR analysis of JAZ1, TT8, and MYB75 in (A). Total RNAs were extracted from leaves of N. benthamiana coinfiltrated with the constructs in (A). The primers specific for amplification of the Arabidopsis JAZ1, TT8, and MYB75 are shown in Supplemental Table 1 online. Nicotiana Actin was used as the internal control. Error bars represent se.

(D) GUS has no effect on the transcriptional function of TT8 and MYB75. LUC activity was detected 50 h after coinfiltration of constructs as indicated. Quantitative analysis of luminescence intensity is presented. Error bars represent se.

(E) COR attenuates JAZ1 inhibition of the transcriptional function of TT8 and MYB75. N. benthamiana leaves were coinfiltrated with Agrobacterium strains carrying the indicated constructs. CK, buffer treatment of coinfiltration of TT8/MYB75/ProDFR–LUC; +JAZ1, buffer treatment of coinfiltration of JAZ1/TT8/MYB75/ProDFR–LUC; +JAZ1+COR, treatment with 5 μM COR of coinfiltration of JAZ1/TT8/MYB75/ProDFR–LUC. Quantitative analysis of luminescence intensity is presented. Error bars represent se.

(F) Real-time PCR analysis for DFR, LDOX, UF3GT, and GL2 in Arabidopsis wild-type (WT), JAZ1-3A, and coi1-2 plants treated without (CK) or with 100 μM MeJA for 8 h. ACTIN8 was used as the internal control. Error bars represent se.

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trichomes and the leaf epidermal cells where trichomes are differentiated for quantitative real-time PCR analysis: the expression of **JAZ1** and **JAZ8** was also detected, consistent with a role for **JAZ** in affecting trichome initiation (Figure 8C). As the level of **JAZ** transcripts is not necessarily correlated with the level of **JAZ** proteins (Thines et al., 2007), the expression levels of **JAZ** proteins in trichomes and epidermal cells remain to be determined.

Previous studies showed that **MYC2** (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007), a target gene of **JAZ** proteins (Chini et al., 2007), is rapidly induced upon JA treatment (Chung et al., 2008; Pauwels et al., 2008). We found that, among the WD-repeat/bHLH/MYB components, **MYB113**, **MYB75**, and **MYB114** were rapidly upregulated to ~29.6-, 6.5-, and 4.6-fold in response to JA induction within 60 min (Figure 9); **TT8**, **GL3**, **MYB90**, and **GL1** were gradually induced by JA within 8 h (Figure 9); however, **EGL3** was not obviously upregulated by JA treatment (Figure 9). We further found that JA-induced expression of the bHLH factors **TT8** and **GL3** and all the MYB members (**MYB113**, **MYB75**, **MYB114**, **MYB90**, and **GL1**) was severely attenuated in **coi1-2** (Figure 9), suggesting that expression of these WD-repeat/bHLH/MYB components is regulated by JA in a **COI1**-dependent manner.

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**Figure 8.** WD-Repeat/bHLH/MYB Complexes Are Required for JA-Regulated Anthocyanin Accumulation and Trichome Initiation.

(A) Eleven-day-old *Arabidopsis* seedlings of wild-type (Col-0 and Landsberg erecta), JAZ1Δ3A, coi1-2, tt8, gl3, eg3, gl3 eg3, gl3 eg3 tt8, and ttg1 grown on MS medium supplied without (CK) or with 25 μM MeJA.

(B) Anthocyanin contents of the seedlings in (A). FW, fresh weight. Error bars represent SE (n = 3).

(C) Real-time PCR analysis of **JAZ1** and **JAZ8** expression in trichomes and epidermal cells. The trichomes and epidermal cells were isolated from *Arabidopsis* leaves using sharp forceps under a microscope. **ACTIN8** was used as the internal control. Expression levels of the genes in leaves were standardized as one unit in the real-time PCR analysis. Error bars represent SE.

(D) Images of trichome in wild-type Col-0 (WT), JAZ1Δ3A, coi1-2, and ttg1 plants treated without (CK) or with MeJA. The fifth true leaves were chosen to observe trichomes using an environmental scanning electron microscope. No trichomes were observed in ttg1. Bars = 500 μm.

(E) Total trichome number in the fifth true leaves described in (D). Error bars represent SE (n ≥ 12).
Overexpression of MYB75 Rescues Anthocyanin Accumulation in the coi1 Mutant

The JAZ proteins, which accumulate to high levels in the coi1 mutant (Thines et al., 2007), interact with their downstream transcription regulators, including MYC2 (Chini et al., 2007), to repress JA responses (Katsir et al., 2008a; Fonseca et al., 2009a). The accumulated JAZ proteins in the coi1 mutant may attenuate transcriptional function of bHLH and MYB factors, including MYB75, TT8, GL3, and GL1 (Figures 6 and 7), essential components of the WD-repeat/bHLH/MYB complexes required for anthocyanin accumulation and trichome formation. We examined whether overexpression of MYB75 could rescue anthocyanin accumulation in the coi1 mutant.

The transgenic line pap1-D overexpressing MYB75 (alternatively named as PAP1) (Borevitz et al., 2000) was crossed with coi1-1 and coi1-2 to generate the coi1-1 pap1-D and coi1-2 pap1-D plants with high expression of MYB75. As expected, the anthocyanin content levels were low in untreated wild-type seedlings and in the coi1 seedlings with or without JA induction but high in the JA-treated wild-type seedlings (Figures 10A and 10B). The transgenic pap1-D seedlings accumulated anthocyanin at high levels even without JA induction and at very high levels in response to JA induction (Figures 10A and 10B).

As shown in Figure 10, overexpression of MYB75 in the coi1 background largely rescued anthocyanin accumulation. coi1-1 pap1-D and coi1-2 pap1-D constitutively accumulated anthocyanin at high levels. We noticed that coi1-2 pap1-D showed increased anthocyanin accumulation in response to JA treatment, although the level remained much lower than that in JA-treated pap1-D seedlings. The responsiveness to JA signal in...
probably resulted from the leakiness of coi1-2, a weak allele of COI1 (Xu et al., 2002). In conclusion, these results demonstrate that overexpression of MYB75 suppresses the defect of anthocyanin accumulation in the coi1 mutants.

Consistent with the anthocyanin accumulation results, the anthocyanin biosynthetic genes DFR, LDOX, and UF3GT in coi1-2 pap1-D were upregulated to high levels (Figure 10D). In coi1-2 pap1-D, but not in coi1-2, a high expression level was detected for TT8 (Figure 10C), a bHLH member of the WD-repeat/bHLH/MYB complex that regulates expression of several anthocyanin biosynthetic genes (Nesi et al., 2000; Baudry et al., 2004). These data showed that overexpression of MYB75 obviously suppressed the defect in JA-induced expression of the bHLH factor TT8 and the anthocyanin biosynthetic genes (DFR, LDOX, and UF3GT) in coi1-2.

Taken together, our results suggest that MYB75 acts downstream of COI1 to regulate JA-induced anthocyanin accumulation positively and that overexpression of MYB75 restores anthocyanin accumulation in the coi1 mutants.

**Overexpression of EGL3 or GL3 Rescues Trichome Initiation in coi1-2**

GL3 and EGL3 play a dual role in regulation of JA-induced trichome initiation and anthocyanin accumulation (Zhang et al., 2003; Yoshida et al., 2009). We induced overexpression of GL3 and EGL3 in the coi1-2 background to examine the effects of elevated GL3 and EGL3 on trichome initiation and anthocyanin accumulation.

As shown in Figures 11A and 11B, the trichome number in coi1-2 was reduced compared with that in the wild type, and the
JA induction of trichomes was severely attenuated in coi1-2. The transgenic lines coi1-2 GL3OE and coi1-2 EGL3OE that overexpress GL3 or EGL3 (see Supplemental Figure 5 online) obviously activated trichome initiation even without JA induction (Figures 11A and 11B). Overexpression of GL3 or EGL3 in the wild type resulted in a dramatic increase in trichome formation under all circumstances without (Morohashi et al., 2007) or with JA induction (Figures 11A and 11B). These results suggest that GL3 and EGL3 act downstream of COI1 to regulate JA-induced trichome initiation positively and that overexpression of GL3 or EGL3 restores trichome initiation in coi1-2.

As the transcription factors GL3 and EGL3 were found to regulate both trichome initiation and anthocyanin accumulation (Zhang et al., 2003; Broun, 2005), we further investigated whether overexpression of GL3 or EGL3 could rescue anthocyanin accumulation in the coi1-2 mutant. As expected, an obvious increase in anthocyanin accumulation, in response to JA induction, was detected in the wild type but not in coi1-2 (Figures 10 and 11C). The transgenic lines coi1-2 GL3OE and coi1-2 EGL3OE exhibited a mild increase in anthocyanin compared with coi1-2 (Figure 11C), indicating that overexpression of GL3 or EGL3 could slightly restore anthocyanin accumulation in coi1-2. However, no obvious increase in anthocyanin was detected in coi1-2 GL3OE and coi1-2 EGL3OE compared with the obvious restoration caused by the MYB75 overexpression in coi1-2 pap1-D (Figures 10 and 11C). Similar data were observed for overexpression of GL3 or EGL3 in the wild type: the transgenic lines GL3OE and EGL3OE exhibited a mild increase in anthocyanin but not as strong as that in the pap1-D transgenic plant with overexpression of MYB75 (Figures 10 and 11C).

Taken together, overexpression of GL3 or EGL3 was able to rescue trichome initiation dramatically (Figures 11A and 11B) and restore anthocyanin accumulation in coi1-2 slightly but not dramatically (Figure 11C). These results imply that GL3 and EGL3 may activate distinct downstream cascades and different feedback loops to regulate trichome initiation and anthocyanin accumulation separately.

**DISCUSSION**

The JAZ proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) are speculated to repress JA-regulated plant responses through interaction and attenuation of their downstream transcription factors (Katsir et al., 2008a; Chung et al., 2009; Fonseca et al., 2009a). The bHLH transcription factors MYC2/3/4 and R2R3 transcription factors MYB21/24 act as direct targets of JAZ proteins (Chini et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Song et al., 2011) to mediate JA-regulated root growth inhibition and stamen development, respectively (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Song et al., 2011). Here, we identified three bHLH transcription factors (TT8, GL3, and EGL3) and two MYB transcription factors (MYB75 and GL1), essential components of
WD-repeat/bHLH/MYB complexes, as new targets of JAZ proteins. We also showed that JAZ proteins attenuate the transcriptional function of WD-repeat/bHLH/MYB complexes to regulate JA-induced anthocyanin accumulation and trichome initiation negatively in Arabidopsis.

A bHLH protein (EGL3, GL3, or TT8), a R2R3 MYB protein (GL1, MYB75, MYB90, MYB113, MYB114, or MYB23) and the WD-repeat protein TTG1 form WD-repeat/bHLH/MYB complexes to regulate anthocyanin biosynthesis and trichome initiation (Zimmermann et al., 2004; Gonzalez et al., 2008; Zhao et al., 2008). Previous studies showed that JA induces anthocyanin accumulation through upregulation of late anthocyanin biosynthetic genes DFR, LDOX, and UF3GT (Shan et al., 2009) and that JA enhances trichome formation through influence on GL3 expression (Yoshida et al., 2009). It was still unclear whether and how the WD-repeat/bHLH/MYB complexes are regulated in the JA signaling pathway to stimulate anthocyanin accumulation and trichome initiation. In this study, we demonstrated that the WD-repeat/bHLH/MYB complexes are regulated through interaction of JAZ proteins with the bHLH members (TT8, GL3, and EGL3) and the R2R3 MYB members (GL1 and MYB75). It remains to be elucidated whether JAZ proteins interact with the remaining R2R3 MYB members (MYB23, MYB90, MYB113, and MYB114) of the complexes.

Previous studies showed that R3-type MYB transcription factors (such as TRY and MYBL2) repress trichome formation and anthocyanin accumulation through their interaction with bHLH members of WD-repeat/MYB/bHLH complexes: the R3 MYB transcription factors compete with R2R3 MYB members (GL1 or MYB75) for the interaction domain located at the N terminus of the bHLH members (GL3, EGL3, and TT8) to repress the transcriptional function of the WD-repeat/MYB/bHLH complex (Schellmann et al., 2002; Esch et al., 2003; Zhang et al., 2003; Matsui et al., 2008). By contrast, JAZ proteins interact with C terminus of both R2R3 MYB and bHLH members to attenuate the transcriptional function of WD-repeat/bHLH/MYB complexes (Figures 4, 6, and 7). JA mediates anthocyanin accumulation and trichome initiation probably through activation of transcriptional function of WD-repeat/bHLH/MYB complexes by removal of the JAZ repression on R2R3 MYB and bHLH members (Figures 6 and 7). Molecular, genetic, and physiological analysis showed that the JAZ1/J3A transgenic plant and the coi1-2 mutant, which highly accumulate JAZ proteins, exhibited repression on JA-induced expression of the anthocyanin biosynthetic genes DFR, LDOX, and UF3GT (Figure 7F), target genes of the WD-repeat/bHLH/MYB complexes, and displayed a reduction in JA-induced anthocyanin accumulation and trichome initiation (Figure 8).

Consistent with this, overexpression of MYB75 in the coi1-2 mutant overcame the JAZ repression to restore JA-induced anthocyanin accumulation (Figure 10), and overexpression of GL3 or EGL3 in coi1-2 surmounted the JAZ repression to rescue trichome initiation (Figure 11).

GL3 and EGL3 play important roles in both anthocyanin accumulation and trichome formation (Zhang et al., 2003; Gonzalez et al., 2008). However, we found that overexpression of GL3 or EGL3 was able to rescue trichome initiation in coi1-2 fully (Figures 11A and 11B) but restore anthocyanin accumulation in Figure 12. A Simplified Model for JA Action in Regulation of Anthocyanin Accumulation and Trichome Initiation in Arabidopsis.

(A) The Arabidopsis JAZ proteins (JAZ), by virtue of their C-terminal Jas domain, interact with the C-terminal domains of bHLH transcription factors (TT8, GL3, and EGL3) and MYB proteins (MYB75 and GL1), the essential components of WD-repeat/bHLH/MYB complexes, to attenuate transcriptional function of WD-repeat/bHLH/MYB complexes and therefore inactivate downstream genes (early genes), repressing JA-regulated anthocyanin biosynthesis and trichome initiation.

(B) Upon perception of JA signal, COI1 recruits JAZ proteins to the SCFCOI1 complex for ubiquitination and degradation through the 26S proteasome.

(C) Upon degradation of JAZ proteins, the bHLH and MYB components of WD-repeat/bHLH/MYB complexes are subsequently released to activate their respective downstream genes, which are essential for anthocyanin biosynthesis and trichome formation. Other signals (such as cytokinin, gibberellin, Suc, nutrition depletion, light, and cold) may also regulate anthocyanin biosynthesis and trichome initiation by directly or indirectly regulating WD-repeat/bHLH/MYB complexes through the bHLH and MYB components as well as the WD-repeat protein TTG1.

[See online article for color version of this figure.]
coi1-2 only slightly, not obviously (Figure 11C), which suggests that GL3 and EGL3 mediate JA-regulated anthocyanin accumulation and trichome formation through different downstream signal cascades and distinct feedback loops. Consistent with this speculation, we found that the double mutants lacking both GL3 and EGL3 showed a complete loss in trichome initiation but just a moderate reduction in JA-induced anthocyanin accumulation (Figure 8) (Yoshida et al., 2009). Furthermore, the R3-MYB transcription factor MYBL2 (Dubos et al., 2008; Matsui et al., 2008), which was upregulated by GL3 (Morohashi and Grotewold, 2009), was found to function as a negative regulator of GL3, EGL3, and TT8 to inhibit GL3/EGL3/TT8-mediated anthocyanin accumulation but not to repress GL3/EGL3-mediated trichome initiation due to lack of MYBL2 expression in trichomes (Dubos et al., 2008; Matsui et al., 2008). It is possible that, in the coi1-2 GL3OE and coi1-2 EGL3OE lines, the elevated GL3 or EGL3 is able to activate fully the trichome cascade essential for trichome initiation but is unable to activate efficiently the distinct anthocyanin cascade.

We propose a model to elucidate the molecular mechanism for JA function in regulating anthocyanin accumulation and trichome formation (Figure 12). JAZ proteins directly interact with C terminus of both bHLH (TT8, GL3, and EGL3) and R2R3 MYB components (MYB75 and GL1) of WD-repeat/bHLH/MYB complexes (Figures 1 to 5), attenuate their transcriptional function (Figures 6 and 7), and therefore repress JA-regulated anthocyanin accumulation and trichome initiation (Figure 8). Upon JA treatment or JA biosynthesis stimulated by diverse developmental and environmental signals, the JA signal induces degradation of JAZ proteins through the SCFCO1-26S proteasome pathway (Chini et al., 2007; Thines et al., 2007; Chung et al., 2009); the WD-repeat/bHLH/MYB complexes are then released to activate their respective downstream transcriptional cascades, leading to anthocyanin biosynthesis and trichome formation. Many other signals, including environmental signals (cold, high light, and insect attack) and endogenous signals (cytokinins and gibberellins), were shown to regulate anthocyanin accumulation or trichome formation (Deikman and Hammer, 1995; Gan et al., 2007; Cominelli et al., 2008; Loreti et al., 2008; Rowan et al., 2009; Yoshida et al., 2009; Yu et al., 2010). These signals may influence anthocyanin accumulation and trichome initiation also through directly or indirectly regulating WD-repeat/bHLH/MYB complexes by virtue of bHLH and R2R3 MYB components as well as the WD-repeat protein TTG1.

METHODS

Plant Materials and Growth Conditions

The Arabidopsis thaliana coi1-1 (Xie et al., 1998), coi1-2 (Xu et al., 2002), and JAZ1J3A (Thines et al., 2007) mutants were described as previously. Columbia-0 (Col-0) was used as the wild-type control. The mutants tt8 (Salk_082999 with T-DNA insertion in the second intron), gl3 (gl3-1) (Payne et al., 2000), egl3 (Salk_077439), ttg1 (ttg1-1) (Walker et al., 1999), pap1-D (Borevitz et al., 2000), and gir (Herman and Marks, 1989) were all obtained from the ABRC.

The JAZ1J3A transgene was maintained by genetic cross of the male-sterile JAZ1J3A transgenic plants with wild-type pollen grains. The harvested seeds from the cross were germinated to produce a population with 1:1 segregation of the wild type and JAZ1J3A transgene. The JAZ1J3A transgenic seedlings were identified by PCR amplification with the primer pair 35S promoter-F/JAZ1J3A-R (see Supplemental Table 1 online) when grown on Murashige and Skoog (MS) medium or screened by observation of JA-insensitive phenotypes (Thines et al., 2007) when grown on MS medium supplied with MeJA.

Arabidopsis seeds were disinfected and plated on MS medium (Sigma-Aldrich) supplemented with 3% sucrose, chilled for 3 d at 4°C, and transferred to a growth house under a 16-h-light (22-24°C)/8-h-dark (17-19°C) photoperiod. Nicotiana benthamiana was grown under a 16-h-light (28°C)/8-h-dark (22°C) photoperiod. All experiments were repeated at least three times.

Y2H Screening and Y2H Assays

The Matchmaker LexA two-hybrid system (Clontech) was used for Y2H screening of the Arabidopsis cDNA library (Xu et al., 2002). Arabidopsis JAZ1 CDNA was cloned into pLexA vector to fuse with DNA BD. Yeast transformants were exhaustively screened on 2% SD/Gal/Raf/X-gal (-Ura/-His/-Trp/-Leu) based on the manufacturer’s instructions (Clontech).

For Y2H assay, all the JAZ cDNAs and their domain derivatives were cloned into pLexA, and the full-length or domain deletion forms of GL3/GL5/TT8/MYB75/GL1 were cloned into pB42AD. Primers used for the vector construction are presented in Supplemental Table 1 online. All the constructs were sequence verified. All the pLexA constructs were proved to have no self-activation by β-galactosidase assay in yeast. The indicated construct pairs were transformed into yeast (Saccharomyces cerevisiae) strain EGY48 and cultured at 30°C. Successful yeast transformants were cultured in liquid SD minimal medium (-Ura/-His/-Trp) at 230 rpm/30°C for ~24 h to the final concentration (OD600 = 1.2 to ~1.5). Then, 1 mL of yeast strain was centrifuged and resuspended in 0.3 mL of distilled water. Five microliter of the suspension cells were plated on SD/Gal/Raf/X-gal (-Ura/-His/-Trp/-Leu) induction medium in 96-well plates. Y2H images were taken 3 d after incubation at 30°C. Experiments were repeated three times. The expressed proteins in different yeast strains were analyzed by immunblot experiments. Proteins fused with the activation domain were detected using anti-HA antibody (Sigma-Aldrich). Proteins fused with the LexA DNA binding domain were detected using anti-LexA antibody.

BIFC Assays

Full-length coding sequences of Arabidopsis JAZ1, JAZ10, TT8, GL3, EGL3, MYB75, and GL1 were individually cloned into the pDONR207 vector (Invitrogen) through Gateway reaction (Invitrogen) and subsequently recombined into the binary YFP BiFC vectors (Song et al., 2011) so that they were fused with the N- or C-terminal fragment of YFP (nYFP or cYFP) to generate JAZ1/JAZ10/GL1/GL3/MYB75-nYFP and cYFP-TT8/GL3/EGL3/MYB75/GL1 plasmids. Primers used for plasmid construction are presented in Supplemental Table 1 online.

These constructs were transformed into Agrobacterium tumefaciens strain GV3101 through electroporation. The Agrobacterium strains containing different constructs were incubated, harvested, and resuspended in infiltration buffer (10 mM MES, 0.2 mM acetylsyringone, and 10 mM MgCl2) to an ultimate concentration of OD600 = 0.5. Equal volumes of different combinations of Agrobacterium strains were mixed and co-infiltrated into the same N. benthamiana leaf with a needleless syringe. Plants were placed at 24°C for 50 h before detection of YFP fluorescence.

YFP fluorescence was detected with Zeiss microscope (LSM710) and analyzed by the ZEN 2009 software. For staining of the nuclei, 10 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) was infiltrated into N. benthamiana leaves 2 h before observation. For the exogenous COR treatment in Figures 6G and 6H, infiltration buffer containing 5 μM COR (+COR) or
 solvent buffer as control) was infiltrated into the *N. benthamiana* leaf area coexpressing the indicated constructs 10 h before YFP fluorescence observation. All experiments were repeated at least three times.

**Transient Expression Assays**

The transient expression assays (Matsui et al., 2008; Shang et al., 2010) were performed in *N. benthamiana* leaves. The DFR promoter was fused with luciferase reporter gene (*P_{DFR-LUC}* through the KpnI and SmaI sites (Shang et al., 2010). JAZ1 was cloned into the modified pCAMBIA1300 vector (Cambia) with four CaMV35S promoters. *TT8* and *MYB75* were cloned into the pROK2 vector (Xu et al., 2002) through SmaI and SacI sites. Primers used for these constructs are shown in Supplemental Table 1 online. pBl121 vector (Clontech) was used for the transient expression of GUS.

The *Agrobacterium* strains containing different constructs were incubated, harvested, and resuspended in infiltration buffer (10 mM MES, 0.2 mM acetosyringone, and 10 mM MgCl₂) to an ultimate concentration of OD₆₀₀ = 0.5. Equal volumes of different combinations of *Agrobacterium* strains were mixed and coinfiltrated into *N. benthamiana* leaves by a needleless syringe. Plants were placed at 24°C for 5 h before CCD imaging. For the exogenous COR treatment in Figure 7E, the infiltration buffer containing 5 μM COR (+COR) (or solvent buffer as control) was infiltrated into the *N. benthamiana* leaf area coexpressing the indicated constructs 10 h before CCD imaging. We used a low-light cooled CCD imaging apparatus (Andor iXon) to acquire the image. The leaves were sprayed with 100 μM luciferin and were placed in darkness for 5 min before luminescence detection. The computational method for counting luminescence intensity was described previously (Shang et al., 2010). Five independent determinations were assessed. Error bars represent SE. All experiments were repeated with five independent biological replicates with similar results.

**Generation of GL3 and EGL3 Transgenic Plants**

To obtain *Arabidopsis* GL3 and EGL3 overexpression transgenic plants in wild-type and *coi1-2* background, the full-length coding sequences of GL3 and EGL3 were amplified using the primers indicated in Supplemental Table 1 online and cloned into the modified pCAMBIA1300 vector (Cambia) containing a CaMV35S promoter through the SalI and SpeI sites. These constructs were introduced into the *COI1/coi1-2* heterozygous plants using the *Agrobacterium*-mediated flower dip method (Clough and Bent, 1998).

Three overexpression transgenic lines for each gene (GL3 and EGL3) in wild-type and *coi1-2* background were identified and characterized. Results from one transgenic line of GL3 (and EGL3) in wild-type and *coi1-2* background was representatively displayed in the article.

**In Vitro Pull-Down Assays**

The coding region of JAZ1 was cloned into the pMAL-c2x (NEB) vector using the primers presented in Supplemental Table 1 online through EcoRI and SalI sites. This construct was transformed into *E. coli* to express MBP-JAZ1 protein. MBP and MBP-JAZ1 proteins were purified from *E. coli* using Ni affinity chromatography according to Yan et al. (2009).

Full-length CDSs of GL3 and TT8 were cloned into the modified pROK2-myc vector (Xu et al., 2002) through SacI and SalI sites for fusion with six myc tags. These constructs were sequence verified and transformed into *Agrobacterium*. The *Agrobacterium* strains were cultured, harvested, and resuspended in infiltration buffer (10 mM MES, 0.2 mM acetosyringone, and 10 mM MgCl₂) and then infiltrated into *N. benthamiana* leaves. After 50 h, 5 g of the *N. benthamiana* leaves transiently expressing myc-GL3 or myc-TT8 were harvested for total protein extraction in a RB buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.8, 25 mM imidazole, 0.1% [v/v] Tween 20, 10% [v/v] glycerol, EDTA-free complete miniprotease inhibitor cocktail, and 20 mM 2-mercaptoethanol). The extracted total protein was concentrated to 300 μL.

Coomassie Brilliant Blue was used to confirm protein amount. About 50 μg of purified MBP and MBP-JAZ1 were incubated with 100 μL amylose resin beads for 2 h at 4°C. These amylose resin beads were then washed five times with 1 mL RB buffer and incubated with 100 μL of concentrated total proteins containing myc-GL3 or myc-TT8 for 2 h. After washing five times with 1 mL RB buffer, the mixture was resuspended in SDS loading buffer, boiled for 5 min, separated on 15% SDS-PAGE, and immunoblotted using anti-c-myc antibody (Roche).

**Anthocyanin Measurement**

*Arabidopsis* seeds were plated on MS medium without or with 25 μM MeJA for 11 d and then were used for anthocyanin measurement with the method described previously (Shan et al., 2009). To show the quantity of anthocyanin, \( A_{520} - A_{650} \) per gram fresh weight was used. All experiments were repeated at least three times.

**Real-Time PCR Analysis**

For real-time PCR analysis, *Arabidopsis* seedlings were grown on MS medium for 3 weeks and then were treated with or without 100 μM MeJA for 0, 1, 2, 8, and 12 h. The treated seedlings were harvested for RNA extraction and subsequent reverse transcription. Real-time PCR analyses were performed with the RealMasterMix (SYBR Green I) (Takara) using the ABI7500 real-time PCR system. *ACTIN8* was used as the internal control. The primers used for real-time PCR analysis are presented in Supplemental Table 1 online.

For real-time PCR analysis of JAZ gene expression, trichomes with epidermis were isolated from 4-week-old wild-type *Arabidopsis* leaves using sharp forceps under a microscope and further used for RNA extraction and subsequent reverse transcription. *ACTIN8* was used as the internal control.

For real-time PCR analysis of EGL3 and GL3 in transgenic plants overexpressing of EGL3 and GL3, respectively, 3-week-old seedlings of transgenic plants were used for RNA extraction and subsequent reverse transcription. *ACTIN8* was used as the internal control.

The *N. benthamiana* leaves for the BIFC assays and the transient assays were also used for RNA extraction and subsequent expression analysis after coinfiltration of construct combinations for 50 h. Nicotiana *Actin* was used as the internal control. All the primers specifically used for real-time PCR analysis are presented in Supplemental Table 1 online.

All the real-time PCR analyses were repeated for three biological replicates with similar results. Error bars represent SE.

**Trichome Measurement**

The method for trichome measurement was modified according to Yoshida et al. (2009). *Arabidopsis* seeds on MS medium were chilled for 3 d at 4°C and transferred to a growth room under a 16-h-light (−22°C)/8-h-dark (−17°C) photoperiod for 6 d. The seedlings were subsequently transferred onto fresh MS medium in 15-cm-diameter plates. In the center of the 15-cm-diameter plate, a piece of filter paper was placed into an eppendorf tube cap. MeJA (150 nmol) was then dropped onto the filter paper for evaporation. The seedlings were cultured for two additional weeks. The fifth truth leaf was used for calculation of total trichome numbers by environmental scanning electron microscopy (FEI Quanta 200). At least 12 leaves for each genotype were analyzed. The experiments were repeated three times.
The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: JAZ1 (AT1G19180), JAZ2 (AT1G74950), JAZ3 (AT3G17860), JAZ4 (AT1G48500), JAZ5 (AT1G17380), JAZ6 (AT1G27450), JAZ7 (AT2G34600), JAZ8 (AT1G30135), JAZ9 (AT1G70700), JAZ10 (AT5G12220), JAZ11 (AT3G43440), JAZ12 (AT5G20900), MYC2 (AT1G32640), TT8 (AT4G09820), GL3 (AT3G41315), EGL3 (AT1G63650), MYB75 (AT1G56650), MYB90 (AT1G66390), MYB113 (AT1G66370), MYB114 (AT1G66380), GL1 (AT3G27920), GL2 (AT1G78840), TTG1 (AT5G42520), DFR (AT5G42800), LDOX (AT4G22880), UFGT (AT5G54060), ACTIN8 (AT1G49240), and Nicotiana Actin (EU938079).

Supplemental Data

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

Supplemental Figure 1. Immunoblot Analysis of 12 Arabidopsis JAZ Proteins in Yeast.

Supplemental Figure 2. Negative Controls for the BiFC Experiments.

Supplemental Figure 3. Interaction of Arabidopsis TT8 with MYB75 in BiFC Assays.

Supplemental Figure 4. GUS Protein Does Not Affect the Transcriptional Function of TT8 and MYB75.

Supplemental Figure 5. Gene Expression Analysis of Transgenic Plants.

Supplemental Table 1. Primers Used for Vector Construction and Gene Expression Analysis.

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The Jasmonate-ZIM-Domain Proteins Interact with the WD-Repeat/bHLH/MYB Complexes to Regulate Jasmonate-Mediated Anthocyanin Accumulation and Trichome Initiation in *Arabidopsis thaliana*

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