Regulation of Jasmonate-Mediated Stamen Development and Seed Production by a bHLH-MYB Complex in Arabidopsis

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Stamens are the plant male reproductive organs essential for plant fertility. Proper development of stamens is modulated by environmental cues and endogenous hormone signals. Deficiencies in biosynthesis or perception of the phytohormone jasmonate (JA) attenuate stamen development, disrupt male fertility, and abolish seed production in *Arabidopsis thaliana*. This study revealed that JA-mediated stamen development and seed production are regulated by a bHLH-MYB complex. The IIIe basic helix-loop-helix (bHLH) transcription factor MYC5 acts as a target of JAZ repressors to function redundantly with other IIIe bHLH factors such as MYC2, MYC3, and MYC4 in the regulation of stamen development and seed production. The *myc2 myc3 myc4 myc5* quadruple mutant exhibits obvious defects in stamen development and significant reduction in seed production. Moreover, these IIIe bHLH factors interact with the MYB transcription factors MYB21 and MYB24 to form a bHLH-MYB transcription complex and cooperatively regulate stamen development. We speculate that the JAZ proteins repress the bHLH-MYB complex to suppress stamen development and seed production, while JA induces JAZ degradation and releases the bHLH-MYB complex to subsequently activate the expression of downstream genes essential for stamen development and seed production.

INTRODUCTION

As the male reproductive organ in plants, stamens are essential for fertility, and defects in stamen development can result in male sterility (Sanders et al., 1999). The development of stamens, comprising filament elongation, anther dehiscence, and pollen maturation at various floral developmental stages (Regan and Moffatt, 1990; Smyth et al., 1990), is regulated by diverse environmental cues and endogenous hormone signals, including gibberellin and jasmonate (JA) (Ma, 2005; Plackett et al., 2011; Song et al., 2013b).

JAs are a class of lipid-derived phytohormone (Browse, 2009; Wasternack and Hause, 2013) that regulate various essential plant defense responses and diverse developmental processes (Howe and Jander, 2008; Sun et al., 2011; De Geyter et al., 2012; Farmer and Mueller, 2013; Xin and He, 2013; Kazan and Lyons, 2014; Song et al., 2014b). Deficiencies in the biosynthesis or perception of JA attenuate stamen development, disrupt male fertility, and abolish seed production in various plant species, including both monocot (Acosta et al., 2009; Yan et al., 2012; Cai et al., 2014) and dicot plants (Feys et al., 1994; McConn and Browse, 1996). Specifically, *Arabidopsis thaliana* mutants deficient in JA perception, signaling, and biosynthesis, such as *coronatine insensitive1* (*coi1*), *JAZ1Δ3A*, *fad3 fad7 fad8*, *defective in anther dehiscence1*, *allene oxide synthase*, and *oxophytodienoate reductase3* (*opr3*), are defective in filament elongation, anther

dehiscence, and pollen maturation at floral stage 13 and unable to produce seeds (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002; von Malek et al., 2002; Thines et al., 2007; Chung et al., 2010; Huang et al., 2014).

The Arabidopsis R2R3 MYB transcription factors MYB21, MYB24, and MYB57 (Mandaokar et al., 2006; Yang et al., 2007; Cheng et al., 2009) function redundantly to regulate stamen development. Another R2R3 MYB factor, MYB108, may act downstream in the regulation of anther dehiscence (Mandaokar and Browse, 2009). JA induces the expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108* (Mandaokar et al., 2006), while the JAZ repressors interact with and repress MYB21, MYB24, and MYB57 to modulate JA-regulated stamen development (Song et al., 2011).

The JAZ repressors regulate various transcription factors to influence diverse JA responses (Cheng et al., 2011; Niu et al., 2011; Pauwels and Goossens, 2011; Qi et al., 2011; Song et al., 2011, 2013a; Zhu et al., 2011; Kazan and Manners, 2012; Fonseca et al., 2014). The subgroup IIIe basic helix-loop-helix (bHLH) transcription factors MYC2, MYC3, and MYC4, which are direct targets of JAZ proteins (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011), form homodimers/heterodimers and function redundantly to regulate JA-mediated root growth inhibition, defense against insects, resistance to pathogen infection, and secondary metabolism (Boter et al., 2004; Lorenzo et al., 2004; Yadav et al., 2005; Dombrecht et al., 2007; Chen et al., 2011; Fernández-Calvo et al., 2011; Hong et al., 2012; Schweizer et al., 2013; Chico et al., 2014).

Here, we found that the IIIe bHLH transcription factor MYC5 is a target of JAZ repressors and functions redundantly with the other IIIe bHLH factors MYC2, MYC3, and MYC4 in the regulation of stamen development and seed production. Moreover, these IIIe bHLH factors interact with the R2R3 MYB transcription factors MYB21 and MYB24 to form a bHLH-MYB transcription complex

and cooperatively regulate stamen development. Our results thus identify a bHLH-MYB complex responsible for the regulation of JA-mediated stamen development and seed production.

RESULTS

JAZ Proteins Interact with MYC5

We exhaustively screened an Arabidopsis cDNA library using JAZ1 protein as bait in the yeast two-hybrid (Y2H) system (Qi et al., 2011; Song et al., 2011, 2013a) and found that a bHLH transcription factor, MYC5, interacted with JAZ proteins (Figures 1A and 1B). As shown in Figure 1B, Y2H analysis showed that MYC5 exhibited interaction with JAZ1, JAZ2, JAZ5, JAZ6, JAZ8, JAZ10, and JAZ11 in yeast. Yellow fluorescent protein (YFP)-based bimolecular fluorescence complementation (BiFC) assays showed that coexpression of JAZ1-nYFP (JAZ1 fusion with the N-terminal part of YFP) or JAZ10-nYFP with cYFP-MYC5 (MYC5 fusion with the C-terminal part of YFP) reconstructed strong YFP signals in the nuclei (Figure 1C; Supplemental Figure 1), demonstrating the interaction of JAZ1 and JAZ10 with MYC5 in planta. Further domain analysis showed that the N terminus of MYC5 containing the JAZ-interaction domain (Fernández-Calvo et al., 2011) is critical for interaction with JAZ proteins (Figures 1A and 1B), whereas the C-terminal part of JAZ11 harboring the Jas domain is necessary for JAZ interaction with MYC5 (Figures 1A and 1D). Taken together (Figure 1), these Y2H and BiFC results demonstrate that MYC5 is a target of JAZ proteins.

The Subgroup IIIe bHLH Factors MYC2, MYC3, MYC4, and MYC5 Function Redundantly to Regulate Stamen Development and Seed Production

Subcellular localization analysis showed that MYC5 was localized in the nucleus (Supplemental Figure 2A), where JAZ proteins are also found (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). Expression pattern analysis with P_{MYC5}-GUS (for β-glucuronidase) reporter and quantitative real-time PCR showed that MYC5 was expressed in various tissues (Supplemental Figures 2B and 2C), with strong expression in flower and stamen (Supplemental Figure 2B), implying a possible role for MYC5 in the regulation of flower development. However, myc5 exhibited normal development in flower and stamen (Figure 2; Supplemental Figure 3).

Previous phylogenetic analysis showed that the IIIe bHLH factors MYC5, MYC2, MYC3, and MYC4 share high identity and similarity at the amino acid level (Heim et al., 2003). We generated a series of the double, triple, and quadruple mutants through genetic crosses among myc2, myc3, myc4, and myc5 to examine whether MYC5 would exhibit redundancy with MYC2, MYC3, and MYC4.

Interestingly, compared with the wild type, all of the triple mutants, including myc2 myc3 myc4, myc2 myc3 myc5, myc2 myc4 myc5, and myc3 myc4 myc5, exhibited defects in stamen development at floral stage 13: their anthers failed to dehisce (Figure 2A; Supplemental Figure 4A), and the pollen grains were unviable and unable to germinate in vitro (Figure 2B; Supplemental Figure 4B). The anther dehiscence and pollen maturation were

delayed in the triple mutants (Figure 2; Supplemental Figure 4). Furthermore, phenotypic comparison among these triple mutants showed that they exhibit similar severity in delayed stamen development, suggesting that MYC2, MYC3, MYC4, and MYC5 play equal roles in regulating stamen development. In contrast with the triple mutants, the single mutants and all the double mutants (myc2 myc5, myc3 myc5, myc4 myc5, myc2 myc3, myc2 myc4, and myc3 myc4) exhibited no obvious defects in stamen development (Figure 2; Supplemental Figures 4 and 5A).

Compared with the triple mutants, the myc2 myc3 myc4 myc5 quadruple mutant exhibited more severe defects in stamen development. In addition to the indehiscent anther and unviable pollen grains (Figures 2A, 2B, and 2D), the filament in myc2 myc3 myc4 myc5 did not elongate normally at floral stage 13 (Figures 2C and 2E), which is similar to coi1-1 and the myb21-3 myb24 double mutant (Figures 2A to 2C) (Song et al., 2011). At floral stage 14, in contrast with the normal stamen development in the wild type and the triple mutants, the anthers in the myc2 myc3 myc4 myc5 quadruple mutant failed to dehisce (similar to coi1-1 and myb21 myb24) (Figure 2A). The majority of the pollen grains in the quadruple mutant did not germinate in vitro (a few germinated pollen grains exhibited shorter pollen tubes) (Figure 2B). At floral stage 15, the anthers in myc2 myc3 myc4 myc5 dehisced (Figure 2A) and released viable pollen (Figures 2B and 2D), which ensured that myc2 myc3 myc4 myc5 was fertile and set seeds (Figures 2F to 2H).

Compared with the wild type, the siliques in the triple mutants contained somewhat fewer seeds (Figure 2G; Supplemental Figure 5B), while seed production was severely reduced in the myc2 myc3 myc4 myc5 quadruple mutant (Figures 2G and 2H).

Taken together (Figure 2; Supplemental Figures 4 and 5), these results demonstrated that the IIIe bHLH transcription factors MYC2, MYC3, MYC4, and MYC5 function redundantly to regulate stamen development and seed production.

Overexpression of MYC5 or MYC3 Can Partially Rescue Stamen Development in coi1-1

As the IIIe bHLH transcription factors act as targets of JAZ proteins (Figure 1) (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011) to mediate JA-regulated stamen development (Figure 2; Supplemental Figures 4 and 5), we next investigated whether the overexpression of MYC5 or MYC3 is able to overcome JAZ inhibition and rescue stamen development in the coi1-1 mutant, in which the JAZ repressors are highly accumulated (Chini et al., 2007; Thines et al., 2007).

We generated coi1-1 plants overexpressing MYC5 (coi1-1 MYC5OE) and found that, among 42 individual transgenic lines, 4 lines showed partially recovered male fertility (2 representative lines are shown in Figure 3). In contrast with coi1-1, the transgenic plants coi1-1 MYC5OE1 and coi1-1 MYC5OE2 at floral stage 13 harbored fertile stamens, in which the filament could elongate and the anthers could dehisce to release viable pollen that could geminate in vitro (Figures 3A and 3B). The mature coi1-1 MYC5OE1 and coi1-1 MYC5OE2 plants could set small amounts of seed (Figures 3C and 3D).

In the coi1-1 plants overexpressing MYC3 (coi1-1 MYC3OE), 4 out of 34 lines exhibited partially recovered stamen development
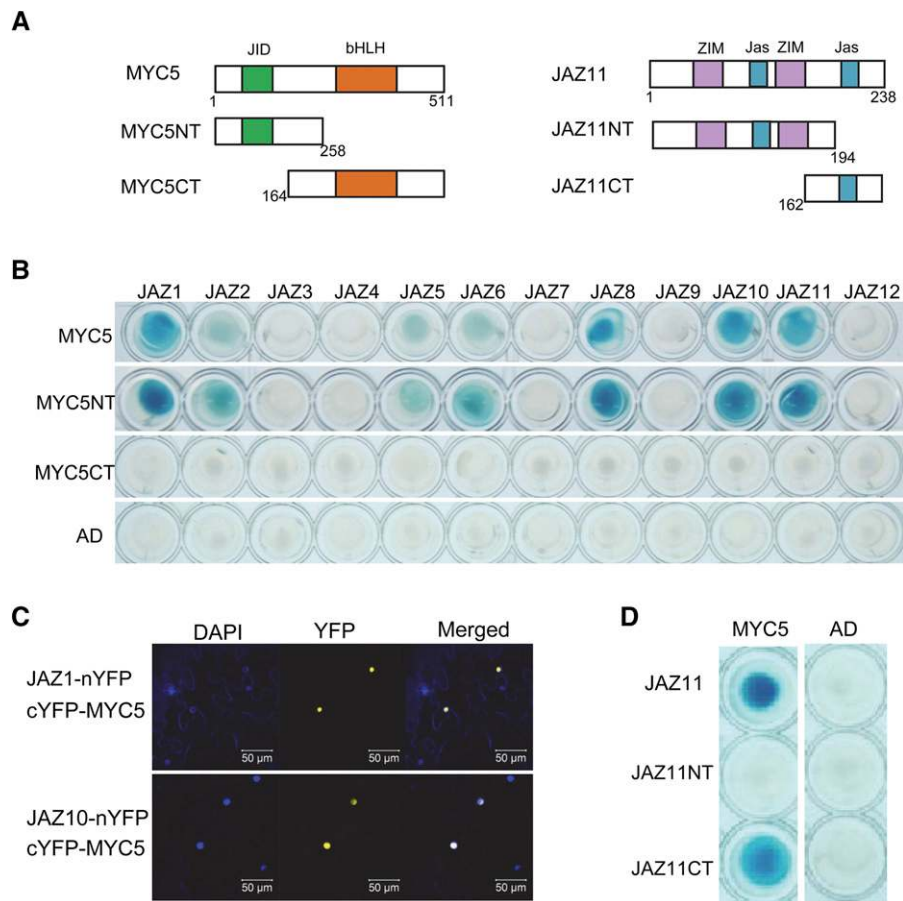
Figure 1. JAZ Proteins Interact with MYC5.

(A) Schematic diagrams show domain constructs of MYC5 and JAZ11. The diagrams display the conserved JAZ-interaction domain (JID; green), bHLH domain (orange), ZIM domain (purple), and Jas domain (blue). The numbers indicate positions of the first and last amino acids of the domain constructs.
(B) Y2H assay to test the interactions of 12 Arabidopsis JAZs with MYC5 and its related domains (as shown in **[A]**). JAZs were fused with the LexA DNA binding domain in pLexA, and MYC5 and its domains were fused with the activation domain (AD) in pB42AD. Interactions (represented by blue color) were assessed on 2% Gal/1% raffinose/SD/-Ura/-His/-Trp/-Leu/X-β-Gal medium.
(C) BiFC assay to detect the interactions of JAZ1 and JAZ10 with MYC5. JAZ1, JAZ10, and MYC5 were fused with the N-terminal fragment of YFP (nYFP) or the C-terminal fragment of YFP (cYFP) to generate JAZ1-nYFP, JAZ10-nYFP, and cYFP-MYC5. YFP fluorescence was detected 50 h after coexpression of the indicated construct pairs in leaves of *N. benthamiana*. The nuclei are indicated by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.
(D) Y2H assay to test the interactions of JAZ11 domain constructs (as shown in **[A]**) with MYC5. MYC5 was fused with the activation domain, and different JAZ11 domains were fused with the DNA binding domain.

(2 representative lines are shown in Figure 4). Taken together (Figures 3 and 4), these results demonstrate that high expression of *MYC5* or *MYC3* is able to relieve JAZ inhibition and partially recover stamen development in the *coi1-1* mutant.

MYC2, MYC3, MYC4, and MYC5 Are Required for JA-Inducible Expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108*

Previous studies showed that JA induces the expression of the R2R3 MYB transcription factors *MYB21*, *MYB24*, *MYB57*, and *MYB108*, which are essential for JA-regulated stamen development (Mandaokar et al., 2006; Cheng et al., 2009; Mandaokar and

Browse, 2009; Song et al., 2011). We further examined whether the IIIe bHLH factors MYC2, MYC3, MYC4, and MYC5 are required for the JA-inducible expression of these R2R3 MYB transcription factors.

As shown in Figure 5A, the expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108* was partially decreased in the flower buds of *coi1-1* and the *myc2 myc3 myc4 myc5* quadruple mutant compared with that of the wild type, suggesting that COI1 and the IIIe bHLH factors are required for the expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108*.

Moreover, JA treatment can induce the expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108* in the flower buds of the wild type (Figure 5A). In contrast with the JA-treated wild type, we found that,
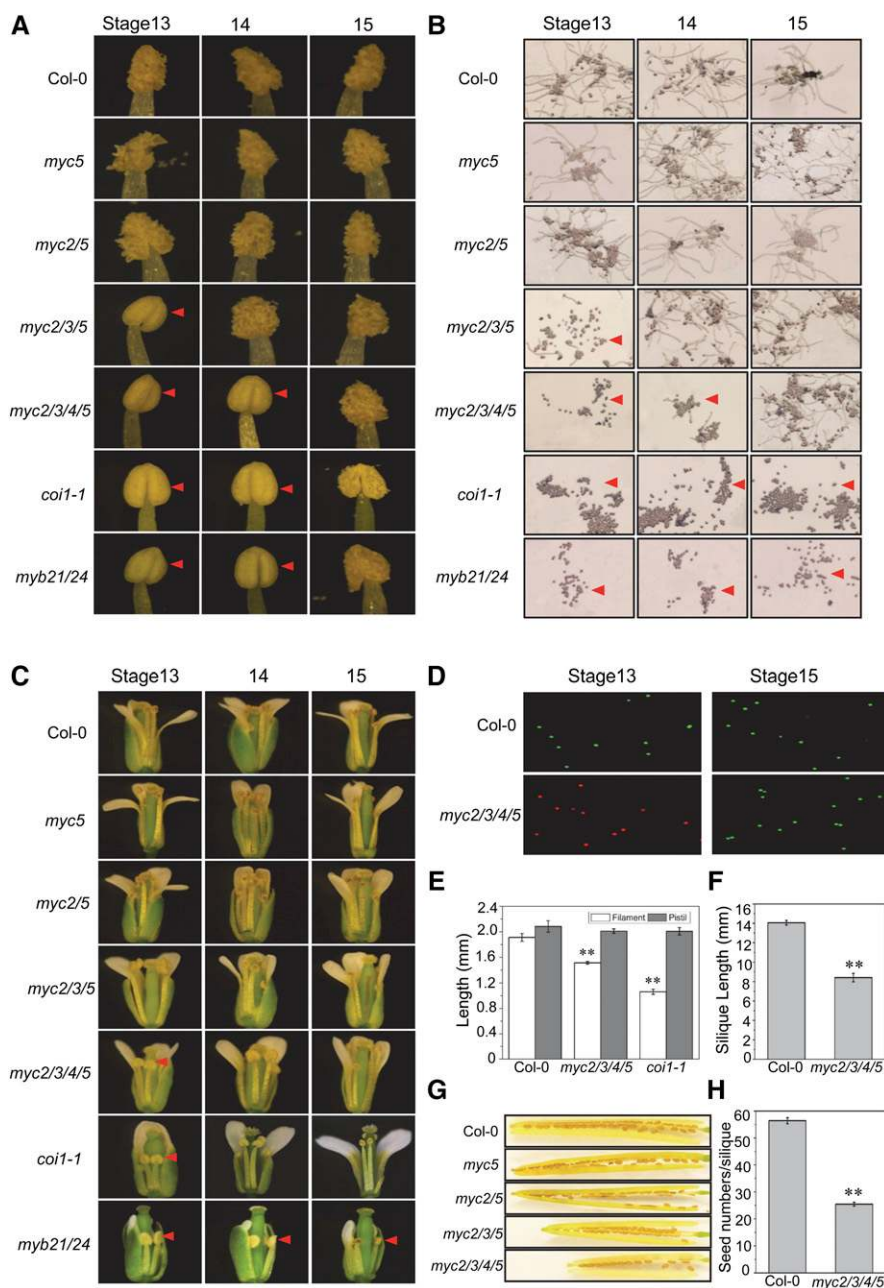
Figure 2. The bHLH Subgroup IIIe Factors Function Redundantly to Regulate Stamen Development.

(A) Phenotypic observation of anther dehiscence of the indicated genotypes at floral stage 13, 14, or 15. Red arrowheads represent indehiscent anthers.

(B) In vitro germination of pollen grains from the flowers at floral stage 13, 14, or 15 in the indicated genotypes. Red arrowheads indicate pollen grains deficient in germination in vitro.

(C) Comparison of flowers at floral stage 13, 14, or 15 in the Col-0 wild type, *myc5*, *myc2 myc5* (*myc2/5*), *myc2 myc3 myc5* (*myc2/3/5*), *myc2 myc3 myc4 myc5* (*myc2/3/4/5*), *coi1-1*, and *myb21-3 myb24* (*myb21/24*). Red arrowheads indicate shorter filaments.

(D) Staining of pollen grains from Col-0 and *myc2 myc3 myc4 myc5* (*myc2/3/4/5*) flowers at floral stage 13 or 15 with fluorescein diacetate and propidium iodide. Viable pollen grains are green, and unviable pollen grains are red.

(E) Filament length and pistil length at floral stage 13 in the indicated genotypes. Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance compared with the Col-0 wild type (**P < 0.01).

(F) Silique length of Col-0 and *myc2 myc3 myc4 myc5* (*myc2/3/4/5*). Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance compared with the Col-0 wild type (**P < 0.01).

(G) Siliques from Col-0, *myc5*, *myc2 myc5* (*myc2/5*), *myc2 myc3 myc5* (*myc2/3/5*), and *myc2 myc3 myc4 myc5* (*myc2/3/4/5*).

(H) Seed numbers per silique of Col-0 and *myc2 myc3 myc4 myc5* (*myc2/3/4/5*). Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance compared with the Col-0 wild type (**P < 0.01).
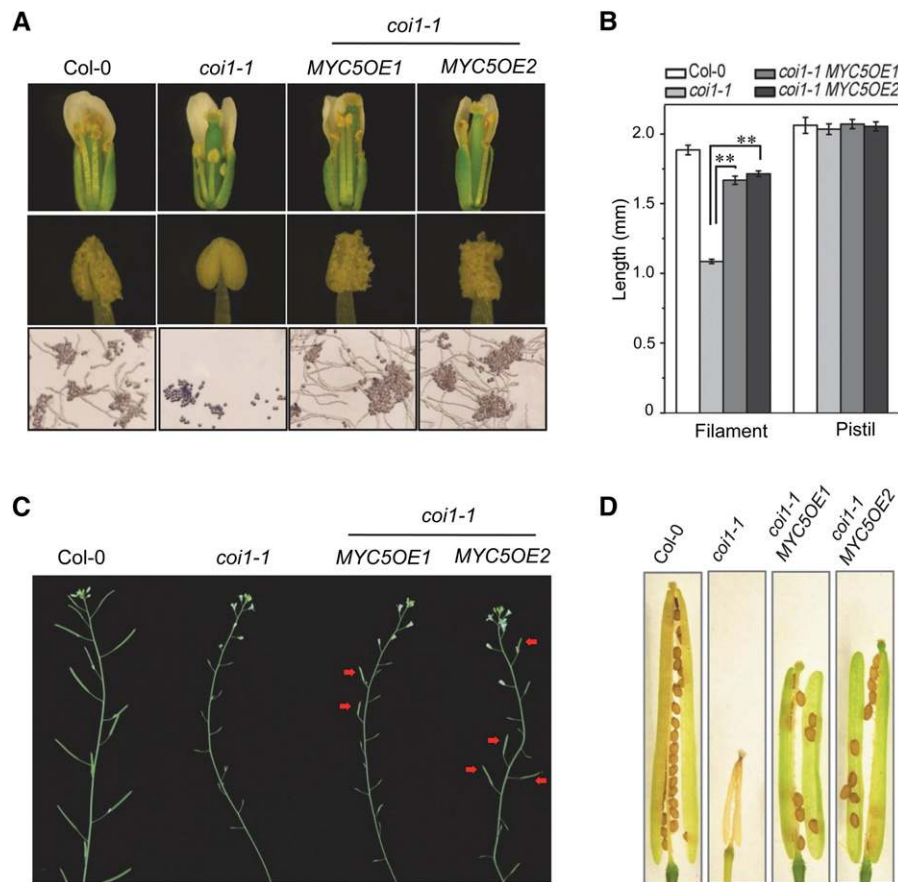
Figure 3. Overexpression of *MYC5* Partially Restores Stamen Development in *coi1-1*.

(A) Comparison of flowers, anthers, and in vitro germination of pollen grains from flowers at floral stage 13 in the Col-0 wild type, *coi1-1*, and *coi1-1* plants overexpressing *MYC5* (*coi1-1 MYC5OE1* and *coi1-1 MYC5OE2*).
(B) Filament length and pistil length in flowers at floral stage 13 from Col-0, *coi1-1*, and *coi1-1* plants overexpressing *MYC5* (*coi1-1 MYC5OE1* and *coi1-1 MYC5OE2*). Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance compared with *coi1-1* (**P < 0.01).
(C) Main inflorescences in the indicated genotypes. Red arrows indicate fertile siliques in *coi1-1 MYC5OE1* and *coi1-1 MYC5OE2*.
(D) Comparison of seed set in the indicated genotypes.

similar to *coi1-1*, the *myc2 myc3 myc4 myc5* quadruple mutant was clearly insensitive to the JA-inducible expression of the R2R3 MYB transcription factors (Figure 5A). Consistent with these results, JA treatment could not rescue stamen development in *myc2 myc3 myc4 myc5* and *coi1-1* (Figure 5B), as indicated by filament elongation, anther dehiscence, and pollen germination in vitro at floral stage 13, while the application of JA was able to rescue stamen development in the JA-deficient mutant *opr3* (Figure 5B).

Furthermore, we found that the overexpression of *MYC5* or *MYC3*, which partially restored stamen development in *coi1-1* (Figures 3 and 4), mildly but clearly restored the expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108* in fertile young flower buds of the *coi1-1 MYC5OE* and *coi1-1 MYC3OE* plants (Figures 5C and 5D). In addition, we applied the ERF-associated amphiphilic repression (EAR) motif-based chimeric repressor-silencing technology (Hiratsu et al., 2003) to overexpress the MYC5 chimeric repressors (MYC5-EAR) in wild-type plants, which disrupted

stamen development, abolished male fertility, and suppressed the expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108* in the flower buds of the sterile *MYC5-EAR* plants (Supplemental Figure 6). Our results are consistent with similar observations of *MYC5-SRDX* transgenic plants reported recently (Figueroa and Browse, 2015).

Taken together (Figures 2 to 5; Supplemental Figures 4 and 5), these results demonstrated that the IIIe bHLH factors MYC2, MYC3, MYC4, and MYC5 function redundantly to regulate stamen development and mediate the JA-inducible expression of the *MYB* genes (*MYB21*, *MYB24*, *MYB57*, and *MYB108*).

The IIIe bHLH Factors Interact with the MYB Transcription Factors MYB21 and MYB24

Having showed that the expression of these R2R3-MYB factors is regulated by the IIIe bHLH factors (MYC2, MYC3, MYC4, and MYC5) (Figure 5), we next investigated whether
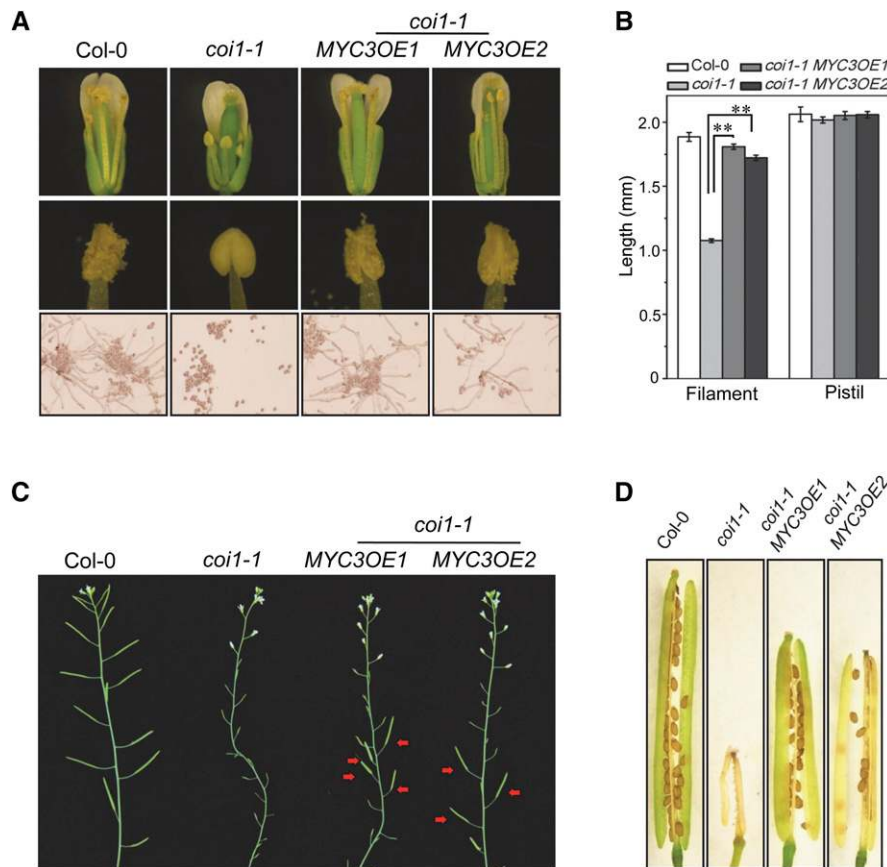
Figure 4. Overexpression of *MYC3* Partially Restores Stamen Development in *coi1-1*.

(A) Comparison of flowers, anthers, and in vitro germination of pollen grains from flowers at floral stage 13 in the Col-0 wild type, *coi1-1*, and *coi1-1* plants overexpressing *MYC3* (*coi1-1 MYC3OE1* and *coi1-1 MYC3OE2*).

(B) Filament length and pistil length in flowers at floral stage 13 from Col-0, *coi1-1*, and *coi1-1* plants overexpressing *MYC3* (*coi1-1 MYC3OE1* and *coi1-1 MYC3OE2*). Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance compared with *coi1-1* (**$P < 0.01$).

(C) Main inflorescences in the indicated genotypes. Red arrows indicate fertile siliques in *coi1-1 MYC3OE1* and *coi1-1 MYC3OE2*.

(D) Comparison of seed set in the indicated genotypes.

the R2R3-MYB factors, such as MYB21 and MYB24, interact with the IIIe bHLH factors.

We used MYC5 as the representative and examined its interaction with MYB21 and MYB24. As both MYB21 and MYB24 exhibited autoactivation in the Y2H system, we used MYB21NT and MYB24NT with deletion of the C-terminal activation domain (NYW^G/$_S$M/$_V$DD^I/$_L$W^S/$_P$ motif) to examine their interaction with MYC5 in the Y2H system (Figure 6A). As shown in Figure 6B, both MYB21NT and MYB24NT interacted with MYC5 in yeast. Moreover, MYB21NT and MYB24NT interacted with MYC5NT but not with MYC5CT (Figure 6B). We next performed coimmunoprecipitation (Co-IP) assays to test the interaction of MYC5 with MYB21 in planta. Our Co-IP assays showed that flag-MYC5 could coimmunoprecipitate with myc-MYB21 (Figure 6C).

We also employed BiFC assays to verify the interaction of MYC5 with MYB21 and MYB24 in planta. The coexpression of MYC5-nYFP with cYFP-MYB21 or cYFP-MYB24 in *Nicotiana*

benthamiana leaves produced strong YFP signals (Figure 6D), but the negative controls did not (Supplemental Figure 1), suggesting that MYC5 interacts with MYB21 and MYB24 in planta. Similar results were observed for the coexpression of MYC2-nYFP, MYC3-nYFP, and MYC4-nYFP with cYFP-MYB21 or cYFP-MYB24 (Figure 6D; Supplemental Figure 1).

Taken together (Figure 6), these Y2H, Co-IP, and BiFC assays demonstrate that IIIe bHLH factors (MYC2, MYC3, MYC4, and MYC5) interact with MYB21 and MYB24 to form the bHLH-MYB transcription complex.

JAZ1 Attenuates the Transcriptional Activation Function of the bHLH-MYB Complex

Since the IIIe bHLH proteins (MYC2, MYC3, MYC4, and MYC5) and the MYB proteins (MYB21 and MYB24) are targets of JAZ proteins, we examined whether the JAZ interactions repress the transcriptional activation activity of these components.
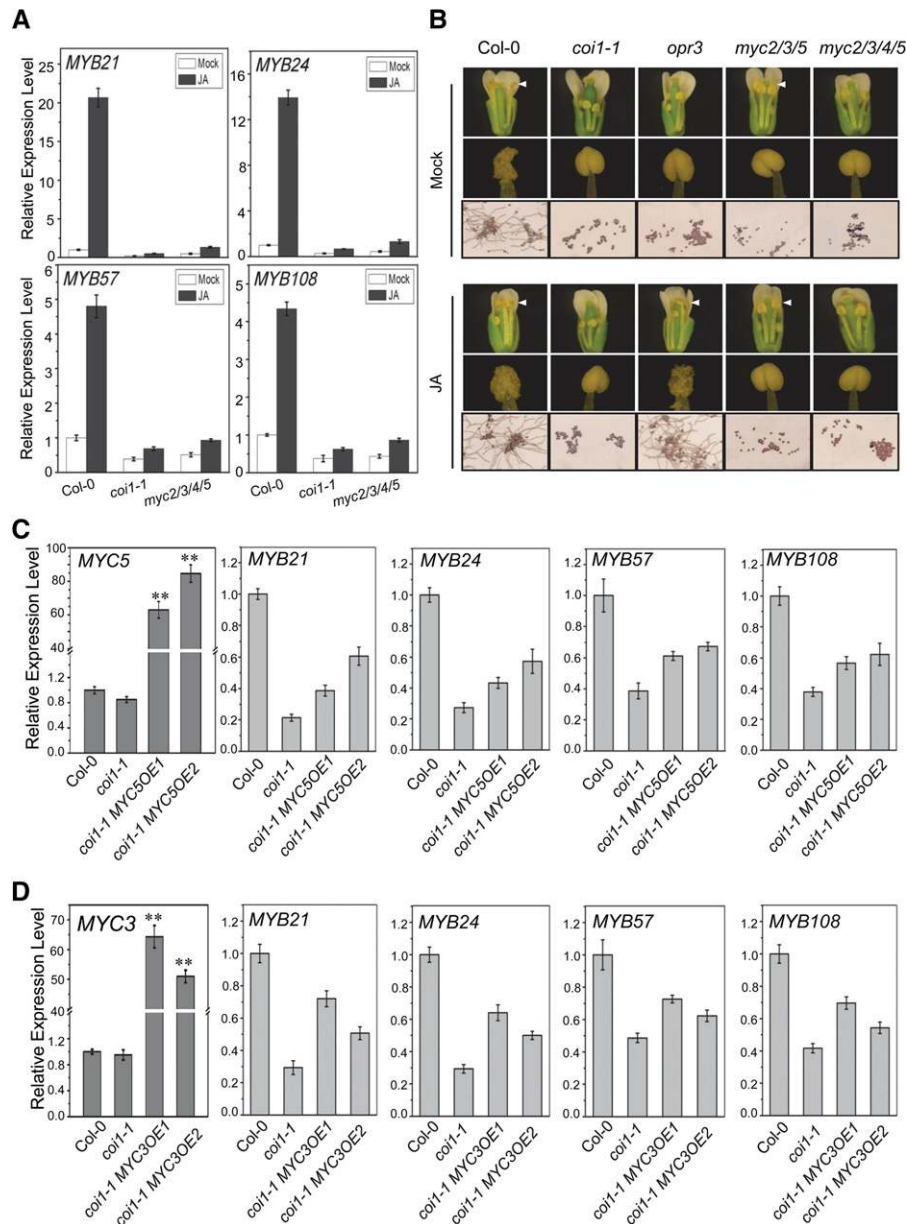
Figure 5. MYC2, MYC3, MYC4, and MYC5 Are Required for JA-Inducible Expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108*.

(A) RT-qPCR analysis for *MYB21*, *MYB24*, *MYB57*, and *MYB108* in young flower buds of the Col-0 wild type, *coi1-1*, and *myc2 myc3 myc4 myc5* (*myc2/3/4/5*) treated without (Mock) or with methyl jasmonate (JA). *ACTIN8* was used as the internal control. Values are means ± SE from three biological replicates.
(B) Comparison of flowers, anther dehiscence, and in vitro germination of pollen grains at floral stage 13 in Col-0, *coi1-1*, *opr3*, *myc2 myc3 myc5* (*myc2/3/5*), and *myc2 myc3 myc4 myc5* (*myc2/3/4/5*) treated without (Mock) or with methyl jasmonate (JA). White arrowheads indicate fully elongated filaments.
(C) and **(D)** RT-qPCR analysis of gene expression in Col-0, *coi1-1*, and *coi1-1* plants overexpressing *MYC5* (*coi1-1 MYC5OE1* and *coi1-1 MYC5OE2*) **(C)** or *coi1-1* plants overexpressing *MYC3* (*coi1-1 MYC3OE1* and *coi1-1 MYC3OE2*) **(D)**. Expression of *MYC5* **(C)** and *MYC3* **(D)** was detected in 3-week-old plants, and expression of *MYB21*, *MYB24*, *MYB57*, and *MYB108* was tested in young flower buds of the indicated genotypes using *ACTIN8* as the internal control. Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance compared with the Col-0 wild type (**P < 0.01).

The fusion of MYC2 with GAL4DB (GAL4DB-MYC2) driven by the 35S promoter was used as an effector, and the *GUS* gene under the control of four copies of GAL4 DNA binding site [GAL4(4x)-D1-3(4x)] was used as a reporter, whereas the *luciferase* (*LUC*) gene driven by the 35S promoter was used as the internal control (Figure 7A). The expression of GAL4DB-MYC2 clearly increased the GUS:LUC ratio, while coexpression of JAZ1 with GAL4DB-MYC2 clearly decreased
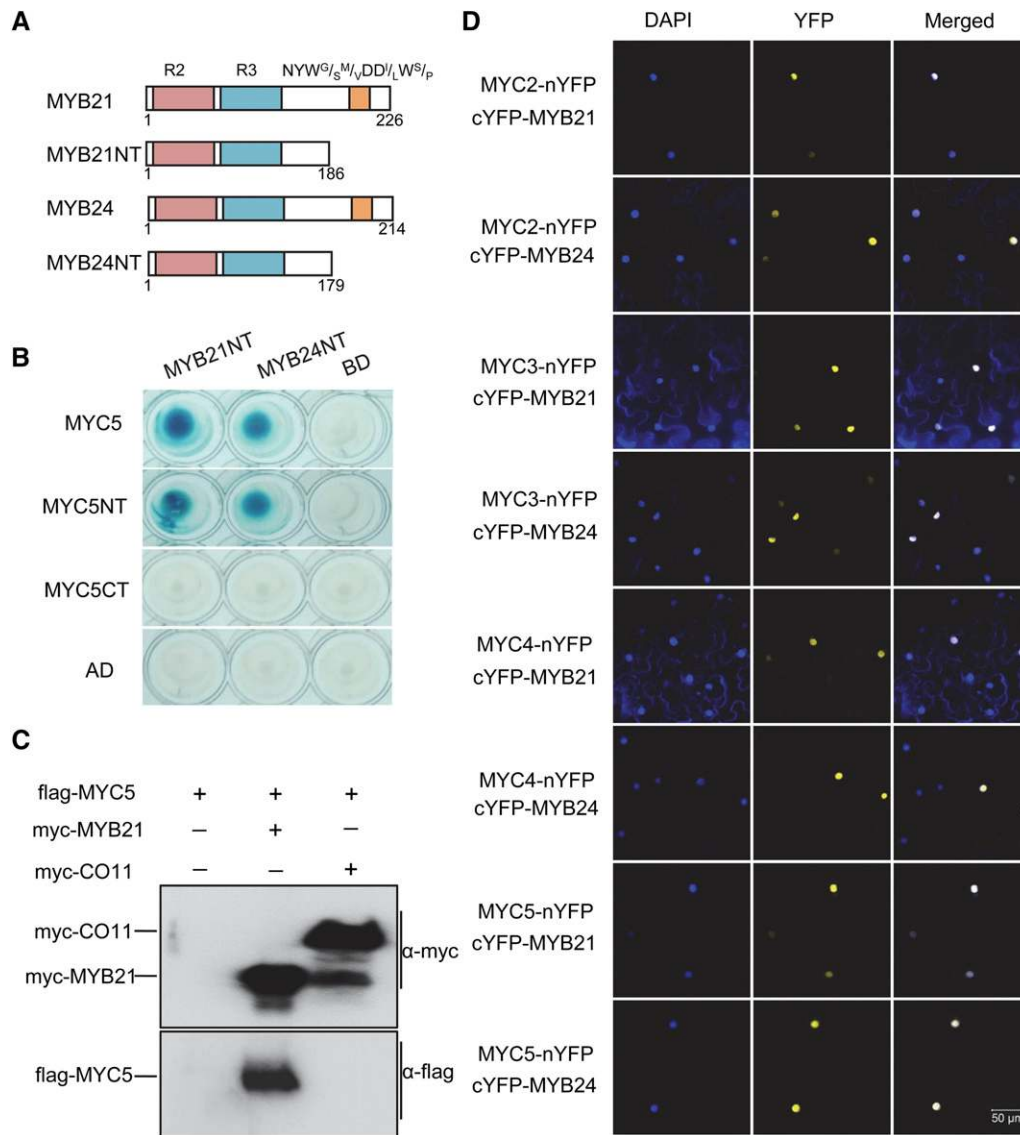
Figure 6. MYC2, MYC3, MYC4, and MYC5 Interact with MYB21 and MYB24.

(A) Schematic diagrams of MYB21, MYB24, and their N-terminal domains (MYB21NT and MYB24NT). MYB21NT and MYB24NT contain the conserved R2 (red) and R3 (blue) domains but not the NYW^G/$_S$M/$_V$DD^I/$_L$W^S/$_P$ motif (orange).
(B) Y2H assay to show interactions between MYB21NT/MYB24NT and MYC5. MYB21NT and MYB24NT were fused with the DNA binding domain (BD), and MYC5 was fused with the activation domain (AD).
(C) Co-IP assay to verify the interaction between MYC5 and MYB21 in vivo. Flag-MYC5 was coexpressed without (empty control) or with myc-MYB21 or myc-COI1 (negative control) in *N. benthamiana* leaves. The total protein extracts from *N. benthamiana* leaves transiently expressing flag-MYC5, flag-MYC5 plus myc-MYB21, or flag-MYC5 plus myc-COI1 were immunoprecipitated using the anti-c-myc antibody-conjugated agarose and were blotted with anti-flag or anti-c-myc antibody.
(D) BiFC assay to detect interactions of MYC2, MYC3, MYC4, and MYC5 with MYB21 and MYB24. MYC2, MYC3, MYC4, and MYC5 were fused with nYFP, and MYB21 and MYB24 were fused with cYFP. The nuclei are indicated by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.

the GUS:LUC ratio (Figure 7B; Supplemental Figure 7). The results suggested that MYC2 acts as a transcriptional activator, while JAZ1 attenuates the transcriptional activation function of MYC2. Similarly, we observed that JAZ1 can inhibit the transcriptional activity of MYC3, MYC4, MYC5, MYB21, and MYB24 (Figure 7).

In summary, these results (Figures 1 and 7) suggest that JAZ proteins repress the transcriptional activation function of the IIIe bHLH (MYC2, MYC3, MYC4, and MYC5) and MYB (MYB21 and MYB24) components of the bHLH-MYB complex.

MYC2, MYC3, MYC4, and MYC5 Function Cooperatively with MYB21 and MYB24 to Regulate Stamen Development and Seed Production

To genetically investigate whether the IIIe bHLH factors (MYC2, MYC3, MYC4, and MYC5) cooperate with MYB21 and MYB24 to regulate stamen development, we used the *myc2 myc3 myc4 myc5* quadruple mutant and the *myb21-t1 myb24* double mutant (with a weak allele for *myb21*) (Cheng et al., 2009) to generate the *myc2 myc3 myc4 myc5 myb21-t1 myb24* sextuple mutant.

As shown in Figure 8, the *myc2 myc3 myc4 myc5 myb21-t1 myb24* sextuple mutant plants were completely male-sterile: they exhibited short filaments, failure of anther dehiscence, and unviable pollen at the late floral stage (Figures 8A to 8C). By contrast, *myc2 myc3 myc4 myc5* and *myb21-t1 myb24* harbored fertile anthers at floral stage 15 and exhibited partial

fertility (Figures 8A to 8C). These results demonstrated that the IIIe bHLH factors (MYC2, MYC3, MYC4, and MYC5) and the MYB factors (MYB21 and MYB24) cooperatively mediate JA-regulated stamen development and seed production.

DISCUSSION

We previously revealed that JAZ repressors interact with and repress the MYB transcription factors MYB21, MYB24, and MYB57 to modulate JA-regulated stamen development (Song et al., 2011). In this study, we elucidated that the IIIe bHLH transcription factor MYC5 acts as a target of JAZ repressors to function redundantly with the other IIIe bHLH factors MYC2, MYC3, and MYC4 in the regulation of stamen development and seed production. Moreover, these IIIe bHLH factors interact with the MYB transcription factors MYB21 and MYB24 to form the
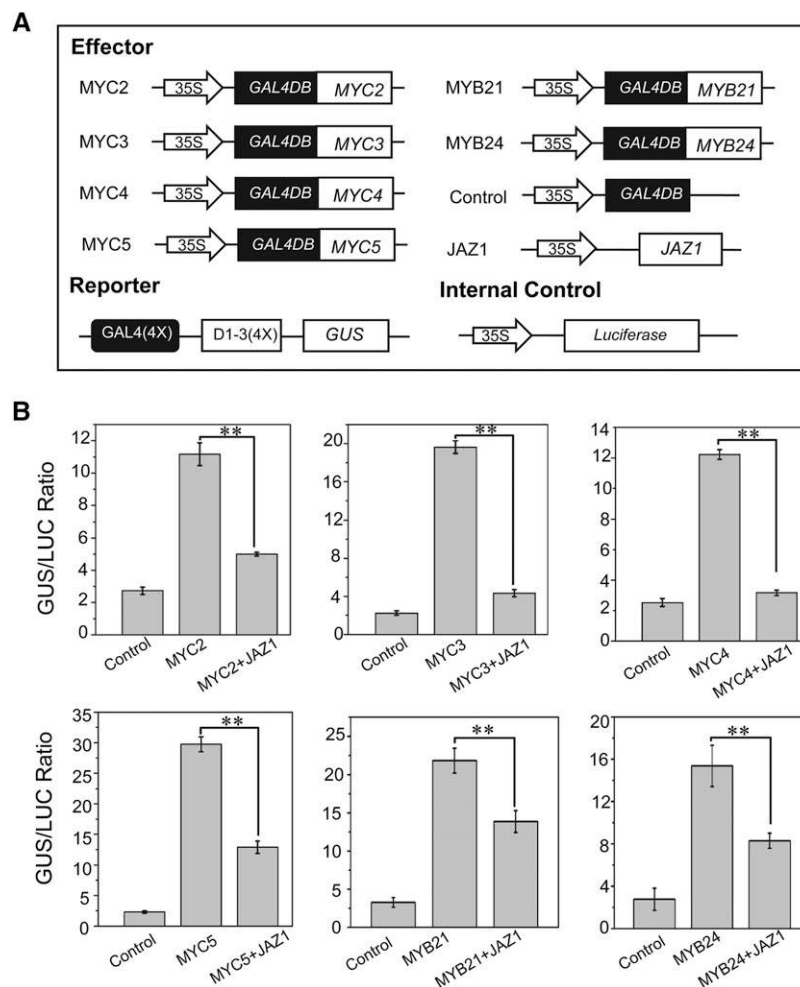

Figure 7. JAZ1 Attenuates the Transcriptional Activation Function of MYC2, MYC3, MYC4, MYC5, MYB21, and MYB24.

(A) Schematic diagrams of the constructs used in the transient expression assays in **(B)**.
(B) Transient expression assays show that JAZ1 inhibits the transcriptional activation function of MYC2, MYC3, MYC4, MYC5, MYB21, and MYB24. Values are means ± SE from three biological replicates. Asterisks represent Student's *t* test significance between pairs indicated with brackets (**P < 0.01).
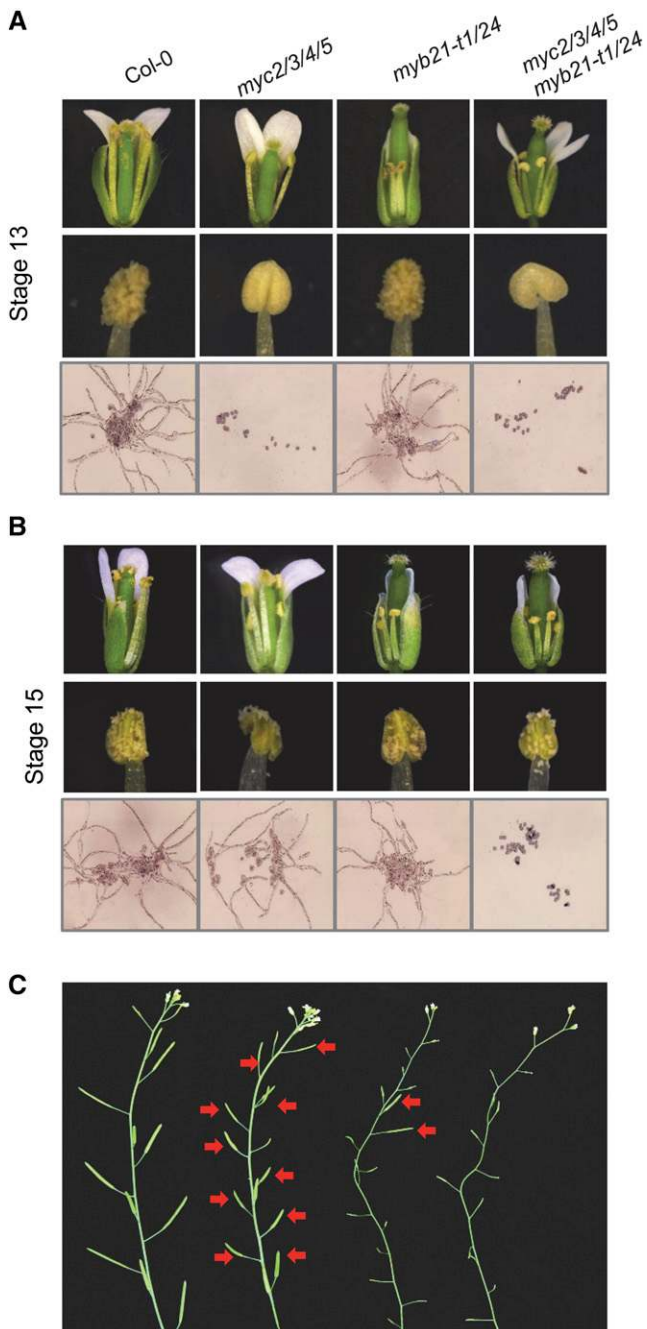
bHLH-MYB transcription complex and cooperatively regulate stamen development.

Our results suggest that, as diagrammed in Figure 9, JAZ repressors interact with and repress the bHLH-MYB complex to suppress stamen development. In response to environmental cues or developmental signals (such as flowering), plants biosynthesize JA that is perceived by its receptor COI1 to induce JAZ degradation and release the bHLH-MYB complex, which subsequently regulates the expression of downstream genes (as well as some members of the bHLH-MYB complex) to mediate stamen development. Based on our current findings together with our previous observations (Cheng et al., 2009), we speculate that gibberellin also acts through the bHLH-MYB complex to regulate stamen development, although additional unidentified components are probably involved: DELLAs inhibit the expression of JA biosynthetic genes and repress the biosynthesis of JA in flowers, thereby leading to the suppression of stamen development. DELLAs directly interact with and repress MYC2 (Hong et al., 2012), and it will be interesting to investigate whether the DELLA proteins directly target all the bHLH members (MYC2, MYC3, MYC4, and MYC5) and MYB members (MYB21 and MYB24) to inhibit the transcriptional function of the bHLH-MYB complex for the regulation of gibberellin-regulated stamen development.

We observed that the MYC members positively regulate the expression of the *MYB* genes: the expression of *MYB21*,
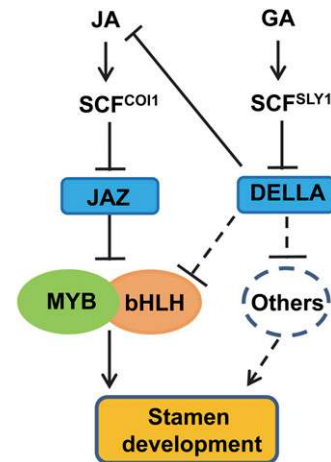

Figure 8. The bHLH and MYB Factors Cooperatively Mediate JA-Regulated Stamen Development.

(A) and **(B)** Phenotypes of flowers, stamens, and in vitro pollen germination in the Col-0 wild type, *myc2 myc3 myc4 myc5* (*myc2/3/4/5*), *myb21-t1 myb24*, and *myc2 myc3 myc4 myc5 myb21-t1 myb24* (*myc2/3/4/5 myb21-t1/24*) at floral stage 13 **(A)** and floral stage 15 **(B)**.
(C) Inflorescences in the indicated genotypes. Red arrows indicate fertile siliques of *myc2 myc3 myc4 myc5* and *myb21-t1 myb24*.

Figure 9. A Simplified Model for JA-Regulated Stamen Development.

JAZ repressors interact with and repress the bHLH-MYB complex consisting of the IIIe bHLH factors MYC2, MYC3, MYC4, and MYC5 (indicated as bHLH) and the R2R3 MYB factors MYB21 and MYB24 (indicated as MYB). JA is perceived by COI1 to induce JAZ degradation and release the bHLH-MYB complex, which regulates the expression of downstream genes (as well as some members of the bHLH-MYB complex) to mediate stamen development. Gibberellin (GA) may also act through the bHLH-MYB complex to regulate stamen development: DELLAs repress the biosynthesis of JA in flowers (Cheng et al. 2009) to suppress JA-regulated stamen development. In addition, DELLAs directly interact with and repress MYC2 (Hong et al. 2012). Further experiments are required to verify whether the interaction of DELLAs with MYC2 or other unidentified components (indicated as Others) modulates gibberellin-regulated stamen development.

MYB24, and *MYB57* was downregulated in the flower buds of the *myc2 myc3 myc4 myc5* quadruple mutant (Figure 5A), and conversely, overexpression of *MYC3* or *MYC5* partially restored the expression of *MYB21*, *MYB24*, and *MYB57* in the flower buds of *coi1-1* (Figures 5C and 5D). Interestingly, the expression of *MYC2* was obviously increased in the flower buds of the *myb21 myb24* double mutants, while the expression of *MYC5* was not elevated (Supplemental Figure 8A). A previous study showed that *myb21 myb24* exhibits elevated JA biosynthesis in flowers (Reeves et al., 2012). It is likely that the elevated JA biosynthesis in *myb21 myb24* may upregulate the expression of *MYC2* but not *MYC5*. Consistent with this idea, we found that treatment with JA significantly induced the expression of *MYC2* but not *MYC5* (Supplemental Figures 8B and 8D). The feedback regulation among the MYCs and MYBs is complicated and requires further experimental verification.

Previous research has shown that bHLH factors (GL3, EGL3, or TT8) interact with R2R3 MYB factors (MYB75 or GL1), together with the WD-repeat protein TTG1, to form a transcription complex, referred to as the WD-repeat/bHLH/MYB complex (Zhang et al., 2003). Within this complex, the bHLH factor (such as GL3) interacts with the MYB factors (MYB75 or GL1) via their N-terminal domains, while JAZ repressors competitively interact with the N-terminal domains of these bHLH and MYB factors to disrupt and repress the WD-repeat/bHLH/MYB complex essential for JA-regulated anthocyanin biosynthesis and trichome initiation (Qi et al., 2011, 2014). JA induces the degradation of JAZs to release this WD-repeat/bHLH/MYB complex, which further activates the expression of the downstream genes to enhance anthocyanin biosynthesis and trigger trichome initiation (Qi et al., 2011).

Analogously to the WD-repeat/bHLH/MYB complex, in our newly identified bHLH-MYB transcription complex, a similar N-terminal interaction also mediates the association of the bHLH factors (MYC2, MYC3, MYC4, and MYC5) with the MYB factors (MYB21 and MYB24). JAZ proteins similarly interact with (Figure 1) (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Song et al., 2011) and repress these bHLH factors and MYB factors (Figure 7) to disrupt the bHLH-MYB complex for the modulation of JA-regulated stamen development. Although interactions occur among multiple bHLH factors (or MYB members) (Fernández-Calvo et al., 2011; Song et al., 2011), it is still unclear whether the bHLH-MYB complex contains a single or multiple bHLH factors (or MYB factors) in planta.

TTG1, a member of the WD-repeat family, acts as a key component of the WD-repeat/bHLH/MYB complex to regulate anthocyanin biosynthesis and trichome initiation (Zhang et al., 2003). Several target genes are directly activated by the WD-repeat/bHLH/MYB complex for the regulation of anthocyanin biosynthesis and trichome initiation (Zhang et al., 2003; Gonzalez et al., 2008; Morohashi and Grotewold, 2009). It remains to be investigated whether, in our newly identified complex, another unidentified WD-repeat protein similarly associates with the IIIe bHLH factors (MYC2, MYC3, MYC4, and MYC5) and MYB factors (MYB21 and MYB24) to regulate stamen development.

The bHLH factors MYC2, MYC3, and MYC4 are known to interact with each other to form homodimers/heterodimers and to associate with MYB factors (MYB28, MYB29, MYB34, MYB51,

MYB76, and MYB122) to regulate glucosinolate biosynthesis (Fernández-Calvo et al., 2011; Schweizer et al., 2013). This study demonstrated that MYC factors (MYC2, MYC3, MYC4, and MYC5) interact with MYB members (MYB21 and MYB24) to form a bHLH-MYB complex for the regulation of stamen development. Identification and characterization of target genes for the bHLH-MYB complex will provide insights into the molecular mechanism underlying JA-regulated stamen development.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* mutants *coi1-1* (Xie et al., 1998), *opr3* (Stintzi and Browse, 2000), and *myc2-2* (Salk_083483) (Boter et al., 2004) were described previously. *myc3* (GK445B11), *myc4* (GK491E10), and *myc5* (Salk_060048C) were obtained from the NASC or ABRC. The double, triple, and quadruple mutants of the bHLH subgroup IIIe factors were generated by genetic crossing with *myc2-2*, *myc3*, *myc4*, and *myc5*.

Arabidopsis thaliana seeds were sterilized with 20% bleach, plated on Murashige and Skoog medium (Sigma-Aldrich), chilled at 4°C for 3 d, and transferred to a growth room under a 16-h (20 to 24°C)/8-h (16 to 19°C) light/dark condition as described previously (Song et al., 2014a). *Nicotiana benthamiana* was grown in a growth room under a 16-h (25 to 28°C)/8-h (22 to 25°C) light/dark condition as described previously (Song et al., 2014a).

Y2H Screening and Y2H Assays

The Y2H screening method was described previously (Song et al., 2013a). For the Y2H assay, JAZ1, JAZ2, JAZ3, JAZ4, JAZ5, JAZ6, JAZ7, JAZ8 JAZ9, JAZ10, JAZ11, JAZ12, and MYC5, or the related domains of JAZ11, MYC5, MYB21, and MYB24, were inserted into pLexA or pB42AD vector, respectively. Primers used for the vector construction are listed in Supplemental Table 1. The methods for yeast transformation and the interaction assay using EGY48 were as described previously (Song et al., 2011). Y2H images were taken 3 d after incubation.

Co-IP Assay

N. benthamiana leaves were infiltrated with *Agrobacterium tumefaciens* strains harboring flag-MYC5, flag-MYC5 plus myc-MYB21, or flag-MYC5 plus myc-COI1 (Xu et al., 2002). At 50 h after infiltration, 3 g of Agrobacterium-infiltrated leaves for each combination was collected. The total protein was extracted with Co-IP buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 50 mM MG132, and complete protease inhibitor cocktail [Roche]) and centrifuged twice at 16,000g at 4°C. The supernatant was concentrated to 400 mL and incubated with the agarose-conjugated anti-myc matrix (Abmart) for 2 h with rotation at 4°C. After washing three times with 1 mL of immuno-precipitation buffer, the agarose beads were denatured in 100 mL of SDS loading buffer. The samples were separated by SDS-PAGE and immunoblotted with the corresponding antibody.

BiFC Assays

For BiFC assays, the coding sequences of Arabidopsis *JAZ1*, *JAZ10*, *MYC2*, *MYC3*, *MYC4*, *MYC5*, *MYB21*, and *MYB24* were inserted individually into the nYFP or cYFP vector through Gateway methods (Invitrogen) (Qi et al., 2011; Song et al., 2011). Primer pairs for vector construction are listed in Supplemental Table 1. The Agrobacterium strains containing the indicated vectors were coinfiltrated into leaves of *N. benthamiana* as

described previously (Song et al., 2011; Qi et al., 2013). After infiltration, plants were placed at 24°C for 50 h before observation.

Reverse Transcription and Quantitative Real-Time PCR

For Supplemental Figure 2C, the expression of MYC5 was analyzed in root, stem, rosette leaf, stem leaf, and flower from 5-week-old Arabidopsis plants. For Figures 5C and 5D and Supplemental Figure 6D, the expression of MYC3 and MYC5 was analyzed in 3-week-old Arabidopsis plants. The expression of MYB21, MYB24, MYB57, and MYB108 in Figures 5C and 5D and Supplemental Figure 6E and the expression of MYC2, MYC3, and MYC5 in Supplemental Figure 8A were analyzed in young flower buds from 5-week-old Arabidopsis plants. For Figure 5A and Supplemental Figures 8B to 8D, the expression of MYB21, MYB24, MYB57, MYB108, MYC2, MYC3, and MYC5 was analyzed in young flower buds from 5-week-old Arabidopsis plants mock treated or treated with 100 μM methyl jasmonate for 4 h. The materials were harvested for RNA extraction, subsequent reverse transcription, and quantitative real-time PCR analysis for the indicated genes. Quantitative real-time PCR analysis was performed with the RealMasterMix (SYBR Green I; Takara) using the ABI7500 real-time PCR system as described previously (Song et al., 2013a). ACTIN8 was used as the internal control. The primers for reverse transcription and quantitative real-time PCR (RT-qPCR) analysis are listed in Supplemental Table 2.

Transcriptional Activity Assays

For Arabidopsis protoplast transient expression assays, MYC2, MYC3, MYC4, MYC5, MYB21, and MYB24 were fused with the GAL4DB under the control of the 35S promoter, and JAZ1 was inserted into the pGreenII 62-SK vector under the control of the 35S promoter (Hellens et al., 2005). The GUS gene driven by four copies of upstream GAL4 DNA binding sites [GAL4(4x)-D1-3(4x)] was used as a reporter construct (Tiwari et al., 2001), and LUC under the control of the 35S promoter was used as the internal control. Preparation of Arabidopsis mesophyll protoplasts and transfection were performed as described previously (Yoo et al., 2007). GUS: LUC ratios are presented. Primers used for plasmid construction are shown in Supplemental Table 1.

Generation of Transgenic Plants

To generate Arabidopsis MYC3 or MYC5 overexpression constructs, the coding sequence of MYC3 or MYC5 was inserted into modified pCAMBIA1300 vector under the control of the 35S promoter. These constructs were transformed into Arabidopsis COI1/coi1-1 plants using the Agrobacterium-mediated floral dip method. To generate Arabidopsis EAR domain-fused MYC5 (MYC5-EAR) overexpression constructs, the coding sequence of MYC5 was inserted into modified pCAMBIA1300 with fusion of the EAR motif (GLDLDLELRLGFA) under the control of the 35S promoter. The construct was transformed into Arabidopsis Columbia-0 (Col-0) wild-type plants using the Agrobacterium-mediated floral dip method. Primer pairs for vector construction are listed in Supplemental Table 1.

GUS Staining

An ~1795-bp promoter region of MYC5 was amplified and inserted into pCAMBIA1391Z vector to drive the GUS reporter gene (P_{MYC5}-GUS). The construct was transformed into Arabidopsis plants using the Agrobacterium-mediated floral dip method. Roots, seedlings, leaves, inflorescences, flowers, and stamens from the plants transgenic for P_{MYC5}-GUS were used for histochemical staining of GUS as described previously (Song et al., 2013a).

Measurement of Pistil and Filament Length

To measure the filament and pistil length, 10 flowers at floral stage 13 (Smyth et al., 1990) for each genotype were harvested, and the pistil and one of the four longer filaments for each flower were measured using a microscope.

Pollen Germination Assays

Pollen germination assays were performed as described previously (Boavida and McCormick, 2007). Pollen grains at the indicated floral stage (Smyth et al., 1990) were dipped into the pollen germination medium (10% [w/v] Suc, 0.01% boric acid, 1 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM KCl, pH 7.5, and 1.5% agar), incubated at 22°C in the dark for 10 h, and finally observed using a microscope.

Subcellular Localization

The coding sequence of MYC5 was inserted into pEGAD vector for fusion with GFP under the control of the 35S promoter to generate the GFP-MYC5 construct. Agrobacterium containing the GFP-MYC5 or GFP construct was resuspended in infiltration buffer and infiltrated into N. benthamiana leaves by a needleless syringe. After infiltration, plants were placed at 24°C for 50 h before GFP observation.

Accession Numbers

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: 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), MYC2 (AT1G32640), MYC3 (AT5G46760), MYC4 (AT4G17880), MYC5 (AT5G46830), MYB21 (AT3G27810), MYB24 (AT5G40350), MYB57 (AT3G01530), MYB108 (AT3G06490), and ACTIN8 (AT1G49240).

Supplemental Data

Supplemental Figure 1. Negative Controls for the BiFC Experiments.

Supplemental Figure 2. Subcellular Localization and Expression Pattern of MYC5.

Supplemental Figure 3. RT-qPCR Analysis of MYC5 Expression in the T-DNA Insertion Mutant.

Supplemental Figure 4. The bHLH Subgroup IIIe Factors Function Redundantly to Regulate Anther Dehiscence and Germinating Ability of Pollen.

Supplemental Figure 5. The bHLH Subgroup IIIe Factors Function Redundantly to Regulate Filament Elongation and Seed Set.

Supplemental Figure 6. Overexpression of MYC5-EAR Leads to Male Sterility.

Supplemental Figure 7. JAZ1 Cannot Attenuate the Basal Activity of GAL4DB.

Supplemental Figure 8. Expression Level of MYC2, MYC3, and MYC5 in myb21-3 myb24 and coi1-1 Mutants.

Supplemental Table 1. Primers Used for Vector Construction.

Supplemental Table 2. Primers Used for RT-qPCR Analysis.

AUTHOR CONTRIBUTIONS

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