Jasmonate Negatively Regulates Stomatal Development in Arabidopsis Cotyledons[1]

Xiao Han,[a,b,c,2] Yanru Hu,[a,2] Gensong Zhang,[b] Yanjuan Jiang,[a] Xiaolan Chen,[b,3] and Diqiu Yu[a,3]

[a]Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
[b]School of Life Sciences, Yunnan University, Kunming, Yunnan 650091, China
[c]University of the Chinese Academy of Sciences, Beijing 100049, China

ORCID IDs: 0000-0001-8163-5777 (X.C.); 0000-0001-7507-7617 (D.Y.).

Stomata are ports that facilitate gas and water vapor exchange between plants and their environment. Stomatal development is strictly regulated by endogenous signals and environmental cues. Jasmonate is an important signal that modulates multiple physiological processes in plants, yet the molecular mechanisms underlying its interactions with other developmental signaling pathways remain poorly understood. Here, we show that jasmonate negatively regulates stomatal development in Arabidopsis (*Arabidopsis thaliana*) cotyledons. Cotyledons of the wild type and stomata-overproliferating mutants (such as *too many mouths-1* and *stomatal density and distribution1-1*) treated with methyl jasmonate exhibit a clear reduction in stomata number. By contrast, blocking endogenous jasmonate biosynthesis or perception enhanced stomatal development. Moreover, three MYC transcription factors involved in jasmonate signaling, MYC2, MYC3, and MYC4, were found to redundantly modulate jasmonate-inhibited stomatal development. A genetic analysis showed that these MYC proteins act upstream of the SPEECHLESS and FAMA transcription factors to mediate stomatal development. Furthermore, jasmonate repression of stomatal development is dependent on these three MYC transcription factors, as stomatal development of the *myc2 myc3 myc4* triple mutant was insensitive to methyl jasmonate treatment. Collectively, our study demonstrates that jasmonate and MYC transcription factors negatively regulate stomatal development in Arabidopsis cotyledons.

Stomata are part of the epidermis of land plants and mediate gas uptake for photosynthesis and water evaporation by transpiration. In Arabidopsis (*Arabidopsis thaliana*), stomata arise from a subset of undifferentiated meristemoid mother cells, which undergo an asymmetric cell division to generate meristemoids. The meristemoids may go through one to three rounds of asymmetric divisions before differentiating into guard mother cells. The guard mother cells undergo a single symmetric division to eventually generate a pair of guard cells surrounding a microscopic pore (Bergmann and Sack, 2007; Pillitteri and Torii, 2012). Stomatal development is strictly organized and regulated by internal and environmental cues. Previous studies using genetic and molecular approaches have identified a number of crucial components regulating stomatal development, such as the ERECTA family of receptor-like kinases ERECTA (ER), ERECTA-LIKE1 (ERL1), and ERL2 (Shpak et al., 2003, 2005; Masle et al., 2005), TOO MANY MOUTHS (TMM, a receptor protein; Yang and Sack, 1995; Nadeau and Sack, 2002), and their related ligands (Hara et al., 2007; Hunt and Gray, 2009; Sugano et al., 2010; Lee et al., 2012, 2015) as well as the MAPK signaling components (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2008). The MAPK cascade comprises the MAPK kinase kinase YODA, the MAPK kinases MKK4/5, and MPK3/6. All these components act as negative regulators to modulate stomatal development, as loss-of-function mutations in these loci lead to massive overproliferation of stomata. In contrast, several downstream basic helix-loop-helix (bHLH) and MYB transcription factors, including SPEECHLESS (SPCH), MUTE, FAMA, SCREAM/INDUCER OF CBF EXPRESSION (ICE), FOUR LIPS, and MYB88, are positively involved in stomatal formation (Lai et al., 2005; Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007; Kanaoka et al., 2008).

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[2] These authors contributed equally to the article.

[3] Address correspondence to chenxiaolan@ynu.edu.cn and ydq@xtbg.ac.cn.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Diqiu Yu (ydq@xtbg.ac.cn).

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Phytohormones also play crucial roles in regulating stomatal development. Gudesblat et al. (2012) showed that brassinosteroids modulate stomatal development in Arabidopsis hypocotyls. Moreover, Kim et al. (2012) found that brassinosteroids inhibit the production of stomata in Arabidopsis cotyledons, as treatment with brassinolide (the most active form of brassinosteroid) reduces stomatal density. By contrast, brassinosteroid-insensitive mutants exhibit a stomatal clustering phenotype. Mechanistic investigations revealed that the GLYCOGEN SYNTHASE KINASE3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) phosphorylates and inhibits YODA, MKK4/5, and SPCH to integrate brassinosteroid signaling and stomatal development (Gudesblat et al., 2012; Kim et al., 2012; Khan et al., 2013). We further showed that auxin transport and activity modulate the development of stomata (Le et al., 2014). Additional phytohormones, such as GAs, ethylene, and abscisic acid (ABA), also mediate stomatal development (Saibo et al., 2003; Tanaka et al., 2013).

Jasmonate is a critical signal that regulates a wide range of physiological processes, including root elongation (Staswick et al., 1992; Feys et al., 1994), anthocyanin accumulation (Franceschi and Grimes, 1991; Shan et al., 2009), male fertility (McConn and Browse, 1996; Sanders et al., 2000), senescence (Ueda and Kato, 1980; Schommer et al., 2008), and stress responses (Howe et al., 1996; Reymond and Farmer, 1998; Browse, 2009). It is perceived by the F-box protein CORONATINE INSENSITIVE1 (COI1), which forms a functional ubiquitin E3 ligase SCFCOI1 and facilitates the degradation of JASMONATE ZIM-DOMAIN (JAZ) proteins via the 26S proteasome pathway (Xie et al., 1998; Xu et al., 2002; Chini et al., 2007; Thines et al., 2007; Yan et al., 2009; Sheard et al., 2010). JAZ proteins are repressors of jasmonate signaling that interact physically with and attenuate the transcriptional functions of downstream transcription factors (Chini et al., 2007; Thines et al., 2007). The bHLH-type MYC transcription factors MYC2, MYC3, and MYC4 are the most extensively studied JAZ-repressed targets and modulate multiple jasmonate responses (Boter et al., 2004; Chini et al., 2007; Dombrecht et al., 2007; Zhai et al., 2013; An et al., 2017).

Despite recent advances in our understanding of the jasmonate signaling pathway, the relationship between jasmonate signaling and other developmental processes or signaling pathways is still not fully understood. In this study, we used molecular and genetic approaches to study the role of jasmonate in regulating stomatal development. Microscopic observations revealed that exogenous jasmonate repressed stomatal development, as the number of stomata in cotyledons of Arabidopsis seedlings germinated on medium with methyl jasmonate (MeJA) was reduced. Conversely, blocking jasmonate biosynthesis and signaling led to enhanced stomatal development, as the number of stomata increased in cotyledons of jasmonate-related mutants, such as *jar1*, *coi1-2*, and the *myc2 myc3 myc4* triple mutant. Moreover, jasmonate repressed stomatal development dependently of the MYC transcription factors. Thus, our results provide evidence that jasmonate and MYC transcription factors are negative regulators of stomatal development in Arabidopsis cotyledons.

RESULTS

Stomatal Development Is Repressed in Cotyledons of Seedlings Germinated on Medium Containing Exogenous Jasmonate

To explore the role of jasmonate in plant development, we examined the cotyledon epidermises of Arabidopsis seedlings germinated on medium with or without MeJA treatment. Microscopic observations revealed that the stomatal index (the number of stomata per total epidermal cells) and stomatal density (the number of stomata per mm^2) on the abaxial surfaces of cotyledons were reduced in MeJA-treated Columbia-0 (Col-0) wild-type seedlings compared with control plants (Figs. 1 and 2, A and B), suggesting that exogenous jasmonate may negatively regulate stomatal development in Arabidopsis cotyledons. To confirm this activity, we compared stomatal development in the cotyledons of three stomata-overproliferating mutants, *tmm-1*, *er erl1 erl2*, and *sdd1-1* (Yang and Sack, 1995; Berger and Altmann, 2000; Shpak et al., 2005), with or without MeJA treatment. *TMM* encodes a transmembrane Leu repeat-containing receptor-like protein that regulates stomatal production and patterning (Nadeau and Sack, 2002). *ER*, *ERL1*, and *ERL2* encode receptor-like kinases that govern the initial decision of protodermal cells to either divide proliferatively to produce pavement cells or divide asymmetrically to generate stomatal complexes (Shpak et al., 2005). *SDD1* encodes a subtilisin-like Ser protease that affects stomatal development and distribution (Berger and Altmann, 2000). As shown in Figure 2C, the stomatal index of MeJA-treated *tmm-1* was significantly lower than that of control plants (Student's *t* test, $P < 0.01$). Exogenous jasmonate also partially rescued the stomatal clustering phenotype of the *tmm-1* mutant. The number of stomata clusters (per mm^2) and the average cluster size of MeJA-treated *tmm-1* were clearly reduced compared with those of control plants (Figs. 1 and 2D). Moreover, the percentage of clustered stomata (the number of stomata in clusters per total number of stomata) in *tmm-1* was affected by MeJA (Fig. 2E). Similarly, the stomatal index, stomatal cluster size, and percentage of clustered stomata in the *er erl1 erl2* and *sdd1-1* mutant cotyledons also were partially rescued by exogenous jasmonate (Figs. 1 and 2, F–K). Taken together, these results suggested a negative role for jasmonate in modulating stomatal development in Arabidopsis cotyledons.

To confirm jasmonate's role in stomatal development, we further analyzed stomatal phenotypes in true leaves of wild-type plants with or without MeJA treatment. Microscopic observations showed that the stomatal index and stomatal density on the abaxial side of true leaves were decreased in MeJA-treated seedlings

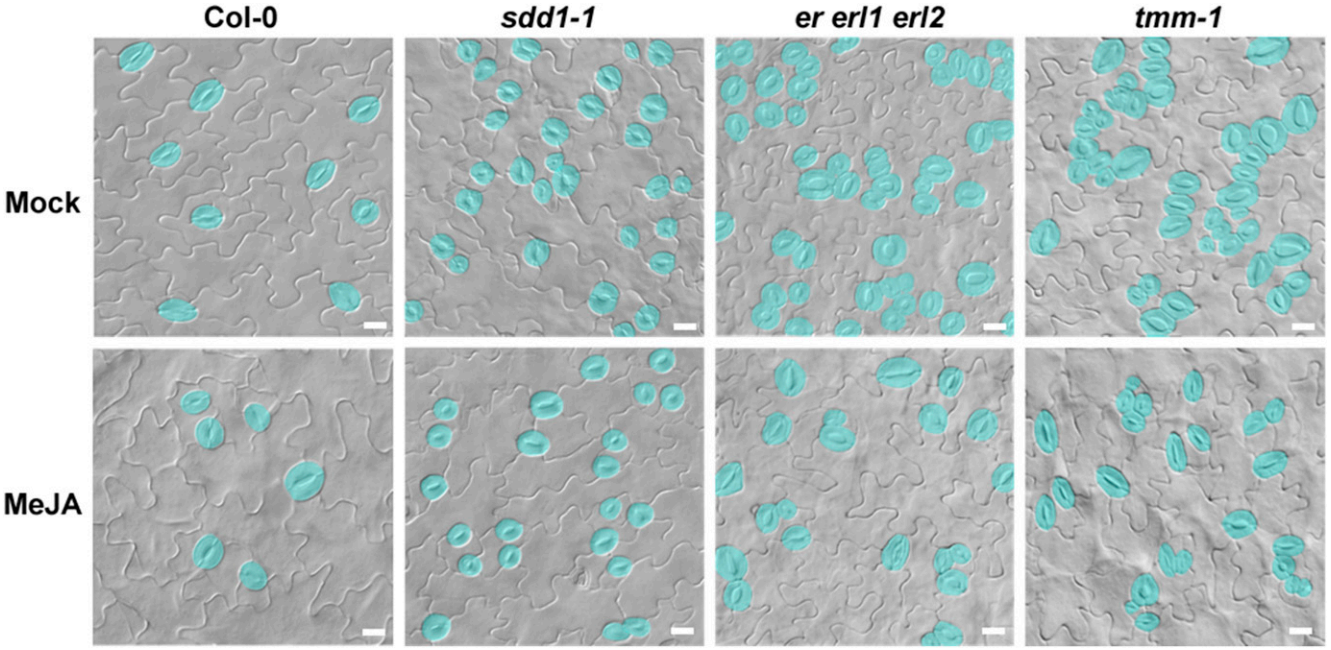

Figure 1. Effect of MeJA on stomatal development in the epidermis of Arabidopsis cotyledons. Differential interference contrast (DIC) images are shown for the cotyledon epidermis of 11-d-old wild type (Col-0), *tmm-1*, *er erl1 erl2*, and *sdd1-1* seedlings germinated on medium with or without 10 μM MeJA. Experiments were performed three times with similar results. Stomata are false colored in blue for easier identification. Bars = 20 μm.

(Supplemental Figs. S1 and S2). In addition, we also analyzed stomatal development in the true leaves of *tmm-1* and *er erl1 erl2* in response to MeJA. As shown in Supplemental Figures S1 and S2, the stomatal index of MeJA-treated *tmm-1* and *er erl1 erl2* was reduced significantly compared with those of control plants (Student's *t* test, $P < 0.01$). Moreover, the number of stomatal clusters, stomatal cluster sizes, and percentage of clustered stomata in the true leaves of *tmm-1* and *er erl1 erl2* mutants also were clearly reduced under MeJA treatment. These findings suggested that exogenous jasmonate also may negatively mediate stomatal development in Arabidopsis true leaves.

The Activities of Several Stomatal Cell Type-Specific Markers Are Suppressed by Exogenous Jasmonate

To verify the regulatory effect of exogenous jasmonate on stomatal development, we characterized the stomatal phenotypes of MeJA-treated plants using several stomatal cell type-specific markers. *E1728* is a mature guard cell-specific GFP marker. In the wild-type background (Col-0), the number of *E1728*-expressing cells was reduced in response to MeJA (Fig. 3, A–C). We then crossed the *E1728* marker into the *tmm-1* mutant background. As shown in Figure 3, D to F, the number of *E1728*-expressing mature stomata also was reduced in the epidermis of *tmm-1* mutant cotyledons under MeJA treatment. These findings, consistent with the results shown in Figures 1 and 2, supported the negative role of exogenous jasmonate in regulating stomatal development.

To further investigate how the stomata in the Col-0 cotyledon epidermis were regulated by MeJA, we examined stomatal differentiation in cotyledons using the *pTMM:TMM-GFP* reporter (Nadeau and Sack, 2002), which marks stomatal lineage cells with the highest expression in meristemoids. As shown in Figure 3, G to I, the number of GFP-positive cells was decreased in the presence of MeJA. We also analyzed the expression of the *pSPCH:SPCH-GFP* reporter (MacAlister et al., 2007), which marks stomatal lineage initiation, in the Col-0 cotyledon epidermis with or without MeJA treatment. Our observations showed that fewer cells of the cotyledon epidermis expressed the stomatal lineage initiation marker *pSPCH:SPCH-GFP* in response to MeJA treatment (Fig. 3, J–L). Similarly, the number of cells expressing the *pFAMA:GFP* reporter, which marks the differentiation of stomatal guard cells (Ohashi-Ito and Bergmann, 2006), also was reduced in the MeJA-treated cotyledons (Fig. 3, M–O). These findings suggested that exogenous jasmonate regulates the number of epidermal cells entering the stomatal lineage.

Stomatal Development Is Enhanced in Jasmonate Biosynthesis- and Perception-Related Mutants

To further determine jasmonate's function in regulating stomatal development, we examined the stomatal phenotype in the cotyledon epidermises of several

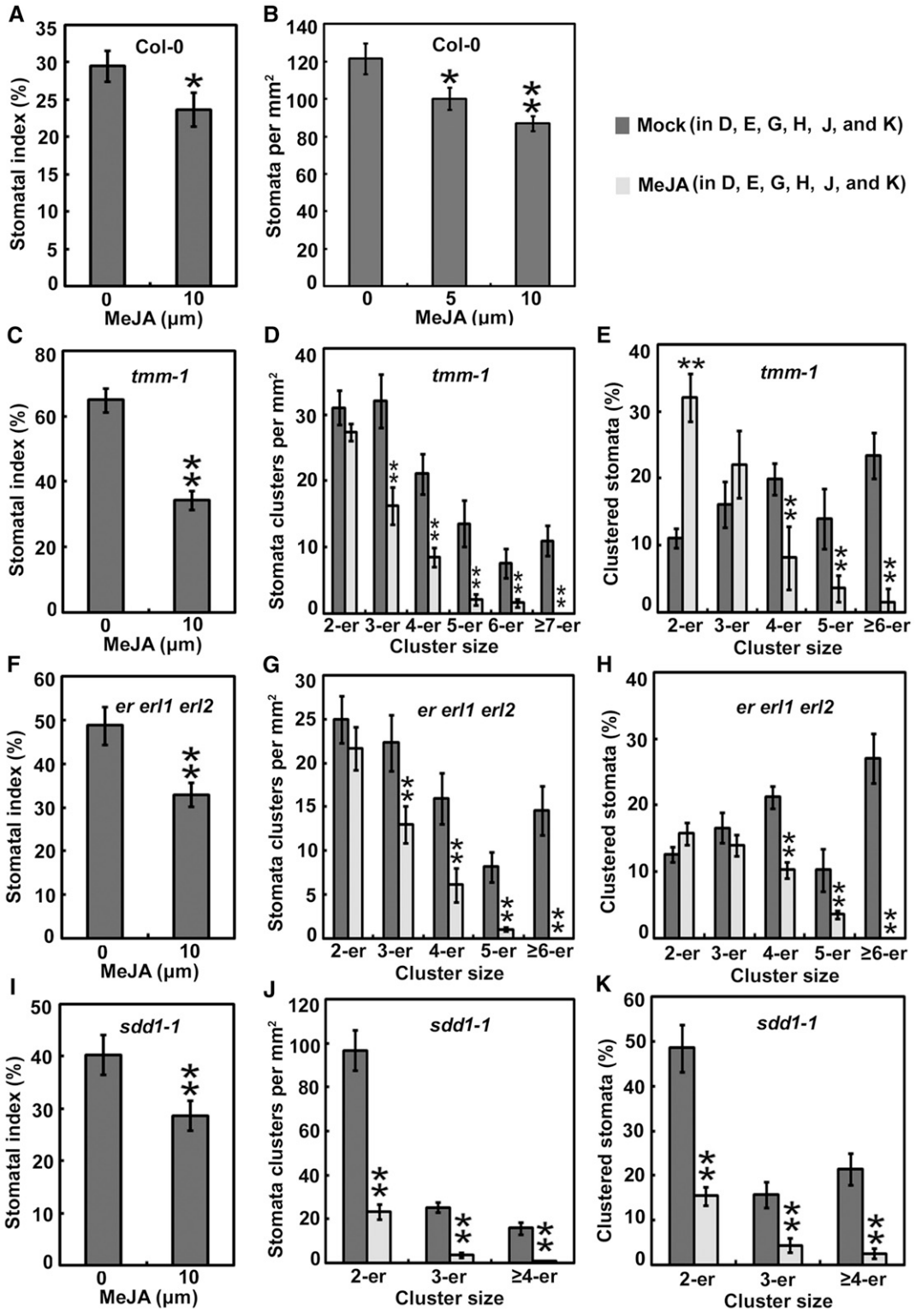

Figure 2. Quantitative analysis of stomata in the epidermis of Arabidopsis cotyledons in response to MeJA. A and B, The stomatal index (A) and stomatal density (B) of 11-d-old wild-type (Col-0) seedlings decreased in response to MeJA treatment. C to E, The stomatal index (C), number of stomatal clusters (D), and percentage of clustered stomata (E) in 11-d-old *tmm-1* seedlings were affected by 10 μM MeJA. F to H, The stomatal index (F), number of stomatal clusters (G), and percentage of clustered stomata (H) in 11-d-old *er erl1 erl2* seedlings were affected by 10 μM MeJA. I to K, The stomatal index (I), number of stomatal clusters (J), and percentage of clustered stomata (K) in 11-d-old *sdd1-1* seedlings were affected by 10 μM MeJA. All statistics were analyzed using

endogenous jasmonate biosynthesis- or perception-related mutants. JASMONATE RESISTANT1 (JAR1) is a jasmonate-amido synthetase that carries out a jasmonate conjugation/activation reaction (Staswick et al., 2002). Microscopic observations revealed that the development of stomata in the *jar1* cotyledon epidermis was enhanced, with a higher stomatal index and more stomatal clusters compared with the wild type (Col-0; Fig. 4, A–C). Moreover, the percentage of clustered stomata in the *jar1* cotyledon epidermis was significantly greater than that in the wild-type control (Fig. 4D). The F-box protein COI1 is the jasmonate receptor and positively regulates jasmonate-mediated processes (Xie et al., 1998; Yan et al., 2009). As shown in Figure 4, the stomatal index, number of stomatal clusters, and percentage of clustered stomata in the *coi1-2* and *coi1-16* mutants were increased compared with those in the wild type. To investigate the molecular characteristics of *coi1-2*, we introduced the *E1728* and *pSPCH*:*SPCH-GFP* markers into the *coi1-2* mutant through crossing. As shown in Figure 5, the *coi1-2* mutant cotyledon epidermis produced many more *E1728*- and *pSPCH*:*SPCH-GFP*-positive cells than the wild type, suggesting that the number of epidermal cells entering the stomatal lineage increased in the *coi1-2* mutant background compared with the wild type. Taken together, these results showed that endogenous jasmonate biosynthesis and perception may negatively modulate stomatal development in Arabidopsis cotyledons.

MYC Transcription Factors Act Upstream of SPCH and FAMA to Modulate Stomatal Development

Having ascertained that jasmonate biosynthesis and perception mediate stomatal development, we then asked whether jasmonate signaling components, such as JAZ repressors and downstream MYC transcription factors, also are involved in this developmental process. JAZ proteins, which directly link jasmonate perception with transcriptional changes, are crucial repressors of jasmonate signaling, with more than 10 members in Arabidopsis (Chini et al., 2007; Thines et al., 2007). To determine whether JAZ proteins are involved in stomatal development, we examined the cotyledon epidermis of the *jaz4 jaz9* double mutant (Hu et al., 2013). As shown in Figure 6, A to C, there were no significant differences in the stomatal index, number of stomatal clusters, and percentage of clustered stomata between the *jaz4 jaz9* double mutant and the wild type. It is very possible that the JAZ proteins function redundantly to regulate stomatal development in Arabidopsis cotyledons, as a similar redundancy of JAZ proteins has been observed in other jasmonate-mediated responses (Chini et al., 2007; Thines et al., 2007). We then queried whether the overexpression of these JAZ proteins affected stomatal development in Arabidopsis cotyledons. To test this possibility, we constructed transgenic plants overexpressing *JAZ1* or *JAZ5* with Jas domain deletion (*JAZ1ΔJas* or *JAZ5ΔJas*), which accumulate large amounts of JAZ1 or JAZ5 repressors and are insensitive to jasmonate (Chini et al., 2007; Thines et al., 2007). Microscopic observations showed that the stomatal index, number of stomatal clusters, and percentage of clustered stomata in the *JAZ1ΔJas* and *JAZ5ΔJas* cotyledon epidermises were clearly higher compared with those in the wild type (Fig. 6), showing that the JAZ1 and JAZ5 proteins promote stomatal development in Arabidopsis cotyledons.

MYC transcription factors (MYC2, MYC3, and MYC4) are the most extensively characterized JAZ-interacting targets and regulate multiple jasmonate-mediated processes (Chini et al., 2007; Dombrecht et al., 2007; An et al., 2017; Du et al., 2017; Yuan et al., 2017). To analyze whether these MYC transcription factors are involved in stomatal development, we first investigated the phenotype of the *myc2* single mutant. As shown in Figure 6, A to C, the stomatal index, number of stomatal clusters, and percentage of clustered stomata in the *myc2* single mutant were similar to those in the wild type. We then examined the cotyledon epidermises of the *myc2 myc4*, *myc3 myc4*, and *myc2 myc3 myc4* double and triple mutants. Our observations revealed that stomatal development was enhanced significantly in the double and triple mutants compared with the wild type and the *myc2* single mutant (Fig. 6), indicating that MYC2, MYC3, and MYC4 act redundantly to negatively regulate stomatal development in Arabidopsis cotyledons. To further confirm the role of MYC transcription factors in stomatal development, we examined stomatal phenotypes in true leaves of *myc2 myc3 myc4*. As shown in Supplemental Figure S3, the development of stomata in *myc2 myc3 myc4* true leaves also was enhanced, with a higher stomatal index and an increase of stomatal density compared with the wild type. To understand the molecular characteristics of MYC transcription factor-mediated stomatal development, we introduced the *E1728* and *pSPCH*:*SPCH-GFP* markers into the *myc2 myc4* mutant through genetic crossing. Consistent with the stomatal phenotype, more cells in *myc2 myc4* double mutant cotyledons expressed the *E1728* and *pSPCH*:*SPCH-GFP* markers (Fig. 5).

It was shown that SPCH directs the first asymmetric division to establish the stomatal cell lineage and that all epidermal cells become pavement cells in the *spch-1* loss-of-function mutant (MacAlister et al., 2007; Pillitteri et al., 2007). To investigate whether the MYC transcription factors genetically interact with SPCH, we

Figure 2. (*Continued.*)
Student's *t* test: *, differences between MeJA-treated plants and control plants are significant ($P < 0.05$); **, differences between MeJA-treated plants and control plants are highly significant ($P < 0.01$). Error bars show the SD ($n = 8$).

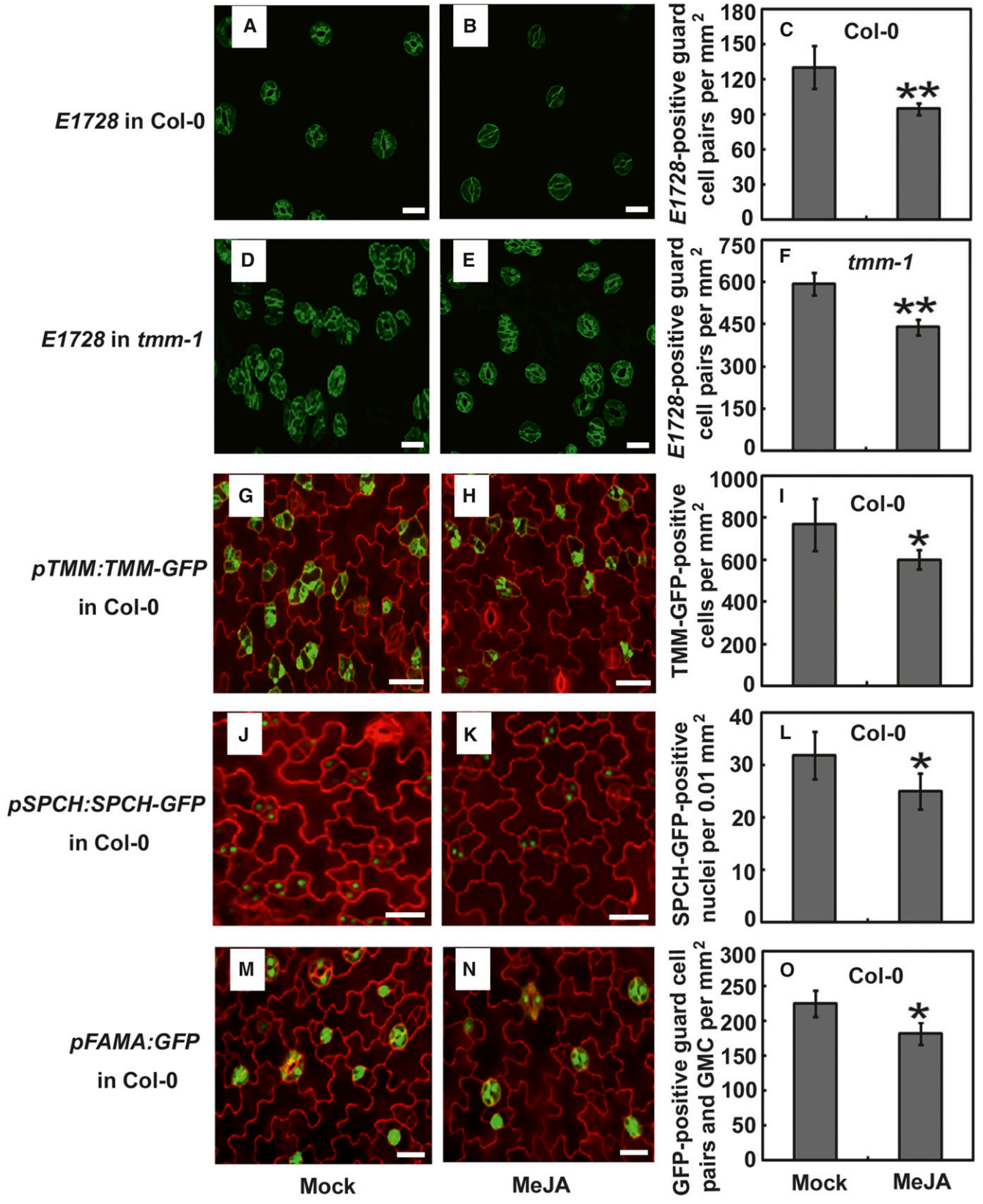

Figure 3. The activities of several stomatal cell type-specific markers were suppressed by MeJA. A and B, Confocal images of the cotyledon epidermis of 7-d-old wild-type plants expressing *E1728* (*E1728* in Col-0) with or without 10 μM MeJA treatment. Experiments were performed three times with similar results. Bars = 20 μm. C, Quantitative analysis of *E1728*-positive guard cell

generated a *spch-1 myc2 myc4* triple mutant through genetic crossing and analyzed its stomatal phenotype. As shown in Figure 6D, the cotyledon epidermis of the *spch-1 myc2 myc4* triple mutant produced no stomata at all, similar to the phenotype of the *spch-1* single mutant. This finding indicated that *SPCH* is epistatic to *MYC2* and *MYC4* in the regulation of stomatal development in Arabidopsis cotyledons. FAMA modulates the final differentiation of guard cells, and its loss-of-function mutation results in reiterative symmetric divisions of guard mother cells (Ohashi-Ito and Bergmann, 2006). Similarly, we also generated a *fama myc2 myc4* triple mutant through crossing and investigated its stomatal phenotype. The stomatal phenotype of the *fama myc2 myc4* triple mutant resembled that of the *fama* single mutant (Fig. 6D; Ohashi-Ito and Bergmann, 2006), suggesting that *FAMA* also is epistatic to MYC2 and MYC4 in the regulation of stomatal development in Arabidopsis cotyledons.

Jasmonate Represses Stomatal Development Dependently on MYC Transcription Factors

Having demonstrated that the MYC transcription factors inhibit stomatal development, we wondered whether jasmonate regulation of stomatal development is dependent on these MYC transcription factors. To test this possibility, we examined the stomatal phenotypes of the *myc2 myc3 myc4* triple mutant and the wild type in response to MeJA treatment. As shown in Figure 7, A and B, the stomatal index, number of stomatal clusters, and percentage of clustered stomata in the cotyledons of the wild type declined as expected in response to MeJA treatment, whereas no obvious decreases were detected in the MeJA-treated *myc2 myc3 myc4* triple mutant. Furthermore, we also examined the stomatal phenotype of the *coi1-2* mutant in the presence of MeJA. The results showed that stomatal development in the cotyledons of the *coi1-2* mutant also was insensitive to MeJA (Fig. 7, A and B). These findings showed that the COI1 receptor and the MYC2, MYC3, and MYC4 transcription factors are essential for jasmonate-regulated stomatal development, indicating that jasmonate modulates stomatal development in a COI1- and MYC transcription factor-dependent manner.

To further understand the regulatory functions of MYC2, MYC3, and MYC4, we used reverse transcription-quantitative PCR (qPCR) to examine the expression levels of the *SPCH*, *MUTE*, and *FAMA* genes in the *myc2 myc3 myc4* triple mutant with or without MeJA treatment. As shown in Figure 7C, transcripts of these genes were more abundant in the *myc2 myc3 myc4* triple mutant compared with the wild type without MeJA treatment. In the presence of MeJA, the expression levels of the *SPCH*, *MUTE*, and *FAMA* genes were decreased in the wild type; however, the transcript levels of these genes did not obviously change in the *myc2 myc3 myc4* triple mutant background with or without MeJA treatment (Fig. 7C). The expression of *MYC2*, *MYC3*, and *MYC4* in the wild-type seedlings with or without MeJA treatment also was analyzed. As shown in Supplemental Figure S4, their expression levels were greater in MeJA-treated seedlings than in control plants. These results suggested that the expression levels of *SPCH*, *MUTE*, and *FAMA* correlate with the altered stomatal development in the *myc2 myc3 myc4* triple mutant.

DISCUSSION

Jasmonate is an important signal that modulates multiple developmental processes and stress responses in plants. In this work, we demonstrated that jasmonate functions as a negative signal in the regulation of stomatal development in Arabidopsis cotyledons. In the presence of exogenous jasmonate (MeJA), the stomatal index and stomatal density on the cotyledons of wild-type seedlings were reduced (Figs. 1 and 2, A and B). Similar results also were observed in three stomata-overproliferating mutants (Figs. 1 and 2). Consistent with these observations, the number of cells expressing several stomatal cell type-specific markers (*E1728*,

Figure 3. (*Continued.*)
pairs in the epidermis of 10-d-old wild-type cotyledons expressing *E1728* with or without 10 μM MeJA treatment. D and E, Confocal images of the cotyledon epidermis of 7-d-old *tmm-1* plants expressing *E1728* (*E1728* in *tmm-1*) with or without 10 μM MeJA treatment. Experiments were performed three times with similar results. Bars = 20 μm. F, Quantitative analysis of *E1728*-positive guard cell pairs in the epidermis of 10-d-old *tmm-1* cotyledons expressing *E1728* with or without 10 μM MeJA treatment. G and H, Confocal images of the cotyledon epidermis of 4-d-old wild-type plants expressing *pTMM:TMM-GFP* (*pTMM:TMM-GFP* in Col-0) with or without 10 μM MeJA treatment. Experiments were performed three times with similar results. Bars = 20 μm. I, Quantitative analysis of TMM-GFP-positive cells in the epidermis of 4-d-old wild-type cotyledons expressing *pTMM:TMM-GFP* with or without 10 μM MeJA treatment. J and K, Confocal images of the cotyledon epidermis of 4-d-old wild-type plants expressing *pSPCH:SPCH-GFP* (*pSPCH:SPCH-GFP* in Col-0) with or without 10 μM MeJA treatment. Experiments were performed three times with similar results. Bars = 20 μm. L, Quantitative analysis of SPCH-GFP-positive nuclei in the epidermis of 4-d-old wild-type cotyledons expressing *pSPCH:SPCH-GFP* with or without 10 μM MeJA treatment. M and N, Confocal images of the cotyledon epidermis of 6-d-old wild-type plants expressing *pFAMA:GFP* (*pFAMA:GFP* in Col-0) with or without 10 μM MeJA treatment. Experiments were performed three times with similar results. Bars = 20 μm. O, Quantitative analysis of GFP-positive guard cell pairs and guard mother cells (GMC) in the epidermis of 6-d-old wild-type cotyledons expressing *pFAMA:GFP* with or without 10 μM MeJA treatment. All statistics were analyzed using Student's *t* test: *, differences between MeJA-treated plants and control plants are significant ($P < 0.05$); **, differences between MeJA-treated plants and control plants are highly significant ($P < 0.01$). Error bars show the SD ($n = 8$).

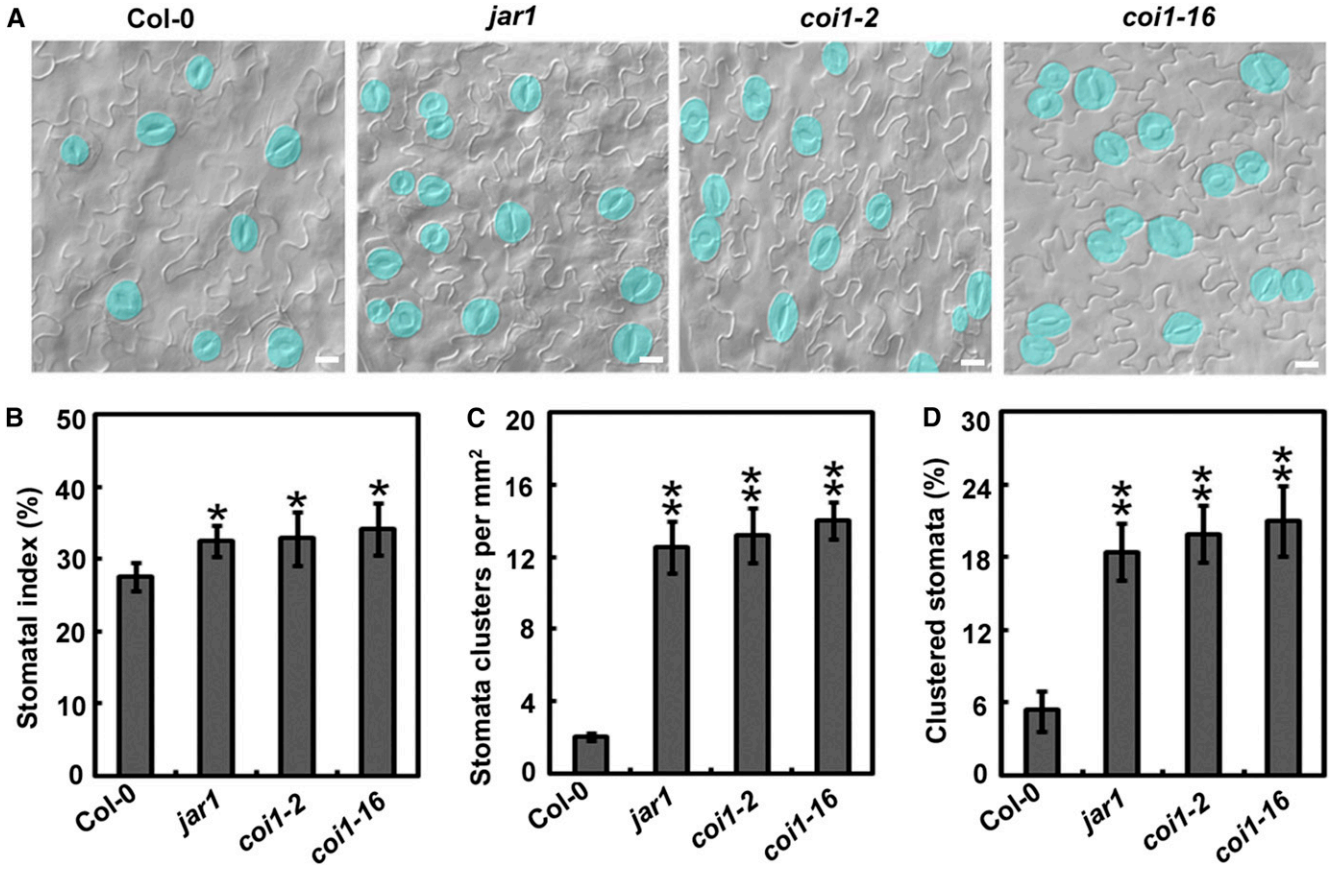

Figure 4. Stomatal phenotypes of mutants involved in jasmonate biosynthesis and perception. A, DIC images of the abaxial cotyledon epidermis of 11-d-old wild-type (Col-0), *jar1*, *coi1-2*, and *coi1-16* plants. Experiments were performed three times with similar results. Stomata are false colored in blue for easier identification. Bars = 20 μm. B to D, Stomatal index (B), number of stomatal clusters (C), and percentage of clustered stomata (D) in *jar1*, *coi1-2*, and *coi1-16*. Statistics were analyzed using Student's *t* test: *, differences between wild-type and mutant plants are significant ($P < 0.05$); **, differences between wild-type and mutant plants are highly significant ($P < 0.01$). Error bars show the SD ($n = 8$).

pSPCH:*SPCH-GFP*, *pFAMA*:*GFP*, and *pTMM*:*TMM-GFP*) was decreased in the presence of exogenous jasmonate (Fig. 3). These results showed that exogenous jasmonate suppresses stomatal development in Arabidopsis cotyledons. In contrast, blocking endogenous jasmonate biosynthesis or signaling enhanced stomatal development in Arabidopsis cotyledons. For example, disruption of the jasmonate receptor COI1 or accumulation of the repressor JAZ1 or JAZ5 resulted in increases of the stomatal index, number of stomatal clusters, and percentage of clustered stomata in cotyledons (Figs. 4–6). Based on these findings, we conclude that jasmonate negatively regulates stomatal development in Arabidopsis cotyledons.

Further examination revealed that the MYC transcription factors MYC2, MYC3, and MYC4, which are direct targets of JAZ repressors, redundantly regulate stomatal development in Arabidopsis cotyledons (Figs. 5 and 6). Moreover, jasmonate represses stomatal development dependently on these MYC transcription factors, as there was no obvious decrease of stomata in the *myc2 myc3 myc4* triple mutant with or without MeJA treatment (Fig. 7), indicating that these MYC transcription factors have a dominant role in jasmonate-inhibited stomatal development. Interestingly, an increasing number of studies have demonstrated that MYC2, MYC3, and MYC4 act redundantly and dominantly to regulate most aspects of jasmonate-signaled processes. For example, Schweizer et al. (2013) showed that MYC2, MYC3, and MYC4 are required for glucosinolate synthesis and resistance to insects. Song et al. (2014) found that MYC2, MYC3, and MYC4 redundantly regulate jasmonate-inhibited apical hook curvature, as the *myc2 myc3 myc4* triple mutant displayed an exaggerated apical hook curvature and was completely insensitive to jasmonate inhibition. Qi et al. (2015b) demonstrated that MYC2, MYC3, and MYC4 activate jasmonate-induced leaf senescence. Furthermore, Qi et al. (2015a) showed in another study that MYC2, MYC3, and MYC4 are redundantly involved in stamen development and seed production. Taken together, these results indicate that the MYC2, MYC3, and

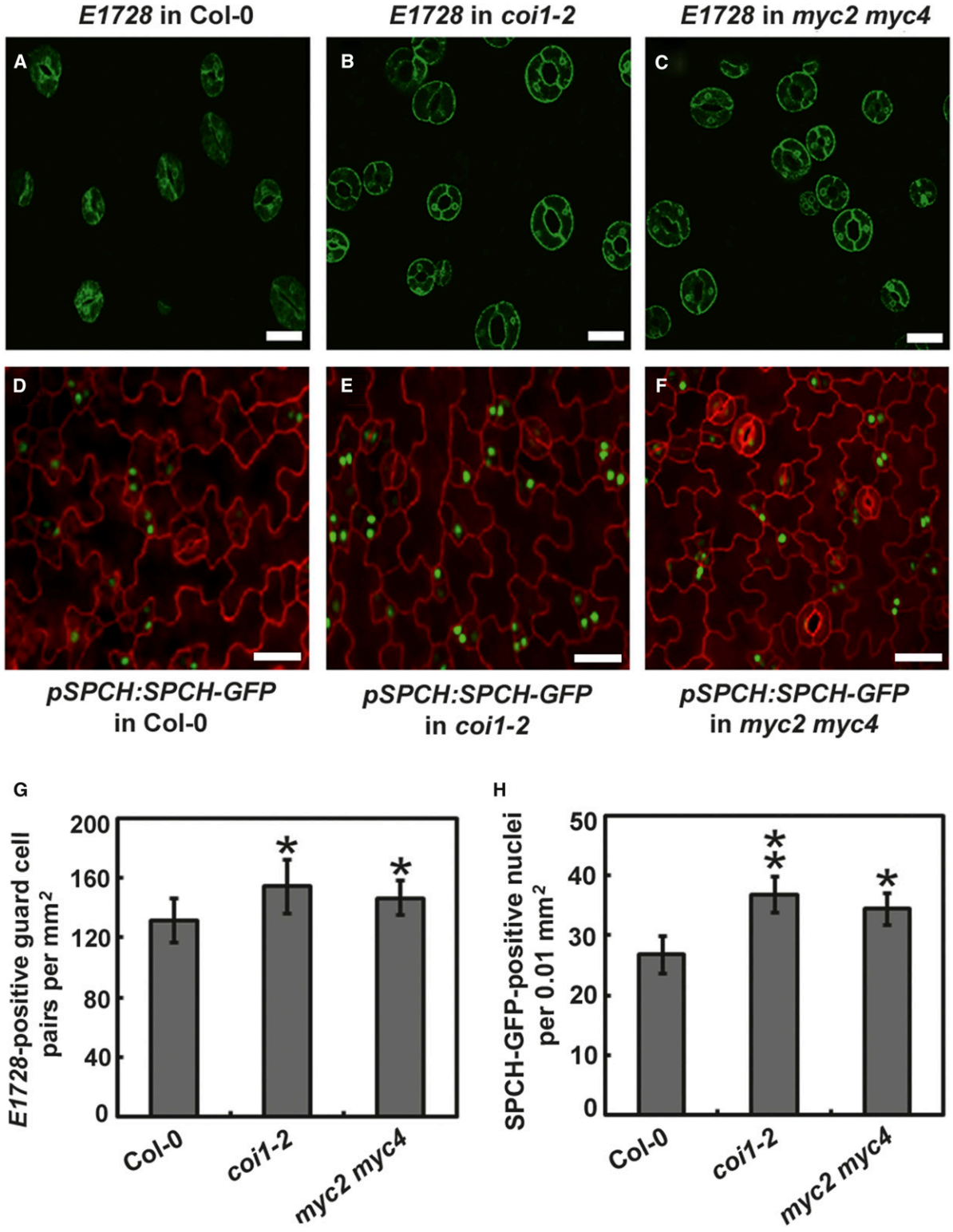

Figure 5. Expression of *E1728* and *pSPCH:SPCH-GFP* in the abaxial cotyledon epidermis of *coi1-2* and *myc2 myc4* mutants. A to C, Confocal images of the cotyledon epidermis of 7-d-old wild-type (Col-0), *coi1-2*, and *myc2 myc4* mutant plants expressing *E1728*. D to F, Confocal images of the cotyledon epidermis of 4-d-old wild-type (Col-0), *coi1-2*, and *myc2 myc4* mutant plants expressing *pSPCH:SPCH-GFP*. Experiments were performed three times with similar results. Bars = 20 μm. G, Quantitative analysis of *E1728*-positive guard cell pairs in the epidermis of 10-d-old wild-type (Col-0), *coi1-2*, and *myc2 myc4* cotyledons expressing *E1728*. H, Quantitative analysis of SPCH-GFP-positive nuclei in the epidermis of 4-d-old wild-type (Col-0), *coi1-2*,

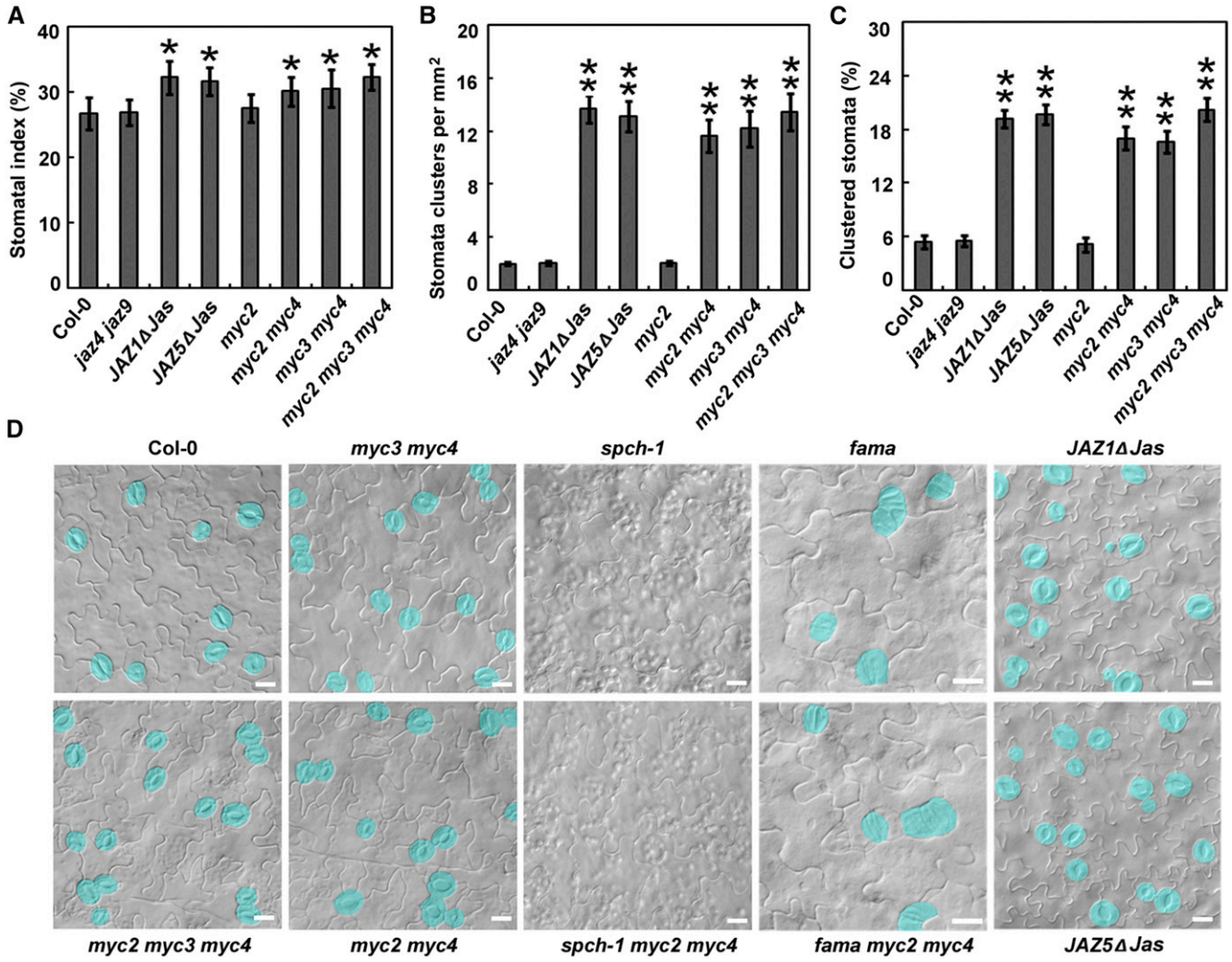

Figure 6. Stomatal phenotypes of JAZ repressor- and MYC transcription factor-related mutants. A to C, Stomatal index (A), number of stomatal clusters (B), and percentage of clustered stomata (C) in various 11-d-old mutant or transgenic overexpression plants. Statistics were analyzed using Student's *t* test: *, differences between wild-type and mutant or transgenic overexpression plants are significant ($P < 0.05$); **, differences between wild-type and mutant or transgenic overexpression plants are highly significant ($P < 0.01$). Error bars show the SD ($n = 8$). D, DIC images of the abaxial cotyledon epidermis of 11-d-old mutant or transgenic overexpression plants. Experiments were performed three times with similar results. Stomata are false colored in blue for easier identification. Bars = 20 μm.

MYC4 transcription factors play central and redundant roles in jasmonate-mediated processes, including stomatal development.

JAZ proteins interact with various transcription factors to regulate diverse jasmonate responses. In addition to MYC2, MYC3, and MYC4, many transcription factors have been identified as JAZ-interacting targets, such as GLABRA3, MYB75, MYB21, bHLH3, ICE1, TARGET OF EAT1, and FILAMENTOUS FLOWER, which regulate jasmonate-mediated trichome development, anthocyanin accumulation, male fertility, stress responses, and flowering (Fernández-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011; Hu et al., 2013; Boter et al., 2015; Zhai et al., 2015). Given that MYC2, MYC3, and MYC4 are involved in most aspects of jasmonate responses, further research is required to

Figure 5. (*Continued.*)
and *myc2 myc4* cotyledons expressing *pSPCH:SPCH-GFP*. Statistics were analyzed by Student's *t* test: *, differences between wild-type and mutant plants are significant ($P < 0.05$); **, differences between wild-type and mutant plants are highly significant ($P < 0.01$). Error bars show the SD ($n = 8$).

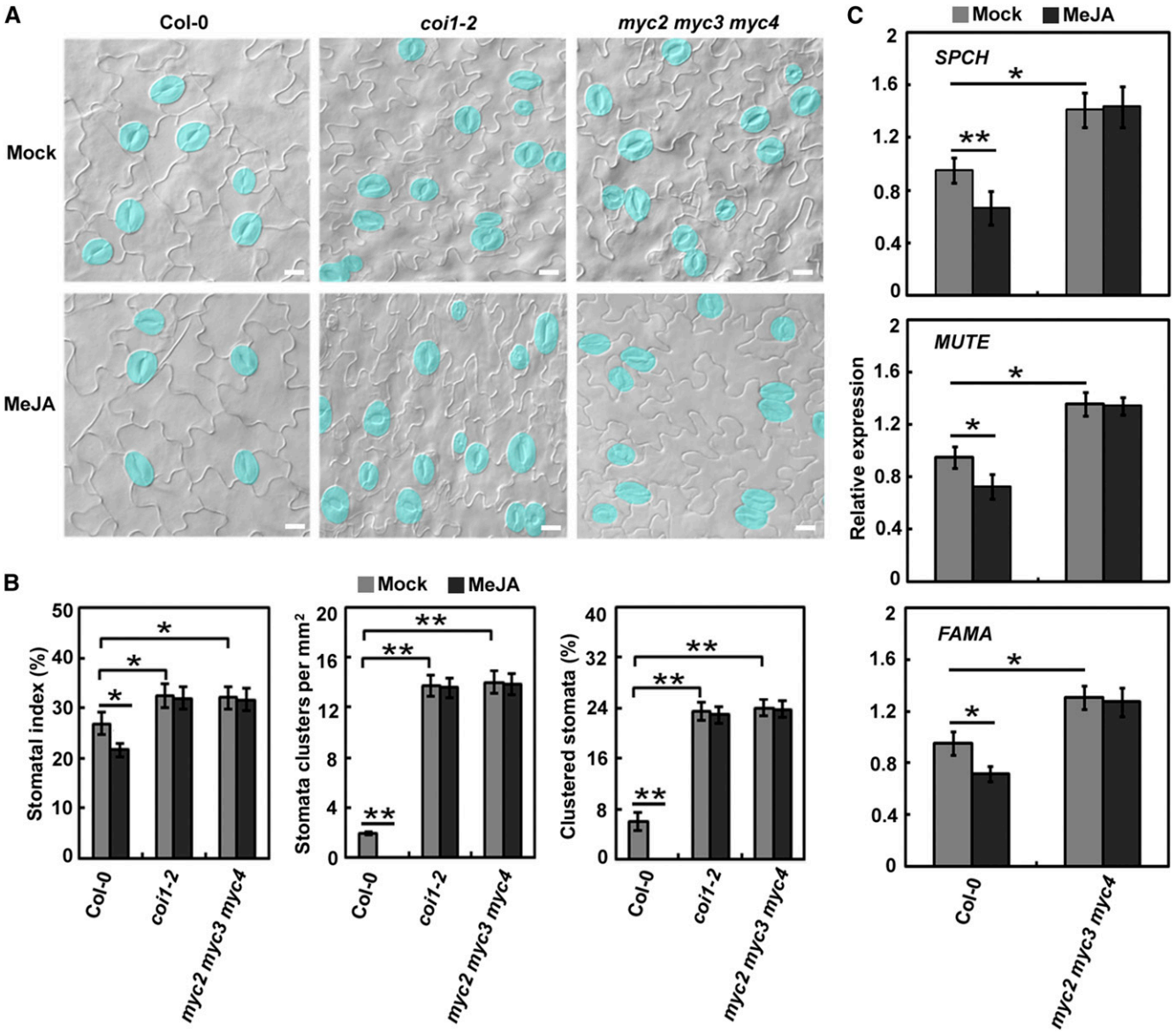

Figure 7. Stomatal development of *coi1-2* and *myc2 myc3 myc4* was insensitive to MeJA. A, DIC images of the abaxial cotyledon epidermis of the wild type (Col-0), *coi1-2*, and *myc2 myc3 myc4* with or without 10 μM MeJA treatment. Experiments were performed three times with similar results. Stomata are false colored in blue for easier identification. Bars = 20 μm. B, Stomatal index, number of stomatal clusters, and percentage of clustered stomata in 11-d-old plants. Error bars show the SD (n = 8). C, Expression of *SPCH*, *MUTE*, and *FAMA* in the wild type (Col-0) and *myc2 myc3 myc4* with or without 10 μM MeJA treatment. *SPCH* and *MUTE* expression was analyzed in 4-d-old seedlings, and *FAMA* expression was analyzed in 6-d-old seedlings. Changes in the expression of the target gene were calculated relative to the expression of the *ACTIN2* (AT3G18780) gene. Error bars show the SD from three independent RNA extractions. All statistics were analyzed using Student's *t* test: *, differences between the wild type and mutant are significant ($P < 0.05$); **, differences between the wild type and mutant are highly significant ($P < 0.01$).

elucidate the relationships among these three MYC transcription factors and other JAZ-interacting proteins, which may shed new light on the molecular basis of jasmonate signal transduction. Interestingly, Pillitteri et al. (2011) found that JAZ10-GFP was expressed in the nucleus through all transitional states of the stomatal lineage but not in fully differentiated guard cells, which indicated that JAZ10 may function in stage switches during stomatal development. In this study, we found that the accumulation of JAZ1 or JAZ5 resulted in increased stomatal density and number of defective clustering stomata on the cotyledon epidermis (Fig. 6), which indicates that multiple JAZ repressors might function redundantly in jasmonate-mediated stomatal development. Further investigation of these JAZ repressors and their potential targets in stomatal

development will enhance our understanding of the stomatal development signaling network involving jasmonate.

In this study, we found that MYC transcription factors act upstream of SPCH and FAMA (two critical controllers of stomatal development) to regulate stomatal development (Fig. 6C). Moreover, our expression analysis showed that these MYC transcription factors repress the expression of the *SPCH*, *MUTE*, and *FAMA* genes, whose transcripts were more abundant in the *myc2 myc3 myc4* triple mutant than in the wild type with or without MeJA treatment (Fig. 7C), implying that jasmonate signaling and stomatal development are integrated. Song et al. (2014) demonstrated that MYC2, MYC3, and MYC4 associate with EIN3 and EIL1 to regulate jasmonate-modulated apical hook curvature and defense responses. MYC2 also was found to interact with DELLA proteins to mediate the cross talk between jasmonate and GA signaling (Hong et al., 2012). Moreover, MYC2 is involved in auxin, ABA, salicylic acid, light, flowering, and circadian clock signaling pathways (Yadav et al., 2005; Chen et al., 2011, 2012; Shin et al., 2012; Chico et al., 2014; Sethi et al., 2014; Schmiesing et al., 2016; Wang et al., 2017). Collectively, these previous results and our findings suggest that MYC2 may function as a modulator integrating jasmonate signaling with other physiological processes. Further investigation of the molecular mechanisms of MYC2-mediated cross talk will enhance our understanding of jasmonate signaling networks.

In a previous study, we found that JAZ proteins interact with ICE1 and ICE2 and that these interactions repress the transcriptional functions of ICE1 and ICE2 (Hu et al., 2013). ICE1 and ICE2 also were shown to be essential for stomatal development in another study (Kanaoka et al., 2008). Thus, jasmonate signaling also might modulate stomatal development via ICE1 and ICE2. It is possible that concurrent regulation of stomatal development by the MYC and ICE transcription factors may be a fine-tuning mechanism for jasmonate signaling. Nevertheless, the outcome of jasmonate regulation was repressed stomatal development under nonstressful conditions in our study (Figs. 1–6). Interestingly, similar dual regulation has been found previously in jasmonate signaling: EIN3 and MYC2 act antagonistically to mediate jasmonate-signaled apical hook curvature and resistance against the necrotrophic fungal pathogen *Botrytis cinerea* (Berrocal-Lobo et al., 2002; Lorenzo et al., 2004; Song et al., 2014; Zhang et al., 2014b), EIN3 and ICE1 antagonistically modulate jasmonate-mediated cold acclimation and freezing tolerance (Zhu et al., 2011; Shi et al., 2012; Hu et al., 2013, 2017), and MYC2 and bHLH subgroup IIId factors (bHLH3, bHLH13, bHLH14, and bHLH17) function oppositely to regulate jasmonate-inhibited root elongation (Lorenzo et al., 2004; Song et al., 2013). Further investigation of these dual-regulation processes will help us understand the precise mechanisms of jasmonate signaling pathways.

This work showed that jasmonate inhibits stomatal development in Arabidopsis cotyledons and that MYC transcription factors modulate the expression of *SPCH*, *MUTE*, and *FAMA* to integrate jasmonate signaling and stomatal development, which extends our knowledge about the regulation of stomatal development by phytohormones. Previous studies have revealed that brassinosteroids function in stomatal development through the phosphorylation and inhibition of YODA, MKK4/5, and SPCH by BIN2 kinase, a critical negative regulator of brassinosteroid signaling (Gudesblat et al., 2012; Kim et al., 2012; Khan et al., 2013). Auxin mediates stomatal development partially through MONOPTEROS repression of the mobile peptide gene *STOMAGEN* in mesophyll cells (Zhang et al., 2014a) and via Aux/IAA proteins in dark-grown seedlings (Balcerowicz et al., 2014). Moreover, GAs, ethylene, and ABA have been reported to modulate stomatal development; nevertheless, their underlying mechanisms remain largely unclear (Saibo et al., 2003; Tanaka et al., 2013). All these findings suggest that the genetic network for the control of stomatal development is complex and tightly regulated by both intrinsic and external signals. The exact molecular mechanisms underlying the interactions among those various signals during stomatal development deserve further investigation.

MATERIALS AND METHODS

Materials and Plant Growth Conditions

Common chemicals were purchased from Shanghai Sangon Biotechnology. The plant hormone MeJA was obtained from Sigma-Aldrich and Taq DNA polymerases from Takara Biotechnology. Arabidopsis (*Arabidopsis thaliana*) seeds were surface sterilized in 20% (v/v) bleach for 15 min, sown on one-half-strength Murashige and Skoog medium containing 1.5% (w/v) Suc, stratified in the dark at 4°C for 3 d, and grown in an artificial growth chamber at 22°C under 120 μmol m^{-2} s^{-1} light with a photoperiod of 16 h of light and 8 h of darkness. The mutants *tmm-1* (Yang and Sack, 1995), *er erl1 erl2* (Shpak et al., 2005), *sdd1-1* (Berger and Altmann, 2000), *spch-1* (MacAlister et al., 2007), *fama* (Ohashi-Ito and Bergmann, 2006), *coi1-2* (Xu et al., 2002), *coi1-16* (Ellis and Turner, 2002), and *jaz4 jaz9* (Hu et al., 2013) were described previously. Double and triple mutants of MYC transcription factors were described by Fernández-Calvo et al. (2011). Other mutants used in this study were *jar1* (CS8072) and *myc2* (SALK_083483). Transgenic plants showing stomatal lineage-specific expression (*pTMM:TMM-GFP*, *pSPCH:SPCH-GFP*, *pFAMA:GFP*, and *E1728* enhancer trap line) were obtained from Fred Sack (University of British Columbia). The *spch-1 myc2 myc4* and *fama myc2 myc4* triple mutants were generated by genetic crossing using standard techniques. All plant materials used for genetic crossing are shown in Supplemental Table S1. The homozygous double or triple mutants generated by crossing were identified by PCR-based genotyping or genomic sequencing. The specific primers used for genotyping are shown in Supplemental Table S2. Moreover, the jasmonate-related mutants were confirmed by MeJA (20 μM)-insensitive phenotypes. GFP fluorescence was confirmed by microscopy.

MeJA Treatment

MeJA was dissolved in 10% ethanol as a 10 mM stock solution. For MeJA treatment on cotyledons, the stock solution was diluted to 5 or 10 μM in one-half-strength Murashige and Skoog medium. Arabidopsis seeds were germinated on MeJA-containing medium, and seedlings were grown in an artificial growth chamber at 22°C under 120 μmol m^{-2} s^{-1} light with a photoperiod of 16 h of light and 8 h of darkness. In the mock treatment, an equal volume of 10% ethanol was added to the medium. For MeJA treatment on true leaves, 5-d-old soil-grown plants were sprayed with 50 μM MeJA solution diluted from the

stock and maintained in a small chamber for 3 h. Then, plants were incubated 2 d before the next treatment in an artificial growth chamber at 22°C under 120 μmol m^{-2} s^{-1} light with a photoperiod of 16 h of light and 8 h of darkness. Those seedlings were treated four times with MeJA before examination.

Microscopy

Images of stomatal phenotypes were obtained from samples stored in Hoyer's solution and visualized using DIC microscopy on a Nikon D-Eclipse C1 microscope. The abaxial epidermises of cotyledons of 11-d-old seedlings and fully expanded true leaves of 20-d-old plants were analyzed. Samples were collected in 70% ethanol, cleared overnight at room temperature, and then stored in Hoyer's solution. A Nikon D-Eclipse C1 laser confocal scanning microscope was used to observe shoot GFP fluorescence from marker gene expression and propidium iodide (Sigma-Aldrich) staining.

Stomatal Count

Samples were fixed in water and observed with an Olympus DP73 microscope. Stomatal density is the number of stomata per unit of area. To count the stomatal density and number of stomatal clusters, five square areas of 0.5 mm^2 were examined for each cotyledon, and the amounts were averaged to yield a predicted stomatal density and number of stomatal clusters per cotyledon. Cotyledons from at least eight different plants were selected for all genotypes. The stomatal index was calculated using the following formula: stomatal index = (number of stomata)/(total epidermal cells) × 100% (Casson et al., 2009). The percentage of clustered stomata was calculated as follows: (total number of stomata in clusters)/(total number of stomata) × 100%. Statistical analysis was performed using Student's *t* test; single asterisks indicate that differences between wild-type and mutant plants are significant ($P < 0.05$), and double asterisks indicate that differences between wild-type and mutant plants are highly significant ($P < 0.01$).

Construction of Overexpression Plants

The coding sequences of JAZ1 and JAZ5 with Jas domain deletion (Thines et al., 2007) were cloned into the pOCA30 vector in the sense orientation behind the cauliflower mosaic virus 35S promoter (Wang et al., 2016). The recombinant plasmids were introduced into *Agrobacterium tumefaciens* (strain EHA105) and used to transform Arabidopsis by the floral dip method (Clough and Bent, 1998). The transformed lines were selected for resistance to kanamycin (50 mg L^{-1}). Moreover, the kanamycin-resistant plants with high expression of *JAZ1* and *JAZ5* were identified by qPCR and further confirmed by MeJA (20 μM)-insensitive phenotypes (Thines et al., 2007). The primers used to amplify these sequences are listed in Supplemental Table S2.

RNA Extraction and qPCR

Total RNA was extracted using Trizol reagent (Invitrogen). qPCR was performed as described previously (Hu and Yu, 2014). Briefly, first-strand cDNA was synthesized from 1.5 μg of DNase-treated RNA in a 20-μL reaction volume using Moloney murine leukemia virus reverse transcriptase (Fermentas) with oligo$(dT)_{18}$ primer. qPCR was performed using 2× SYBR Green I master mix on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. At least three biological replicates for each sample were used for qPCR analysis, and at least three technical replicates were analyzed for each biological replicate. Gene-specific primers used to detect transcripts are listed in Supplemental Table S2. A no-template control was routinely included to confirm the absence of DNA or RNA contamination. Changes in the expression of the target gene were calculated relative to the expression of the *ACTIN2* (AT3G18780) gene. To assess reaction efficiencies, standard curves were created using a 5-fold serial dilution of the cDNA pool. A linear regression between the amount of cDNA template and the cycle threshold value was calculated to obtain a correlation coefficient ($R^2 > 0.97$). The PCR efficiency was determined according to Schmittgen and Livak (2008).

Statistical Analysis

Statistically significant differences in the results (*, $P < 0.05$ or **, $P < 0.01$) are based on Student's *t* test computed by SigmaPlot 10.0. Data are means ± SD of independent replicates. All experiments reported here were repeated at least three times with similar results.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *JAR1*, AT2G46370; *COI1*, AT2G39940; *JAZ1*, AT1G19180; *JAZ4*, AT1G48500; *JAZ5*, AT1G17380; *JAZ9*, AT1G70700; *MYC2*, AT1G32640; *MYC3*, AT5G46760; *MYC4*, AT4G17880; *SPCH*, AT5G53210; *MUTE*, AT3G06120; *FAMA*, AT3G24140; *TMM*, AT1G80080; *SDD1*, AT1G04110; *ER*, AT2G26330; *ERL1*, AT5G62230; *ERL2*, AT5G07180; and *ACTIN2*, AT3G18780.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of MeJA on stomatal development in the epidermis of Arabidopsis fully expanded true leaves.

Supplemental Figure S2. Quantitative analysis of stomata in the epidermis of Arabidopsis fully expanded true leaves in response to MeJA.

Supplemental Figure S3. Stomatal phenotypes of *coi1-2* and *myc2 myc3 myc4* fully expanded true leaves.

Supplemental Figure S4. MeJA-induced expression of *MYC2*, *MYC3*, and *MYC4*.

Supplemental Table S1. Plant materials used for genetic crosses.

Supplemental Table S2. Primers used in this study.

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