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Interaction between MYC2 and ETHYLENE INSENSITIVE3 Modulates Antagonism between Jasmonate and Ethylene Signaling in *Arabidopsis*[C][W]

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Plants have evolved sophisticated mechanisms for integration of endogenous and exogenous signals to adapt to the changing environment. Both the phytohormones jasmonate (JA) and ethylene (ET) regulate plant growth, development, and defense. In addition to synergistic regulation of root hair development and resistance to necrotrophic fungi, JA and ET act antagonistically to regulate gene expression, apical hook curvature, and plant defense against insect attack. However, the molecular mechanism for such antagonism between JA and ET signaling remains unclear. Here, we demonstrate that interaction between the JA-activated transcription factor MYC2 and the ET-stabilized transcription factor ETHYLENE-INSENSITIVE3 (EIN3) modulates JA and ET signaling antagonism in *Arabidopsis thaliana*. MYC2 interacts with EIN3 to attenuate the transcriptional activity of EIN3 and repress ET-enhanced apical hook curvature. Conversely, EIN3 interacts with and represses MYC2 to inhibit JA-induced expression of wound-responsive genes and herbivory-inducible genes and to attenuate JA-regulated plant defense against generalist herbivores. Coordinated regulation of plant responses in both antagonistic and synergistic manners would help plants adapt to fluctuating environments.

INTRODUCTION

Sessile plants have evolved sophisticated mechanisms for integration of endogenous and exogenous signals to regulate their growth, development, and defense responses, which benefits their survival in the changing environment. Both ethylene (ET) and jasmonate (JA) are essential plant hormones that regulate various plant developmental processes and diverse defense responses (Kieber, 1997; Bleecker and Kende, 2000; Guo and Ecker, 2004; Broekaert et al., 2006; Howe and Jander, 2008; Browse, 2009; Shan et al., 2012; Wasternack and Hause, 2013). ET signal is perceived by its receptors ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4) (Hua and Meyerowitz, 1998) to repress CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Kieber et al., 1993), which activates EIN2 (Alonso et al., 1999; Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012) and subsequently stabilizes EIN3 and EIN3-LIKE1 (EIL1) (Chao et al., 1997; Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004) to mediate various ET responses, including hypocotyl growth (Zhong et al., 2012), apical hook formation (Knight et al., 1910; An et al., 2012), root growth (Ortega-Martínez et al., 2007; Růzicka et al., 2007), flowering (Ogawara et al., 2003; Achard et al., 2007), fruit ripening (Burg and Burg, 1962; Theologis et al., 1992), leaf senescence (Gepstein and Thimann, 1981; Li et al., 2013), freezing tolerance (Shi et al., 2012), and resistance against pathogen infection (Alonso et al., 2003; Chen et al., 2009).

JA plays essential roles in the regulation of plant development and defense. Upon perception of JA signal (Fonseca et al., 2009; Yan et al., 2009; Sheard et al., 2010), the F-box protein CORONATINE INSENSITIVE1 (COI1) (Xie et al., 1998; Yan et al., 2009) recruits the JASMONATE ZIM-DOMAIN (JAZ) proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) for degradation, which leads to the release of various downstream factors, including MYC2/JASMONATE INSENSITIVE1 (JIN1), MYC3, and MYC4 (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011), as well as WD-repeat/bHLH/MYB complex (Qi et al., 2011), MYB21, MYB24, and MYB57 (Mandaokar et al., 2006; Song et al., 2011) and the IIId bHLH factors (Nakata et al., 2013; Song et al., 2013b), which regulate diverse JA-mediated functions. These functions include root growth (Dathe et al., 1981; Chen et al., 2011), apical hook formation (Turner et al., 2002), flowering (Robson et al., 2010), stamen development (McConn and Browse, 1996; Song et al., 2011, 2013a), leaf senescence

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(Ueda and Kato, 1980; Shan et al., 2011), secondary metabolism (De Geyter et al., 2012; Schweizer et al., 2013), drought responses (Seo et al., 2011), wounding responses (Mason and Mullet, 1990; Acosta et al., 2013; Mousavi et al., 2013), and defense against pathogen infection (Thomma et al., 1998; Vijayan et al., 1998; Melotto et al., 2006; Rowe et al., 2010; Yang et al., 2012; Zheng et al., 2012) and insect attack (McConn et al., 1997; Hu et al., 2013a).

Previous studies showed that both JA and ET concomitantly and synergistically regulate plant defense against necrotrophic fungi (Penninckx et al., 1998; Thomma et al., 1998; Thomma et al., 1999) and root hair development (Zhu et al., 2006). It is so far reported that such JA-ET signaling synergy is mediated by derepression of ET-stabilized EIN3 and EIL1: JAZ proteins directly interact with and repress EIN3/EIL1, while JA induces JAZ degradation to derepress EIN3 and EIL1 (Zhu et al., 2011). JA-induced EIN3 and EIL1 activation (Zhu et al., 2011) and ET-induced EIN3 and EIL1 stabilization (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004) mediate JA and ET signaling synergy in the regulation of root hair development and resistance against necrotrophic fungal infection.

In addition to their synergistic regulation, JA and ET also act antagonistically in regulating expression of wound-responsive genes (Rojo et al., 1999; Lorenzo et al., 2004) and metabolite biosynthetic genes (Mikkelsen et al., 2003). JA represses apical hook formation (Turner et al., 2002) and positively regulates plant defense against insect attack (Fernández-Calvo et al., 2011; Schweizer et al., 2013), while ET functions oppositely (Guzmán and Ecker, 1990; Mewis et al., 2005, 2006; Bodenhausen and Reymond, 2007). However, the molecular mechanism for such antagonism between JA and ET signaling remains unclear. In this study, we show that MYC2 interacts with EIN3 and EIL1 to repress the transcriptional activity of EIN3 and EIL1 in *Arabidopsis thaliana* and consequently to inhibit ET-regulated apical hook formation; similarly, we found that EIN3 and EIL1 interact with and repress MYC2, further attenuate JA-induced expression of wound-responsive genes and herbivory-inducible genes, and inhibit plant defense against insect attack. This molecular, biochemical, and genetic evidence reveals that interactions of the JA-activated transcription factor MYC2 with the ET-stabilized transcription factors (EIN3 and EIL1) repress their respective transcriptional activities to modulate JA and ET signaling antagonism, which provides insights into how plants integrate various phytohormone signals to coordinately regulate plant development, growth, and defense.

RESULTS

MYC2, MYC3, and MYC4 Function Redundantly to Mediate JA-Inhibited Apical Hook Curvature

The formation of the apical hook helps cotyledons and meristem tissues protrude from the soil without being damaged. Previous studies showed that ET induces hook curvature (Guzmán and Ecker, 1990), whereas JA antagonizes the ET pathway that functions in apical hook formation in etiolated *Arabidopsis* seedlings (Turner et al., 2002). Consistent with previous observations (Turner et al., 2002), 1-aminocyclopropane-1-carboxylic acid (ACC), the ET biosynthesis precursor, enhanced the apical hook curvature, while JA obviously suppressed the ET-enhanced hook curvature of the dark-grown *Arabidopsis* wild-type seedlings (Figure 1A); the *coi1-1* mutant exhibited an exaggerated apical hook curvature (Figure 1A). As expected, the mutants with ET overproduction (*ethylene overproducer1* [*eto1-1*]) or constitutive ET responses (*ctr1-1*) exhibited constitutive exaggerated hook curvature, while the mutants deficient in ET signaling (*ein2-1* and *ein3-1 eil1-3*) displayed obvious reduction in hook curvature (Figure 1A). We further found that the exaggerated hook curvature in *eto1-1* and *ctr1-1* was clearly inhibited by JA (Figure 1A), which indicates that JA functions downstream of *ETO1* and *CTR1* to repress ET-regulated hook curvature.

HOOKLESS1 (HLS1) is a central positive regulator of apical hook development (Lehman et al., 1996; An et al., 2012) (Figures 1A and 1B). The mutants *eto1-1* and *ctr1-1*, with high level of *HLS1* expression, exhibited exaggerated hook curvature, while the mutant *ein3-1 eil1-3*, with low levels of *HLS1* expression, displayed reduced hook curvature (Figures 1A and 1B). ACC treatment induced *HLS1* expression and exaggerated apical hook curvature in the wild type, and ACC-induced *HLS1* expression and hook formation were obviously repressed by JA treatment (Figures 1A and 1C). These results suggest that JA represses *HLS1* expression to inhibit ET-enhanced hook formation and imply that JA acts upstream of *HLS1* to repress hook curvature.

To genetically verify whether JA acts upstream of *HLS1*, we further generated the double mutant *coi1-2 hls1-1* and the triple mutant *coi1-2 ein3 eil1* via genetic cross of *coi1-2* with *hls1-1* or *ein3 eil1*. The results in Figure 2 showed that the *coi1-2* exhibited an exaggerated hook curvature, while no hook was formed in *coi1-2 hls1-1* and *hls1-1*. Similar data were also observed for *coi1-2 ein3 eil1* (Figure 2). Suppression of the exaggerated hook curvature in *coi1-2* by the *hls1-1* and *ein3 eil1* mutations suggests that COI1 acts upstream of the EIN3/EIL1-HLS1 cascade to regulate apical hook formation.

To identify the key components responsible for repression of hook curvature in JA signaling pathway, we examined apical hook phenotypes in JA signaling mutants. As expected, the *coi1-1* mutant exhibited an exaggerated apical hook curvature (Figure 3A) (Turner et al., 2002). *JAZ1Δ3A* transgenic plants, with high levels of JAZ proteins (Thines et al., 2007), also displayed an exaggerated apical hook curvature (Figure 1A). Among the key transcription factors targeted by JAZ proteins, MYB21/MYB24/MYB57 (Song et al., 2011) and WD-repeat/bHLH/MYB complex (Qi et al., 2011) are not involved in the suppression of hook formation, as the *myb21 myb24 myb57* and *gl3 egl3 tt8* mutants exhibited wild-type-like hook curvature (Figure 3A).

Interestingly, the *myc2* single mutant exhibited a mildly exaggerated apical hook curvature compared with the wild type (Figure 3A); the apical hook curvature was clearly enhanced in the double mutants *myc2 myc3* and *myc2 myc4* (Figure 3A), while the triple mutant *myc2 myc3 myc4* displayed the strongest apical hook curvature (Figure 3A), which is similar to that observed in the *coi1* mutant (Figure 3A). The hook curvature of the single or double mutants (*myc2*, *myc2 myc3*, and *myc2 myc4*) could be further inhibited by JA

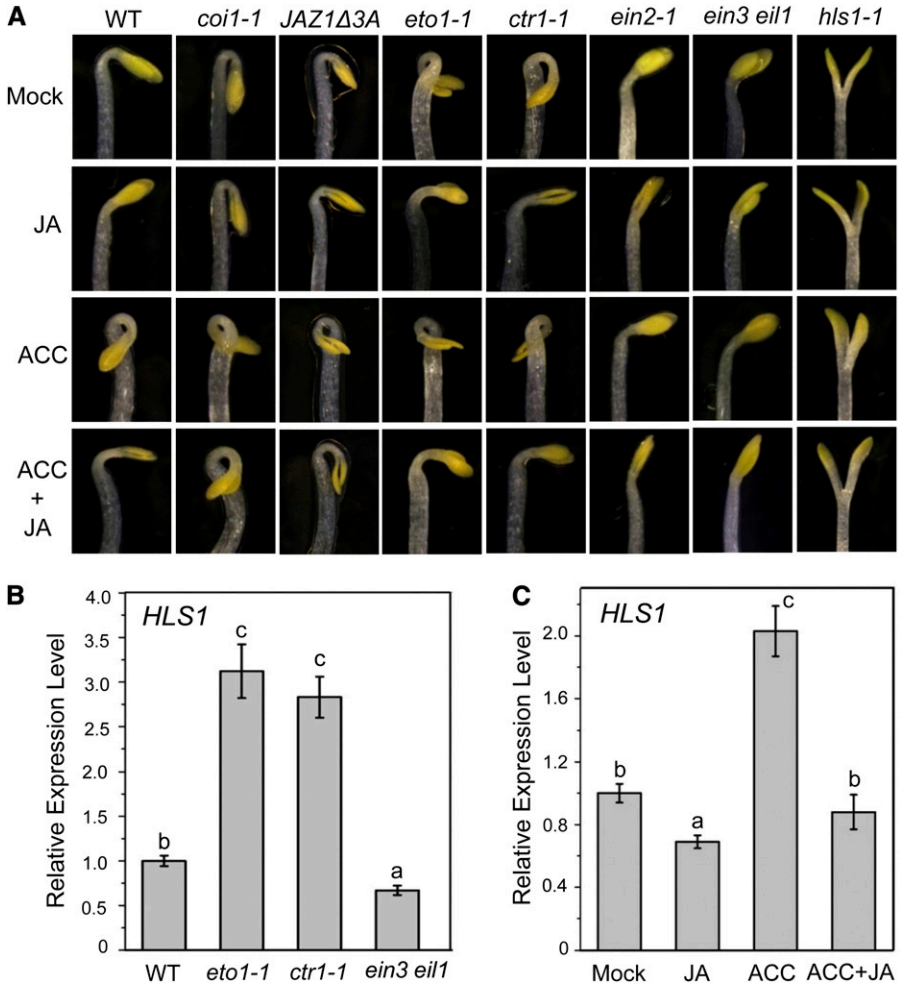

Figure 1. JA Suppresses the ET-induced Apical Hook Formation.

(A) The hook phenotypes of 4-d-old etiolated *Arabidopsis* seedlings Columbia-0 (Col-0; WT), *coi1-1*, *JAZ1Δ3A*, *eto1-1*, *ctr1-1*, *ein2-1*, *ein3-1 ein1-3* (*ein3 eil1*), and *hls1-1* grown in the dark on MS medium supplied without (Mock) or with 5 μM MeJA (JA), 10 μM ACC, or 10 μM ACC plus 5 μM MeJA (ACC+JA).

(B) Real-time PCR analysis for *HLS1* in 4-d-old etiolated Col-0 (WT), *eto1-1*, *ctr1-1*, and *ein3 eil1*. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

(C) Real-time PCR analysis for *HLS1* in 4-d-old etiolated Col-0 (WT) treated with mock, 100 μM MeJA (JA), 100 μM ACC, or 100 μM ACC plus 100 μM MeJA (ACC+JA) for 6 h. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

treatment, whereas the triple mutant (*myc2 myc3 myc4*) was completely insensitive to JA-inhibited hook curvature (Figure 3A). Furthermore, JA was unable to repress ACC-enhanced hook curvature in *myc2 myc3 myc4* (Figure 3A). Consistent with the exaggerated hook curvature, the expression of *HLS1* was upregulated in the mutants *myc2*, *myc2 myc3*, *myc2 myc4*, and *myc2 myc3 myc4* (Figures 3B). Furthermore, ACC-enhanced *HLS1* expression in *myc2 myc3 myc4* was not repressed by JA treatment (Figures 3C).

Taken together, the results in Figure 3 suggest that MYC2, MYC3, and MYC4 function redundantly to mediate JA-inhibited hook curvature.

MYC2, MYC3, and MYC4 Interact with EIN3 and EIL1

Having shown that MYC2, MYC3, and MYC4 function redundantly to repress *HLS1* expression and mediate JA inhibition of ET-enhanced hook curvature (Figure 3), we further found that MYC2, MYC3, and MYC4 were able to interact with EIN3 and EIL1 (Figure 4), activators of *HLS1* (An et al., 2012).

The yellow fluorescent protein (YFP)–based bimolecular fluorescence complementation (BiFC) assays showed that coexpression of EIN3-nYFP (fusion of EIN3 with N-terminal fragment of YFP) or EIL1-nYFP with cYFP-MYC2 (fusion of MYC2 with C-terminal fragment of YFP), cYFP-MYC3, or cYFP-MYC4 produced

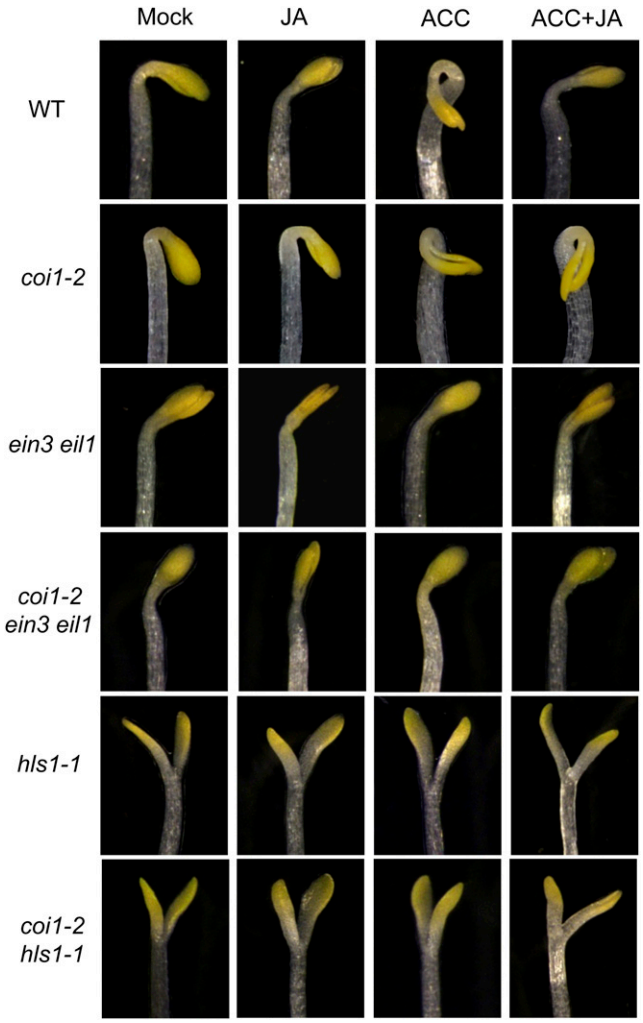

Figure 2. COI1 Acts Upstream of EIN3/EIL1 and HLS1 in Regulation of Apical Hook Formation.

The hook phenotypes of 4-d-old etiolated *Arabidopsis* seedlings Col-0 (WT), *coi1-2*, *ein3 eil1*, *coi1-2 ein3 eil1*, *hls1-1*, and *coi1-2 hls1-1* grown in the dark on MS medium supplied without (Mock) or with 5 μM MeJA (JA), 10 μM ACC, or 10 μM ACC plus 5 μM MeJA (ACC+JA).

strong YFP signals in the nuclei (Figure 4A), while the negative controls did not (Supplemental Figure 1), demonstrating that MYC2, MYC3, and MYC4 interact with EIN3 and EIL1.

We also used pull-down assays to representatively examine the interaction of MYC2 with EIN3 (Figure 4B). Purified maltose binding protein (MBP)–fused MYC2 (MBP-MYC2) resin was incubated with total protein from *Nicotiana benthamiana* leaves with transient expression of flag-tagged EIN3 (flag-EIN3) and separated by SDS-PAGE for immunoblotting with anti-flag antibody. As shown in Figure 4B, the MBP-MYC2 resin could pull down flag-EIN3, suggesting that MYC2 interacts with EIN3.

Furthermore, we performed coimmunoprecipitation (Co-IP) assays to examine the interaction between MYC2 and EIN3 in planta. The flag-EIN3 was coexpressed with myc-tagged MYC2 (myc-MYC2) or myc-COI1, respectively, in leaves of *N. benthamiana*, and the total proteins were then used for coimmunoprecipitation. The results showed that flag-EIN3 was indeed coimmunoprecipitated with myc-MYC2 (Figure 4C), but not with the control protein myc-COI1 (Figure 4C). Taken together, the BiFC assay, pull-down assay, and Co-IP assay consistently demonstrate that MYC2, MYC3, and MYC4 interact with EIN3 and EIL1 (Figure 4).

MYC2 Inhibits Transcriptional Activity of EIN3 and EIL1

Having shown that MYC2, MYC3, and MYC4 interact with EIN3 and EIL1, we then investigated whether such interactions affect the transcriptional activity of EIN3 and EIL1 using an *Arabidopsis* mesophyll protoplast transfection-based transcriptional activity assay (Hellens et al., 2005).

A previous study showed that EIN3 could bind to the promoter of *HLS1* to activate its expression, leading to hook curvature (An et al., 2012). We first examined whether MYC2 affects the influence of EIN3 on *HLS1* transcription. As expected (An et al., 2012), expression of EIN3 dramatically activated the expression of *LUC* driven by the *HLS1* promoter (Figures 5A and 5B). However, coexpression of MYC2 with EIN3 significantly repressed EIN3-activated P_{HLS1}-*LUC* activity (Figure 5B). Similarly, expression of EIL1 activated P_{HLS1}-*LUC* activity, whereas coexpression of MYC2 repressed EIL1-activated P_{HLS1}-*LUC* activity (Figures 5A and 5C). The results in Figures 4 and 5A to 5C demonstrate that MYC2 interacts with EIN3 and EIL1 to interfere with their effect on the transcription of *HLS1*.

Having shown that MYC2 suppresses the effect of EIN3 and EIL1 on *HLS1* transcription, we further examined whether MYC2 could repress the effects of EIN3 and EIL1 on the transcription of another target gene, *ETHYLENE RESPONSE FACTOR1* (*ERF1*) (Solano et al., 1998), a key transcription factor that activates the expression of *PDF1.2* to induce resistance against necrotrophic pathogens (Pré et al., 2008; Zarei et al., 2011). As shown in Figures 5D and 5E, overexpression of EIN3 activated the *ERF1* promoter that controlled expression of the *LUC* gene (P_{ERF1}-*LUC*), whereas such EIN3-activated P_{ERF1}-*LUC* expression was obviously repressed by coexpression of MYC2 (Figures 5D and 5E). Furthermore, we found that expression of EIL1 also activated P_{ERF1}-*LUC* activity, while coexpression of MYC2 repressed the EIL1-activated P_{ERF1}-*LUC* activity (Figures 5D and 5F). Taken together (Figures 4 and 5), these results demonstrate that MYC2 interacts with EIN3 and EIL1 to attenuate their effect on the transcription of their target genes *HLS1* and *ERF1*.

Disruption of EIN3 and EIL1 Suppresses Exaggerated Apical Hook Formation and Resistance against a Necrotrophic Pathogen in *myc2*

In agreement with the observation that MYC2 represses the transcriptional activity of EIN3 and EIL1, abolishment of *MYC2* in planta is expected to derepress EIN3 and EIL1, which would further activate the expression of *HLS1* (essential for hook curvature) and *ERF1* (vital for resistance against *Botrytis cinerea*). Indeed, the *myc2* mutants (e.g., *myc2 myc3*, *myc2 myc4*, and *myc2 myc3 myc4*) showed increased expression of *HLS1* (Figures 3B, 3C, and 6B), and the *myc2* mutant exhibited elevated expression of defensive genes, such as *ERF1*, *OCTADECANOID-RESPONSIVE*

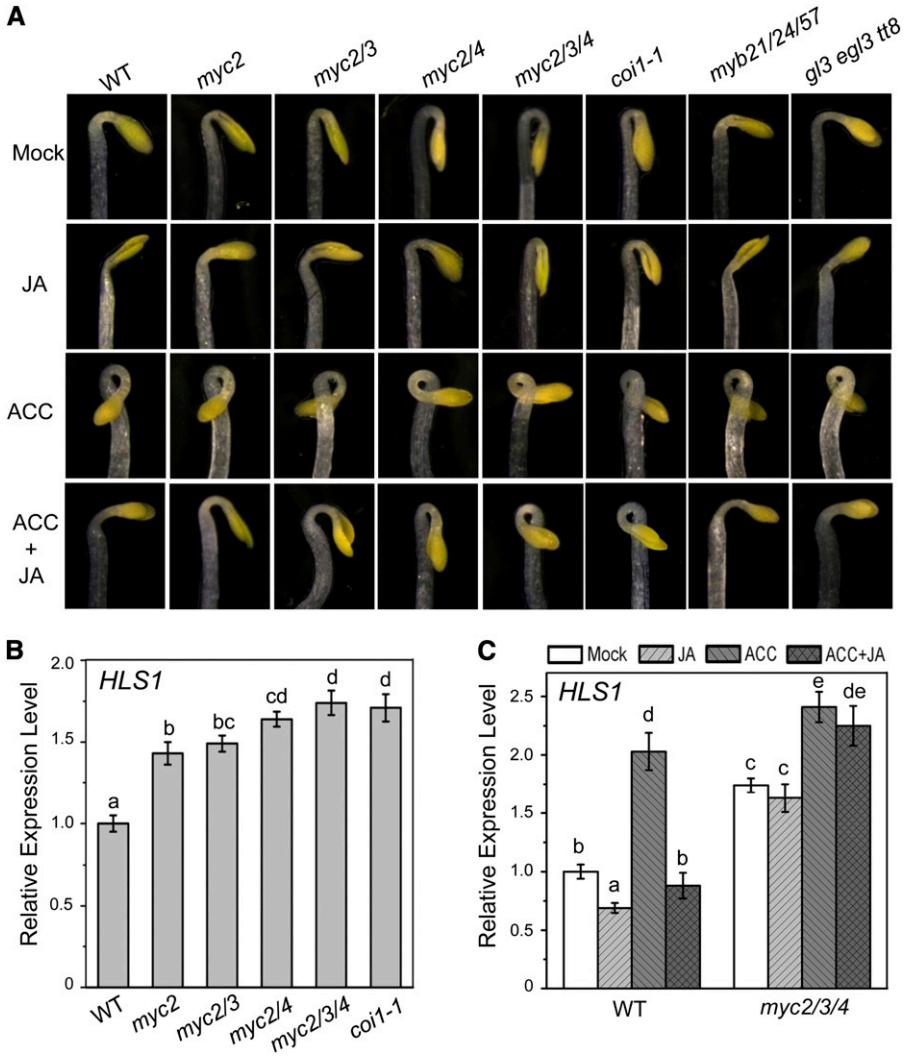

Figure 3. *MYC2*, *MYC3*, and *MYC4* Function Redundantly to Mediate the JA-Inhibited Apical Hook Formation.

(A) The hook phenotypes of 4-d-old etiolated *Arabidopsis* seedlings Col-0 (WT), *myc2-2* (*myc2*), *myc2-2 myc3* (*myc2/3*), *myc2-2 myc4* (*myc2/4*), *myc2-2 myc3 myc4* (*myc2/3/4*), *myb21 myb24 myb57* (*myb21/24/57*), and *gl3 egl3 tt8* grown in the dark on MS medium supplied without (Mock) or with 5 μM MeJA (JA), 10 μM ACC, or 10 μM ACC plus 5 μM MeJA (ACC+JA).

(B) Real-time PCR analysis for *HLS1* in the indicated 4-d-old etiolated seedlings. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

(C) Real-time PCR analysis for *HLS1* in the 4-d-old etiolated Col-0 (WT) and *myc2-2 myc3 myc4* (*myc2/3/4*) treated with mock, 100 μM MeJA (JA), 100 μM ACC, or 100 μM ACC plus 100 μM MeJA (ACC+JA) for 6 h. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

ARABIDOPSIS AP2/ERF59 (*ORA59*) (*ERF1* homolog), and their target gene *PLANT DEFENSIN1.2* (*PDF1.2*) (Figure 7C). Consistent with their gene expression patterns, the *myc2*-related mutants showed exaggerated hook formation (Figures 3A and 6A) and the *myc2* mutant displayed increased resistance against *B. cinerea* (Figures 7A and 7B) (Lorenzo et al., 2004). These results suggest that mutation in MYC2 releases EIN3 and EIL1 to further activate the expression of *HLS1* and *ERF1*, which are vital for hook curvature and disease resistance.

To examine whether *ein3 eil1* is able to suppress the exaggerated hooks in the *myc2*-related mutants, we generated the *myc2 ein3 eil1* and *myc2 myc3 myc4 ein3 eil1* mutants via crossing *myc2*-related mutants with the *ein3 eil1* mutant. The results in Figure 6A show that the exaggerated hook curvature in *myc2*-related mutants was repressed by *ein3 eil1* (Figure 6A). Consistently, the elevated expression of *HLS1* in *myc2* and *myc2 myc3 myc4* was abolished in *myc2 ein3 eil1* and *myc2 myc3 myc4 ein3 eil1* (Figure 6B). Furthermore, the expression of

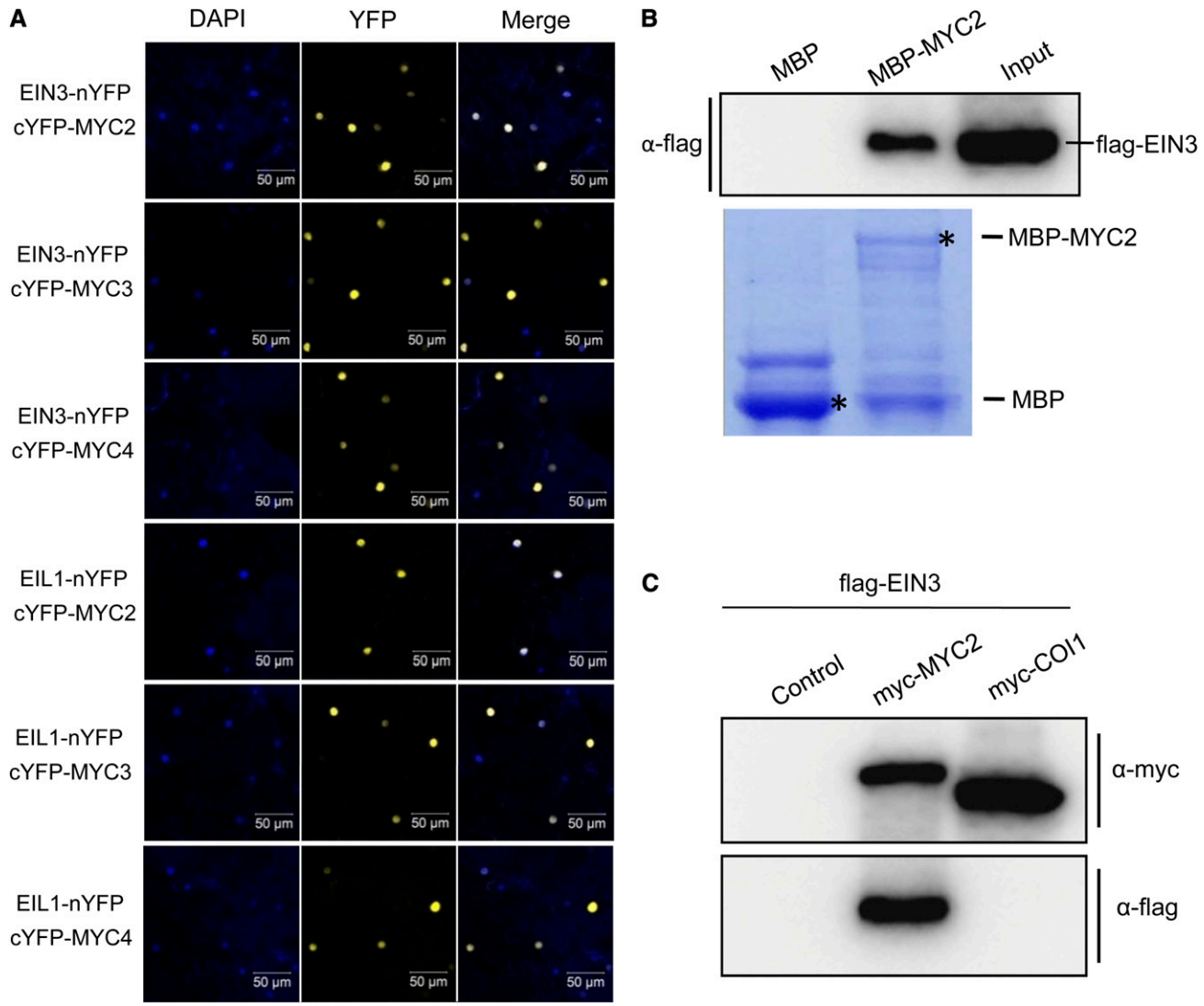

Figure 4. MYC2, MYC3, and MYC4 Interact with EIN3 and EIL1.

(A) BiFC assay to detect the interactions of MYC2, MYC3, and MYC4 with EIN3 and EIL1. EIN3 and EIL1 were fused with the N-terminal fragment of YFP (nYFP) to form EIN3-nYFP and EIL1-nYFP, respectively. MYC2, MYC3, and MYC4 were fused with the C-terminal fragment of YFP (cYFP) to generate cYFP-MYC2, cYFP-MYC3, and cYFP-MYC4. YFP fluorescence was detected in *N. benthamiana* leaves coinfiltrated with the combination of indicated constructs. The positions of nuclei were shown by 4′,6-diamidino-2-phenylindole (DAPI) staining.

(B) In vitro pull-down assay to verify the interaction of MYC2 with EIN3. The purified MBP and MBP-MYC2 fusion protein were incubated with the total protein from *N. benthamiana* leaves with transient expression of flag-EIN3. Bound proteins were washed, separated on SDS-PAGE, and immunoblotted with the anti-flag antibody (α-flag; top panel). The input lane shows the protein level of flag-EIN3 expressed in leaves of *N. benthamiana*. The positions of purified MBP and MBP-MYC2 separated on SDS-PAGE are marked with asterisks (bottom panel; stained by Coomassie blue).

(C) Co-IP assay to verify the interaction of MYC2 with EIN3 in planta. The flag-EIN3 was coexpressed without (Control) or with myc-MYC2 or myc-COI1 in the *N. benthamiana* leaves. The total protein extracts from the *N. benthamiana* leaves with transient expression of flag-EIN3, flag-EIN3 plus myc-MYC2, or flag-EIN3 plus myc-COI1 were immunoprecipitated with the anti-c-myc antibody-conjugated agarose and were further detected by immunoblot using anti-flag antibody and anti-c-myc antibody.

HLS1 in the *myc2 myc3 myc4 ein3 eil1* mutant was not affected by JA and/or ACC treatment (Figure 6C). Taken together (Figures 3 to 6), these results show that MYC2, MYC3, and MYC4 interact with and attenuate EIN3 and EIL1 to repress hook curvature.

We further investigated whether *ein3 eil1* could suppress the increased disease resistance against necrotrophic pathogen *B. cinerea* in *myc2*. As shown in Figures 7A and 7B, after inoculation with spores of *B. cinerea*, the *myc2* mutant exhibited disease resistance, as indicated by the smaller lesion size compared with the wild type, which is similar with previous studies demonstrating that MYC2 negatively regulates resistance against *B. cinerea* (Lorenzo et al., 2004; Zhai et al., 2013). The *ein3 eil1*

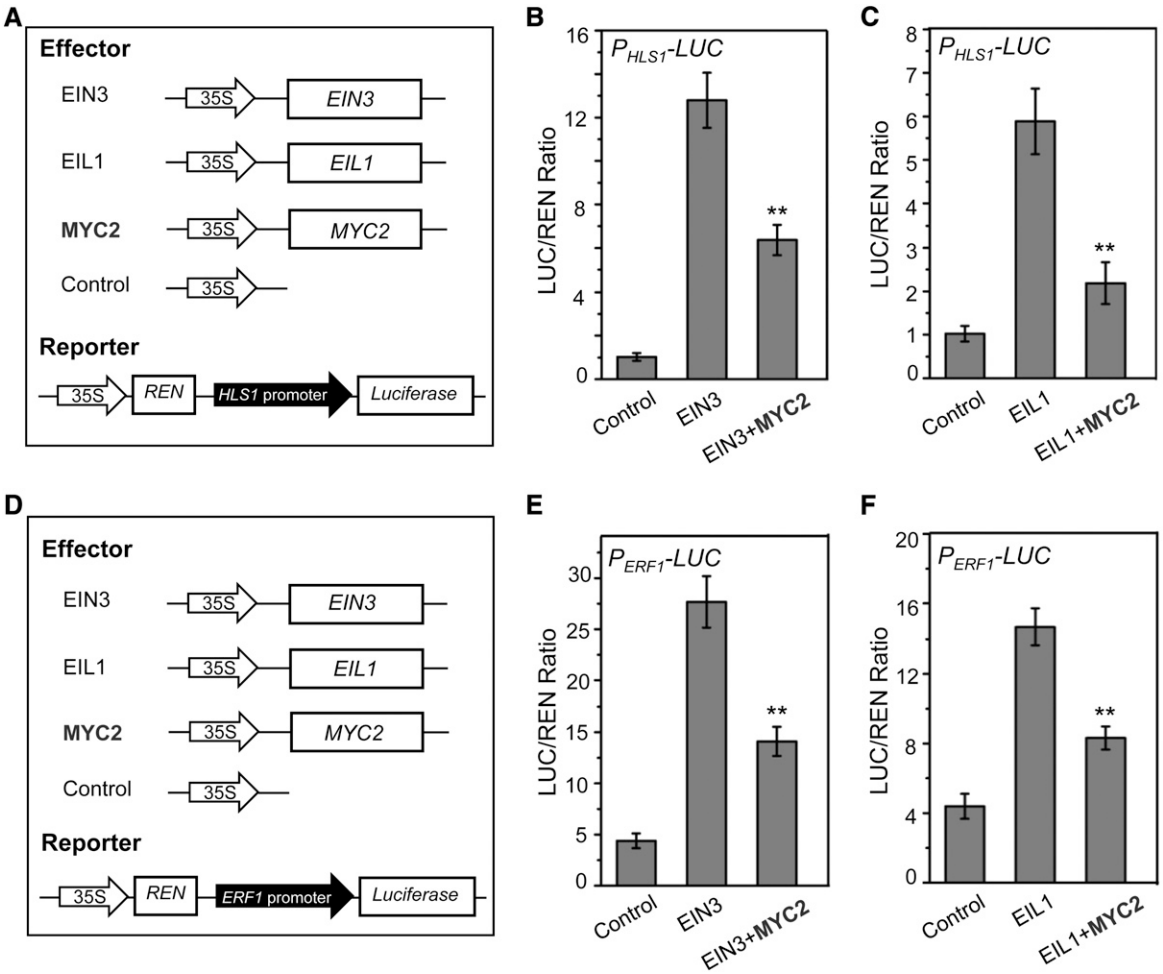

Figure 5. MYC2 Represses Transcriptional Activity of EIN3 and EIL1.

(A) The schematic diagram shows the constructs used in the transient transcriptional activity assays of **(B)** and **(C)**.

(B) and **(C)** Transient transcriptional activity assays show that activation of *HLS1* promoter by EIN3 **(B)** and EIL1 **(C)** is repressed by MYC2. The P_{HLS1}-*LUC* reporter was cotransformed with the indicated constructs. The LUC/REN ratio represents the P_{HLS1}-*LUC* activity relative to the internal control (*REN* driven by 35S promoter). Data are means (±SD) of three biological replicates. Asterisks represent Student's *t* test significance between EIN3 and EIN3+MYC2 or EIL1 and EIL1+MYC2 samples (**P < 0.01).

(D) The schematic diagram shows the constructs used in the transient transcriptional activity assays of **(E)** and **(F)**.

(E) and **(F)** Transient transcriptional activity assays show that activation of *ERF1* promoter by EIN3 **(E)** and EIL1 **(F)** is repressed by MYC2. The P_{ERF1}-*LUC* reporter was cotransformed with the indicated constructs. Data are means (±SD) of three biological replicates. Asterisks represent Student's *t* test significance between EIN3 and EIN3+MYC2 or EIL1 and EIL1+MYC2 samples (**P < 0.01).

double mutant displayed susceptibility, as indicated by the larger lesion size compared with the wild type (Figures 7A and 7B), confirming that EIN3 and EIL1 are required for resistance against *B. cinerea* (Alonso et al., 2003; Zhu et al., 2011). Similar to *ein3 eil1*, the *myc2 ein3 eil1* triple mutant also exhibited larger lesion size (Figures 7A and 7B), demonstrating that *ein3 eil1* blocked the elevated resistance against *B. cinerea* in *myc2*. Consistently, the upregulated expression of defense genes *ERF1*, *ORA59*, and *PDF1.2* (Zarei et al., 2011) in *myc2* was blocked by the *ein3 eil1* mutations (Figure 7C). Taken together (Figures 4, 5, and 7), these results showed that MYC2 interacts with and attenuates EIN3 and EIL1 to repress resistance against the necrotrophic pathogen *B. cinerea*.

In summary, the results in Figures 3 to 7 collectively demonstrate that MYC2 interacts with and represses EIN3 and EIL1 to regulate apical hook formation and resistance against the necrotrophic pathogen *B. cinerea*.

EIN3 and EIL1 Attenuate the Transcriptional Activation Function of MYC2 to Repress Plant Defense against Insect Attack

Having shown MYC2 interacts with and represses EIN3 and EIL1 to attenuate hook formation and disease resistance (Figures 3 to 7), we next explored whether EIN3 and EIL1 conversely

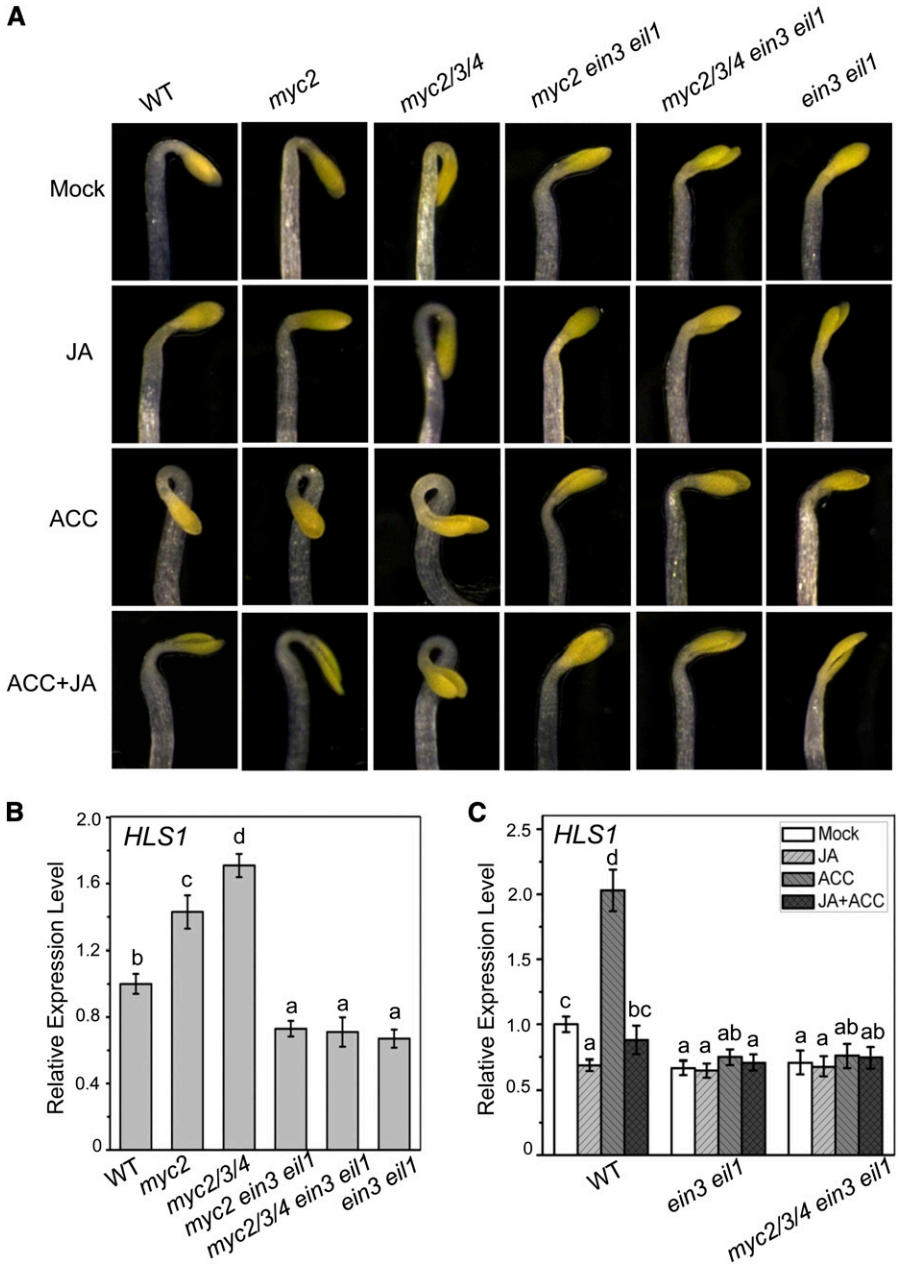

Figure 6. Mutations in EIN3 and EIL1 Block the Exaggerated Hook Curvature of *myc2* and *myc2 myc3 myc4*.

(A) The hook phenotypes of 4-d-old etiolated *Arabidopsis* Col-0 (WT), *myc2-2* (*myc2*), *jin1-2 myc3 myc4* (*myc2/3/4*), *myc2-2 ein3 eil1* (*myc2 ein3 eil1*), *jin1-2 myc3 myc4 ein3 eil1* (*myc2/3/4 ein3 eil1*), and *ein3 eil1* grown in the dark on MS medium supplied without (Mock) or with 5 μM MeJA (JA), 10 μM ACC, or 10 μM ACC plus 5 μM MeJA (ACC+JA).

(B) Real-time PCR analysis for *HLS1* in the indicated 4-d-old etiolated seedlings. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

(C) Real-time PCR analysis for *HLS1* in the indicated 4-d-old etiolated seedlings treated with mock, 100 μM MeJA (JA), 100 μM ACC, or 100 μM ACC plus 100 μM MeJA (JA+ACC) for 6 h. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

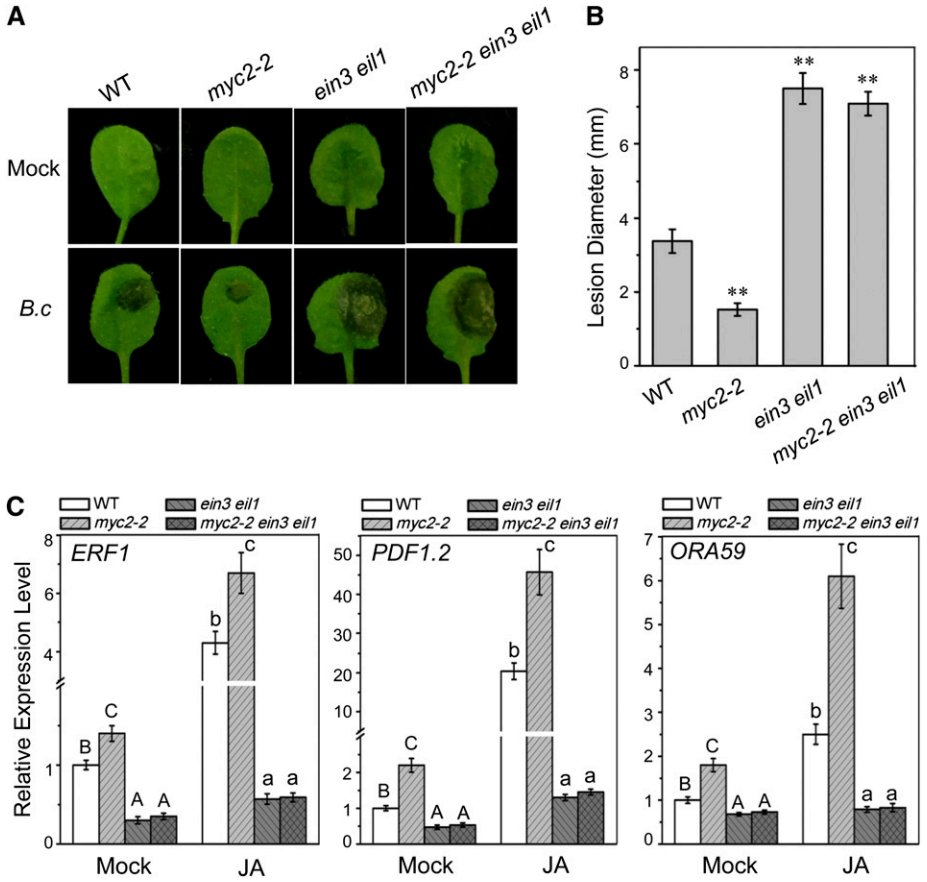

Figure 7. Mutations in EIN3 and EIL1 Repress the Enhanced Resistance against Necrotrophic Pathogen *Botrytis cinerea* in *myc2*.

(A) Symptoms on detached rosette leaves from 3-week-old plants of Col-0 (WT), *myc2-2*, *ein3 eil1*, and *myc2-2 ein3 eil1* at day 2 after inoculation with mock or *B. cinerea* (*B.c*) spores.

(B) The lesion sizes on rosette leaves at day 2 after inoculation with *B. cinerea* spores. Data are means (±SD) of three biological replicates. Asterisks represent Student's *t* test significance compared with the wild type (**$P < 0.01$).

(C) Quantitative real-time PCR analysis of *ERF1*, *PDF1.2*, and *ORA59* in 12-d-old wild type, *myc2-2*, *ein3 eil1*, and *myc2-2 ein3 eil1* treated with mock or 100 μM MeJA (JA) for 6 h. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Different letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$). Capital letters correspond with each other, and lowercase letters correspond with each other.

affect the transcriptional function of MYC2 using the GAL4 DNA binding domain (GAL4DB) and its binding site [GAL4(4X)-D1-3 (4X)-GUS]–based *Arabidopsis* protoplast transient expression system (Tiwari et al., 2001).

The *MYC2* gene was fused with GAL4DB under the control of 35S promoter to generate the effector GAL4DB-MYC2. The β-glucuronidase (*GUS*) gene driven by four copies of GAL4 DNA binding sites [GAL4(4x)-D1-3(4x)] was used as a reporter, whereas the *LUC* gene under the control of 35S promoter was used as the internal control (Figure 8A). Similar with previous observations (Pauwels et al., 2010; Song et al., 2013b), expression of GAL4DB-MYC2 clearly increased the GUS/LUC ratio (Figure 8B). However, coexpression of EIN3 or EIL1 with GAL4BD-MYC2 obviously reduced the GUS/LUC ratio (Figure 8B), suggesting that EIN3 and EIL1 attenuate the transcriptional activation function of MYC2.

To further verify that the EIN3 and EIL1 repress the transcriptional activation function of MYC2, we investigated whether abolishment of EIN3 and EIL1 in planta would derepress MYC2 to enhance the expression of MYC2-regulated genes. Consistent with previous studies (Lorenzo et al., 2004; Fernández-Calvo et al., 2011; Schweizer et al., 2013), our results showed that MYC2 upregulated JA-induced expression of the wound-responsive genes *VEGETATIVE STORAGE PROTEIN1* (*VSP1*), *VSP2*, and *TYROSINE AMINOTRANSFERASE3* (*TAT3*) (Figure 9A) and the herbivore-inducible genes *CYP79B3*, *BRANCHED-CHAIN AMINOTRANSFERASE4* (*BCAT4*), and *BILE ACID TRANSPORTER5* (*BAT5*) (Figure 9B), which are required for the biosynthesis of the secondary metabolites glucosinolates (Zhao et al., 2002; Kliebenstein et al., 2005; Schweizer et al., 2013). Interestingly, the double mutant *ein3 eil1* exhibited upregulated expression of these wound-responsive genes (*VSP1*, *VSP2*, and *TAT3*) as well as herbivory-inducible genes (*CYP79B3*, *BCAT4*, and *BAT5*) when treated with (or even without) JA compared with the wild type (Figures 9A and 9B). Consistent with the expression levels of

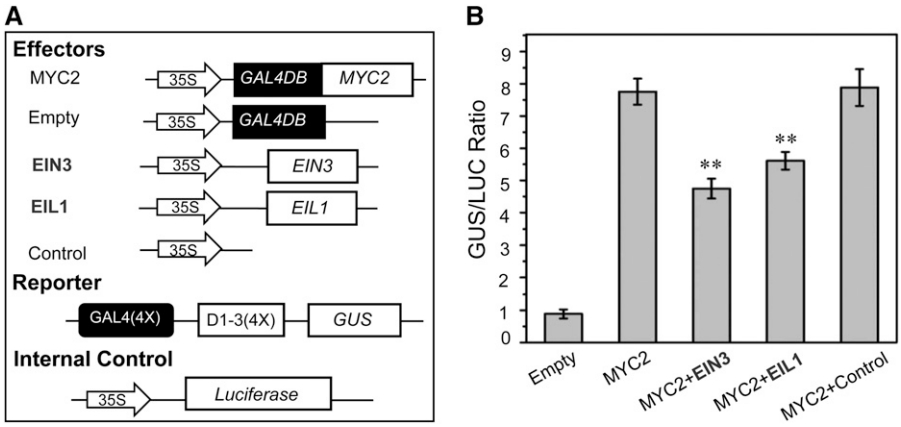

Figure 8. EIN3 and EIL1 Antagonize the Transcriptional Activation Function of MYC2.

(A) The schematic diagram shows the constructs used in the transient expression assays.

(B) Transient expression assays show that MYC2 acts as a transcriptional activator, while EIN3 and EIL1 attenuate the transcriptional activation function of MYC2. Data are means (±SD) of three biological replicates. Asterisks represent Student's *t* test significance between MYC2 and MYC2+EIN3 or MYC2+EIL1 samples (**$P < 0.01$).

wound-responsive and herbivory-inducible genes (Figures 9A and 9B), the *myc2 myc3 myc4* triple mutant, which was almost completely devoid of glucosinolates (Schweizer et al., 2013), exhibited susceptibility to the generalist herbivores *Spodoptera littoralis* (Schweizer et al., 2013) and *Spodoptera exigua* (Figures 9C and 9D), while plant defense against these generalist herbivores was enhanced in the ET-signaling mutants *ein3 eil1* (Figures 9C and 9D), *etr1*, and *ein2* (Stotz et al., 2000; Mewis et al., 2005, 2006; Bodenhausen and Reymond, 2007). These results demonstrate that the abolishment of EIN3 and EIL1 derepresses MYC2, which enhances the expression of wound-responsive and herbivore-inducible genes and elevates plant defenses against generalist herbivores.

Further comparison of the gene expression pattern among the double mutant *ein3 eil1*, the pentuple mutant *myc2 myc3 myc4 ein3 eil1*, and the triple mutant *myc2 myc3 myc4* showed that JA-induced expression of *VSP1*, *VSP2*, *TAT3*, *CYP79B3*, *BCAT4*, and *BAT5* was significantly elevated in *ein3 eil1*, whereas such elevated gene expression was obviously repressed by the *myc2 myc3 myc4* mutations (Figures 9A and 9B). Consistently, plant defense against insect attack was enhanced in *ein3 eil1*, but disrupted by the *myc2 myc3 myc4* mutations (Figures 9C and 9D). These results suggest that mutations in *MYC2*, *MYC3*, and *MYC4* abolish the enhanced expression of wound/herbivore-inducible genes and plant defense against insect attack in *ein3 eil1*.

Taken together (Figures 3 to 9), we demonstrated that the interaction between the JA-activated transcription factors (MYC2, MYC3, and MYC4) and the ET-stabilized transcription factors (EIN3 and EIL1) represses their respective transcriptional activities to modulate the JA and ET signaling antagonism. EIN3 and EIL1 interact with and repress MYC2, MYC3, and MYC4 to attenuate JA-induced expression of wound-responsive and herbivore-inducible genes and to repress plant defense against the generalist herbivores *S. littoralis* and *S. exigua* (Figures 4, 8, and 9). Conversely, MYC2 interacts with and represses EIN3 and EIL1 to inhibit hook formation and disease resistance against a necrotrophic pathogen (Figures 3 to 7).

DISCUSSION

Land plants live in fixed location, often encounter environmental stresses, and maintain plasticity in growth and development to adapt to the fluctuating environment by integrating multiple signals including the endogenous phytohormone signals JA and ET. JA and ET act synergistically to defend against necrotrophic pathogen infection and to promote root hair development (Penninckx et al., 1996; Zhu et al., 2006) via a synergistic regulatory model in which JA induces degradation of JAZ proteins and derepresses ET-stabilized EIN3 and EIL1, which interact with JAZs (Zhu et al., 2011). However, such a synergistic regulatory model is inconsistent with the antagonistic roles of JA and ET signaling in many important processes. For example, JA antagonizes ET to repress apical hook formation (e.g., exaggerated hook formation in the *coi1* mutant) (Figures 1 and 2) (Turner et al., 2002), whereas ET antagonizes JA to repress the expression of wound-responsive genes (*VSP1*, *VSP2*, and *TAT3*) and herbivore-inducible genes (*CYP79B3*, *BCAT4*, and *BAT5*) (Figures 9A and 9B) (Rojo et al., 1999; Mikkelsen et al., 2003) and to attenuate plant defense against generalist herbivores (Figures 9C and 9D) (Stotz et al., 2000; Mewis et al., 2005, 2006; Bodenhausen and Reymond, 2007).

This study reveals a mechanism underlying antagonism between JA and ET signaling. Molecular, biochemical, and genetic evidence suggest an antagonistic regulatory model in which interaction between MYC2, key transcription factor in the JA pathway, and EIN3 and EIL1, master transcription factors in the ET pathway, modulates the JA-ET signaling antagonism (Figure 10). MYC2 interacts with and represses EIN3 and EIL1 to inhibit their effects on the transcription of *HLS1* and *ERF1*, which represses ET-regulated apical hook formation (Figures 10A) and resistance to necrotrophic pathogen (Figures 10B). Conversely, EIN3 and EIL1 interact with and attenuate MYC2, MYC3, and MYC4 to inhibit the expression of wound-responsive and herbivore-inducible genes and to repress JA-regulated plant defense

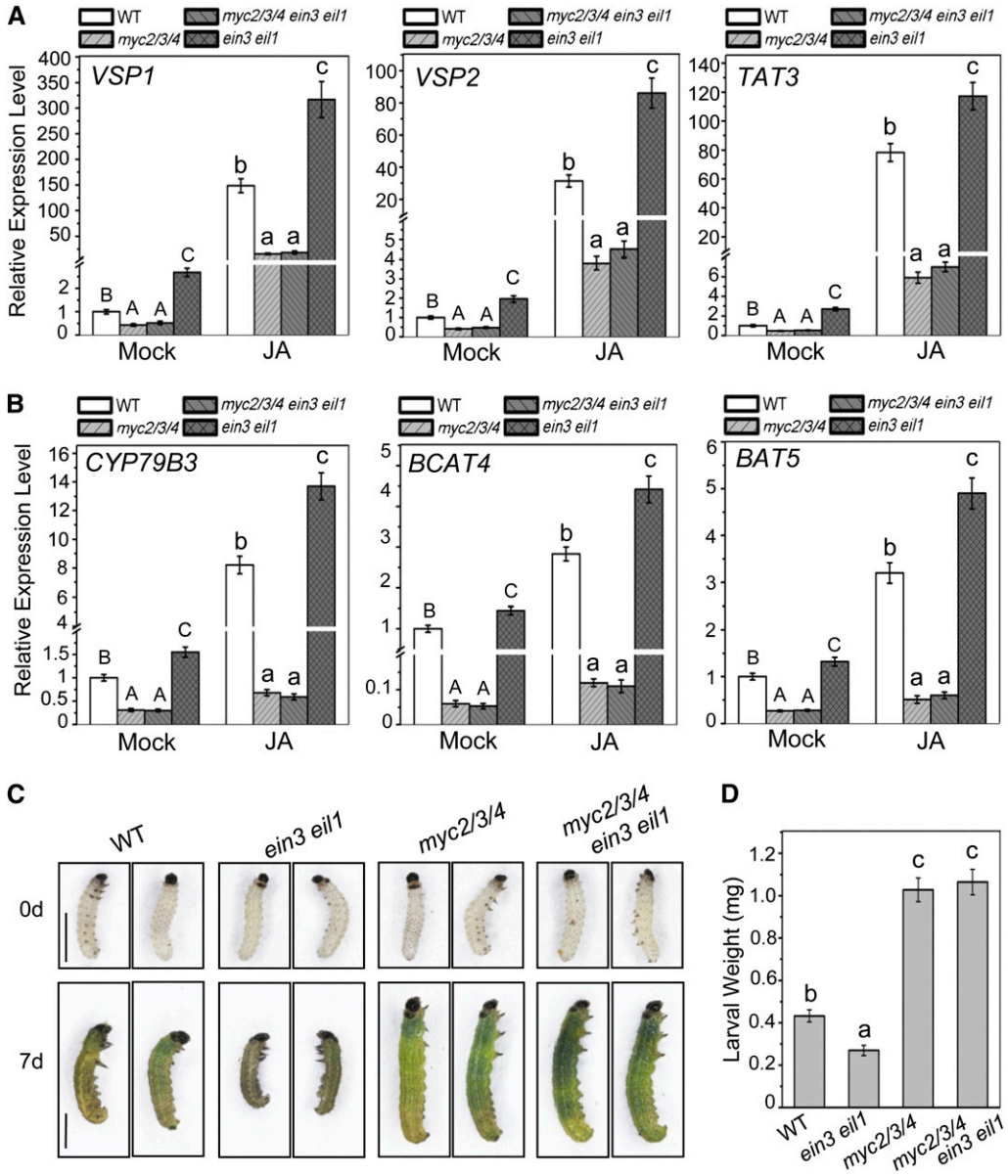

Figure 9. Mutations in MYC2, MYC3, and MYC4 Block the Enhanced Defense against Insect Attack in *ein3 eil1*.

(A) and **(B)** Real-time PCR analysis for *VSP1*, *VSP2*, *TAT3*, *CYP79B3*, *BCAT4*, and *BAT5* in the 12-d-old seedlings Col-0 (WT), *jin1-2 myc3 myc4* (*myc2/3/4*), *jin1-2 myc3 myc4 ein3 eil1* (*myc2/3/4 ein3 eil1*), and *ein3 eil1* treated with mock or 100 μM MeJA (JA) for 6 h. *Actin8* was used as the internal control. Data are means (±SD) of three biological replicates. Different letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$). Capital letters compare with each other, and lowercase letters compare with each other.

(C) Photograph of *S. exigua* larvae before feeding (0 d) and 7 d after feeding (7 d) with wild-type, *ein3 eil1*, *jin1-2 myc3 myc4* (*myc2/3/4*), or *jin1-2 myc3 myc4 ein3 eil1* (*myc2/3/4ein3 eil1*) plants. Bars = 1 mm.

(D) Larval weight of *S. exigua* reared on wild-type, *ein3 eil1*, *jin1-2 myc3 myc4* (*myc2/3/4*), or *jin1-2 myc3 myc4 ein3 eil1* (*myc2/3/4ein3 eil1*) plants for 7 d. Ten larvae as one sample were weighed together to obtain one datum for average weight. Fifty larvae (five independent samples) for each genotype in each biological experiment were used. Values are means (±SD) from three biological replicates. Lowercase letters indicate significant differences by one-way ANOVA analysis with SAS software ($P < 0.05$).

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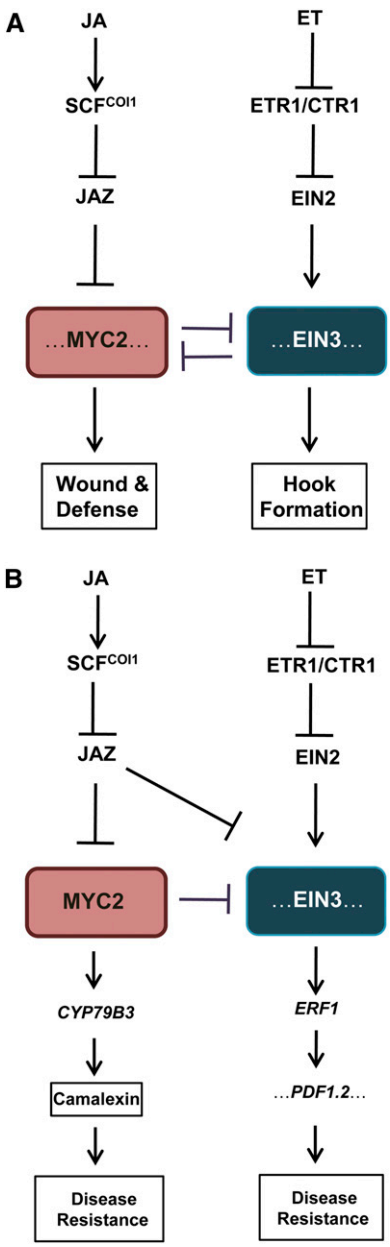

Figure 10. A Simplified Model for JA and ET Signaling Antagonism.

(A) Model for JA and ET antagonistic action in regulating hook curvature, wounding, and defense against insect attack. In response to JA signaling, SCF^{COI1} recruits JAZs for ubiquitination and degradation. MYC2, MYC3, and MYC4 (indicated as MYC2) are then released to interact with and repress EIN3 and EIL1 (indicated as EIN3), which leads to attenuation of ET-enhanced hook curvature. ET signal inactivates the ET receptors (indicated as ETR1) and the negative regulator CTR1 to mediate EIN2 translocation into nucleus and to stabilize EIN3 and EIL1. EIN3 and EIL1 then interact with and repress MYC2, MYC3, and MYC4 to inhibit expression of wound responsive genes (e.g., *VSP1*, *VSP2*, and *TAT3*) and herbivory-inducible genes (e.g., *CYP79B3*, *BCAT4*, and *BAT5*) and suppress JA-regulated plant defense against generalist herbivores *S. littoralis* and *S. exigua* (indicated as wound and defense).

(B) Model for JA and ET crosstalk in regulating plant resistance against necrotrophic pathogen. JAZs and MYC2 interact with and repress ET-stabilized EIN3 and EIL1 (indicated as EIN3). In response to JA signaling, JAZ proteins are degraded to derepress EIN3/EIL1, leading to the increased disease resistance against necrotrophic pathogen *B. cinerea* (indicated as disease resistance) (Zhu et al., 2011). Meanwhile, JA-induced JAZ degradation releases MYC2, which counteracts EIN3 and EIL1 to prevent excessive disease resistance responses. In addition, other factors, including CYP79B3, which is required for biosynthesis of camalexin (Glawischnig et al., 2004; Kliebenstein et al., 2005), may be also regulated by MYC2 to modulate disease resistance. Regulation of plant resistance against *B. cinerea* might be complicated and modulated by the coordinated action of synergistic and antagonistic mechanisms. [See online article for color version of this figure.]

against the generalist herbivores *S. littoralis* and *S. exigua* (Figure 10A).

Consistent with this antagonistic regulatory model, in the *myc2* mutant, the absence of MYC2 fails to repress the transcriptional activity of EIN3 and EIL1, leads to the activation of EIN3/EIL1-regulated gene expression (*HLS1*, *ERF1*, *ORA59*, and *PDF1.2*) and results in enhanced apical hook formation and plant resistance against *B. cinerea* infection (Figures 3 to 7 and 10). On the other hand, in the ET signaling mutants (e.g., *ein3 eil1* and *ein2*), the absence of EIN3 and EIL1 enables MYC2, MYC3, and MYC4 to induce the expression of wound-responsive genes (*VSP1*, *VSP2*, and *TAT3*) (Figure 9A) (Rojo et al., 1999; Lorenzo et al., 2004) and herbivore-inducible genes (*CYP79B3*, *BCAT4*, and *BAT5*), and enhances plant defense against the herbivores *S. littoralis* and *S. exigua* (Figures 4, 8, 9, and 10A) (Stotz et al., 2000; Mewis et al., 2005, 2006; Bodenhausen and Reymond, 2007). In wild-type plants, antagonistic regulation between the ET-stabilized transcription factors (EIN3 and EIL1) and the JA-activated transcription factors (MYC2, MYC3, and MYC4) would lead to suitable expression of MYC2-dependent genes (*VSP1*, *VSP2*, *TAT3*, *CYP79B3*, *BCAT4*, and *BAT5*) and EIN3-regulated genes (*HLS1*, *ERF1*, *ORA59*, and *PDF1.2*), resulting in proper plant responses, such as hook formation and defense against the herbivores *S. littoralis* and *S. exigua*.

MYC2 functions as a key transcription factor to positively regulate diverse JA responses (Kazan and Manners, 2013), including root growth (Boter et al., 2004; Lorenzo et al., 2004), secondary metabolism (Dombrecht et al., 2007; Hong et al., 2012; Schweizer et al., 2013), wound response, and plant defense against insect attack (Zhang and Turner, 2008; Fernández-Calvo et al., 2011; Schweizer et al., 2013). Surprisingly, MYC2 also acts as a negative regulator to repress JA-mediated plant resistance to necrotrophic fungi and pathogenesis-related gene expression (e.g., *PDF1.2*) (Anderson et al., 2004; Lorenzo et al., 2004; Zhai et al., 2013). Such MYC2-regulated susceptibility to necrotrophic fungi seems incompatible with the previously reported synergistic model (Zhu et al., 2011). Our results provide a mechanistic understanding of the long-standing question of how MYC2 represses JA-regulated plant resistance against necrotrophic fungi: MYC2 interacts with and represses EIN3 and EIL1, which inhibits expression of the EIN3/EIL1-dependent defense genes (*ERF1*, *ORA59*, and *PDF1.2*) and consequently depresses plant resistance against necrotrophic pathogen infection.

Coordinated regulation of plant responses in both antagonistic and synergistic manners would help plants adapt to fluctuating environments. Plant resistance against necrotrophic fungi might be modulated by a balance between the previously described synergistic mechanism (interaction of JAZs with EIN3) (Zhu et al., 2011) (Figure 10B) and our antagonistic regulation model (interaction between MYC2 and EIN3) (Figure 10B). Future research should focus on identifying protein domains essential for the interaction between MYC2 and EIN3 and clarifying whether reciprocal repression between MYC2 and EIN3 occurs on promoters of its target genes or disrupts the binding of MYC2 or EIN3 to its respective target promoters, which would help advance our understanding of the reciprocal regulation of the transcriptional functions of MYC2 and EIN3.

JA and ET exhibit opposite effects on many other plant responses. JA enhances anthocyanin accumulation (Qi et al., 2011) and freezing tolerance (Hu et al., 2013b), inhibits seed germination (Miersch et al., 2008), hypocotyl elongation in the light (Chen et al., 2013) and the ozone-induced spreading of cell death (Rao et al., 2000; Tuominen et al., 2004), and delays flowering (Robson et al., 2010). Conversely, ET suppresses anthocyanin accumulation (Jeong et al., 2010) and freezing tolerance (Shi et al., 2012) and enhances seed germination (Linkies et al., 2009; Linkies and Leubner-Metzger, 2012), hypocotyl elongation in the light (Zhong et al., 2012), the ozone-induced spreading of cell death (Overmyer et al., 2000), and flowering (Ogawara et al., 2003). It would be interesting to investigate whether these ET-JA antagonistic actions are mediated by similar interactions between their respective master transcription factors in the JA and ET pathways.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* mutants *coi1-1* (Xie et al., 1998), *coi1-2* (Xu et al., 2002), *JAZ1Δ3A* (Thines et al., 2007), *myc2-2* (Boter et al., 2004), *jin1-2* (Lorenzo et al., 2004), *myc3* (GK445B11) (Fernández-Calvo et al., 2011), *myc4* (GK491E10) (Fernández-Calvo et al., 2011), *jin1-2 myc3 myc4* (Fernández-Calvo et al., 2011), *eto1-1* (Guzmán and Ecker, 1990), *ctr1-1* (Kieber et al., 1993), *ein2-1* (Alonso et al., 1999), *hls1-1* (Lehman et al., 1996), *ein3-1* (Chao et al., 1997), *eil1-3*/Salk_049679 (Binder et al., 2007), *myb21 myb24 myb57* (Cheng et al., 2009), and *gl3 egl3 tt8* (Qi et al., 2011) were previously described. The higher order mutants *myc2-2 myc3*, *myc2-2 myc4*, *myc2-2 myc3 myc4*, *ein3-1 eil1-3*, *coi1-2 ein3-1 eil1-3*, *coi1-2 hls1-1*, *myc2-2 ein3 eil1-3*, and *jin1-2 myc3 myc4 ein3-1 eil1-3* were generated by genetic crosses using standard techniques.

The *Arabidopsis* seeds were sterilized with 20% bleach, plated on Murashige and Skoog (MS) medium, chilled at 4°C for 3 d, and then transferred to a growth room under a 16-h (20 to 24°C)/8-h (16 to 19°C) light/dark photoperiod. For hook phenotype analysis, seeds were sterilized, chilled, and transferred to a growth chamber at 22°C in the dark for 4 d. *Nicotiana benthamiana* was grown in a growth room under a 16-h (25 to 28°C)/8-h (22 to 25°C) light/dark cycle.

BiFC Assay

For the BiFC assays, the full-length coding sequence (CDS) of *Arabidopsis EIN3*, *EIL1*, *MYC2*, *MYC3*, and *MYC4* were cloned into the binary nYFP or cYFP vector through the Gateway system (Invitrogen) (Qi et al., 2011). Primer pairs used for the generation of constructs are listed in Supplemental Table 1. BiFC assays were performed as previously described (Qi et al., 2011). Equal concentrations and volumes of resuspended *Agrobacterium tumefaciens* strain GV3101 harboring the indicated nYFP or cYFP vectors in infiltration buffer (0.2 mM acetosyringone, 10 mM $MgCl_2$, and 10 mM MES) were mixed and coinfiltrated into leaves of *N. benthamiana* using a needleless syringe. Two days after infiltration, the YFP signal was observed using a Zeiss confocal microscope (LSM710). Four hours before observation, 100 μM MG132 was infiltrated into the leaves of *N. benthamiana*.

Pull-Down Assay

MBP-MYC2 (Chen et al., 2011) and MBP proteins were purified from *Escherichia coli* using MBP affinity chromatography according to Qi et al. (2011). The full-length CDS of EIN3 was cloned into the modified pCambia1300 vector under the control of 35S promoter for fusion with three flag tags to generate flag-EIN3. *Agrobacterium* strain harboring flag-EIN3 was infiltrated into *N. benthamiana* leaves. After 50 h, 5 g of *N. benthamiana* leaves transiently expressing flag-EIN3 were harvested for total protein extraction in RB buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.8, 25 mM imidazole, 0.1% [v/v] Tween 20, 10% [v/v] glycerol, EDTA-free complete miniprotease inhibitor cocktail, and 20 mM 2-mercaptoethanol). The extracted total protein was concentrated in centrifugal filter tubes (Millipore) to 400 μL. Coomassie Brilliant Blue was used to confirm the protein amount. About 50 μg of purified MBP and MBP-MYC2 was incubated with 120 μL of amylose resin beads for 2 h at 4°C. These amylose resin beads were then washed five times with 1 mL of RB buffer and incubated with 200 μL of concentrated total proteins containing flag-EIN3 for 2 h at 4°C. After washing five times with 1 mL RB buffer, the mixture was resuspended in SDS loading buffer, boiled for 5 min, separated on 15% SDS-PAGE, and immunoblotted using 1:1000 dilution for anti-flag antibody (Abmart).

Co-IP

N. benthamiana leaves were infiltrated with *Agrobacterium* strains harboring flag-EIN3, flag-EIN3 with myc-MYC2 (Zhai et al., 2013), or flag-EIN3 with myc-COI1 (Yan et al., 2013). Two days after infiltration, 3 g of agroinfiltrated leaves for each combination was collected and homogenized in Co-IP buffer containing 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 16,000*g* at 4°C. The supernatant was concentrated to 400 μL and incubated with the agarose-conjugated anti-myc matrix (Abmart) for 2 h (4°C, with rotation), then washed three times with 1 mL of immunoprecipitation buffer. After denaturation in 100 μL of SDS loading buffer, the samples were loaded into 15% SDS-PAGE gels, subjected to gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore), and immunoblotted with anti-flag antibody (Abmart) and anti-myc antibody, respectively.

Protoplast Transfection Assay

For the transient transcriptional activity assay, constructs harboring the *LUC* gene under the control of the ~1500-bp or ~1523-bp promoter sequences of *HLS1* or *ERF1*, respectively, in the pGreenII 0800-LUC vector were generated as reporters (Hellens et al., 2005). The *renilla luciferase* (*REN*) gene under the control of 35S promoter in the pGreenII 0800-LUC vector was used as the internal control. The CDS sequences of *EIN3*, *EIL1*, and *MYC2* were cloned into the pGreenII 62-SK vector under the control of the 35S promoter and were used as effectors. All primers used for making these constructs are listed in Supplemental Table 1. *Arabidopsis* mesophyll protoplast was prepared and transfected as previously described (Yoo et al., 2007). The firefly LUC and REN activities

were measured using the Dual-Luciferase Reporter Assay System (Promega). LUC/REN ratios were presented.

For transient expression assay, the CDS of *MYC2* fused with GAL4DB (GAL4DB-MYC2) under the control of 35S promoter was used as effector. The GUS gene under the control of four copies of upstream GAL4 DNA binding sites [GAL4(4x)-D1-3(4x)]was used as the reporter (Tiwari et al., 2001; Zhu et al., 2008). The *firefly LUC* gene under the control of 35S promoter was the internal control. Primers used for plasmid construction are shown in Supplemental Table 1. The preparation and subsequent transfection of *Arabidopsis* mesophyll protoplasts were performed as described previously (Yoo et al., 2007). The GUS/LUC ratios were presented.

Infection with Pathogen

Detached leave from 3-week-old plant were inoculated with 5 μL spores of *Botrytis cinerea* (SCL2-4, isolated from tomato in 2011, Shanghai) (Song et al., 2013b) (10^5 spores/mL) suspended in potato dextrose broth (with potato dextrose broth alone as the control), placed in Petri dishes with 0.8% agar, and covered with lids. The lesion diameter from eight leaves for each genotype exhibiting disease symptoms was measured 2 d after inoculation.

Insect Defense Assay with *Spodoptera exigua*

Newly hatched *S. exigua* larvae were placed on 3-week-old plants (10-h-light/14-h-dark photoperiod) of each genotype for 7 d of feeding. Ten surviving larvae were weighted as one sample to obtain one datum for average weight. Fifty surviving larvae (five independent samples in total) for each genotype in each biological experiment were used. The experiment was repeated for three biological replicates.

Quantitative Real-Time PCR

For Figures 1B, 3B, and 6B, *Arabidopsis* seedlings were grown on MS medium at 22°C in the dark for 4 d. For Figures 1C, 3C, and 6C, seedlings were grown on MS medium at 22°C in the dark for 4 d and then were treated with mock, 100 μM methyl jasmonate (MeJA), 100 μM ACC, or 100 μM MeJA plus 100 μM ACC for 6 h. For Figures 7C, 9A, and 9B, seedlings were grown on MS medium for 12 d under a 16-h (20 to 24°C)/8-h (16 to 19°C) light/dark photoperiod and then were treated with mock or 100 μM MeJA for 6 h. These materials were harvested for RNA extraction and subsequent reverse transcription. Real-time PCR analyses were performed using the ABI7500 real-time PCR system with the RealMasterMix (SYBR Green I) (Takara) as described previously (Qi et al., 2011). The primers for real-time PCR analysis are presented in Supplemental Table 2 online. *ACTIN8* was used as the internal control. The experiment was repeated for three biological replicates.

Accession Numbers

The Arabidopsis Genome Initiative numbers for genes mentioned in this article are as follows: COI1 (AT2G39940), MYC2 (AT1G32640), MYC3 (AT5G46760), MYC4 (AT4G17880), JAZ1 (AT1G19180), ETO1 (At3g51770), CTR1 (AT5G03730), EIN2 (AT5G03280), EIN3 (AT3G20770), EIL1 (AT2G27050), ERF1 (AT3G23240), HLS1 (AT4G37580), PDF1.2 (AT5G44420), MYB21 (At3g27810), MYB24 (At5g40350), MYB57 (At3g01530), TT8 (AT4G09820), GL3 (AT5G41315), EGL3 (AT1G63650), VSP1 (AT5G24780), VSP2 (AT5G24770), TAT3 (AT2G24850), ORA59 (AT1G06160), CYP79B3 (At2g22330), BCAT4 (At3g19710), BAT5 (At4g12030), and ACTIN8 (AT1G49240).

Supplemental Data

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

Supplemental Figure 1. The Negative Controls for the BiFC Experiments.

Supplemental Table 1. Primers Used for Vector Construction.

Supplemental Table 2. Primers Used for Quantitative Real-Time PCR Analysis.

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AUTHOR CONTRIBUTIONS

S.S., T.Q., and D.X. designed the research. S.S., H.H., H.G., J.W., D.W., Q.Z., and T.Q. performed research. S.S., X.L., S.Y., C.L., T.Q., and D.X. analyzed data. S.S., T.Q., and D.X. wrote the article.

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