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FILAMENTOUS FLOWER Is a Direct Target of JAZ3 and Modulates Responses to Jasmonate

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The plant hormone jasmonate (JA) plays an important role in regulating growth, development, and immunity. Activation of the JA-signaling pathway is based on the hormone-triggered ubiquitination and removal of transcriptional repressors (JASMONATE-ZIM DOMAIN [JAZ] proteins) by an SCF receptor complex (SCF^{COI1}/JAZ). This removal allows the rapid activation of transcription factors (TFs) triggering a multitude of downstream responses. Identification of TFs bound by the JAZ proteins is essential to better understand how the JA-signaling pathway modulates and integrates different responses. In this study, we found that the JAZ3 repressor physically interacts with the YABBY (YAB) family transcription factor FILAMENTOUS FLOWER (FIL)/YAB1. In *Arabidopsis thaliana*, *FIL* regulates developmental processes such as axial patterning and growth of lateral organs, shoot apical meristem activity, and inflorescence phyllotaxy. Phenotypic analysis of JA-regulated responses in loss- and gain-of-function *FIL* lines suggested that YABs function as transcriptional activators of JA-triggered responses. Moreover, we show that *MYB75*, a component of the WD-repeat/bHLH/MYB complex regulating anthocyanin production, is a direct transcriptional target of FIL. We propose that JAZ3 interacts with YABs to attenuate their transcriptional function. Upon perception of JA signal, degradation of JAZ3 by the SCF^{COI1} complex releases YABs to activate a subset of JA-regulated genes in leaves leading to anthocyanin accumulation, chlorophyll loss, and reduced bacterial defense.

INTRODUCTION

A characteristic feature of plants is their pronounced developmental plasticity in response to environmental fluctuations. To achieve this adaptability, plants rely on signaling molecules or phytohormones to properly balance their growth and development with appropriate responses to environmental cues.

Jasmonates (JAs) are plant-specific oxylipin signaling molecules related to mammal prostaglandins (Browse, 2009). The active form of JA hormone, (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al., 2009b), is perceived through a coreceptor complex formed by the F-box protein CORONATINE-INSENSITIVE1 (COI1) and JASMONATE ZIM DOMAIN (JAZ) proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Sheard et al., 2010). JAZ coreceptors negatively regulate the JA-Ile-signaling pathway by directly interacting with and repressing transcription factors (TFs) that control downstream JA responses, including fertility, root growth, senescence, secondary metabolite accumulation, and defense (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Sheard et al., 2010; Fernández-Calvo et al., 2011; Wasternack and Hause, 2013). Upon JA-Ile perception, JAZ repressors are ubiquitinated by the SCF^{COI1} and targeted for degradation by the 26S proteasome, releasing downstream TFs and thus activating JA responses.

JAZ proteins are characterized by three sequence motifs, namely, a relatively conserved N-terminal motif and the two highly conserved ZIM (central) and Jas (C-terminal) domains (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Fonseca et al., 2009a; Pauwels and Goossens, 2011; Moreno et al., 2013). The ZIM domain is known to mediate homo- and heterodimerization between JAZ proteins and to exert the repressor function of JAZs, which is achieved by the recruitment of the general transcriptional corepressor TPL through the interaction with the NINJA adaptor protein (Pauwels et al., 2010). The C-terminal Jas motif mediates interaction with COI1 and TFs (Chini et al., 2007; Melotto et al., 2008a; Sheard et al., 2010). For a long time, MYC2 was the only transcription factor known to interact directly with JAZ proteins and to regulate early JA-Ile-responsive genes. However, more recently, an array of JAZ targets responsible for different JA-Ile-mediated responses have been identified (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Hu et al., 2013; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Schweizer et al., 2013; Song et al., 2013; Fonseca et al., 2014).

The YABBY (YAB) family of transcription factors regulates various aspects of vegetative and floral development in flowering plants. First identified in *Arabidopsis thaliana*, YABs have an N-terminal zinc finger domain and a C-terminal YAB domain containing a helix-loop-helix motif similar to that found in the high mobility group (HMG) of proteins (Sawa et al., 1999; Siegfried et al., 1999). The zinc finger domain mediates homo- and heterodimerization between the YABs, as well as interactions with other proteins. By contrast, the

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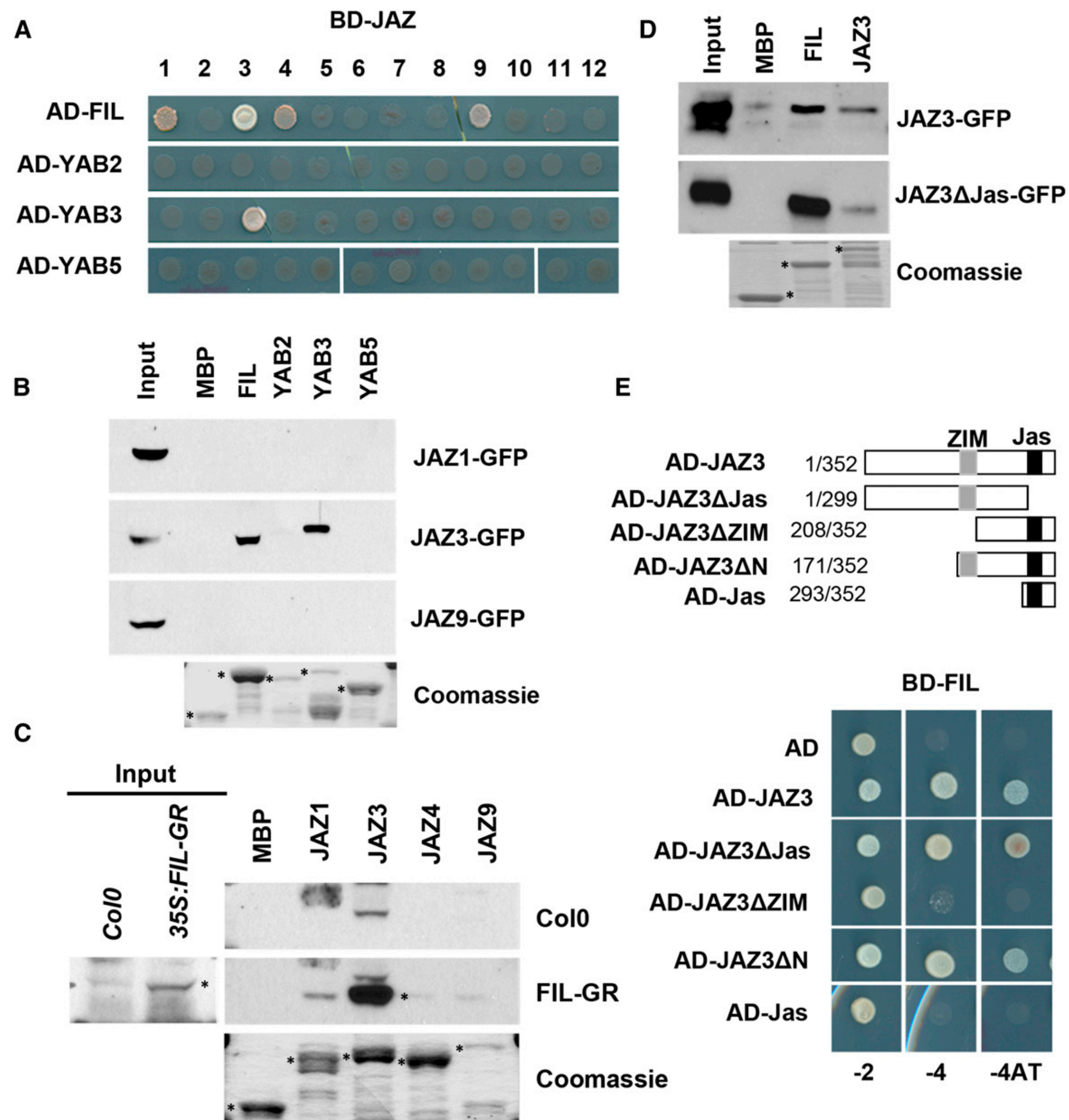

Figure 1. JAZ3 Physically Interacts with FIL and YAB3.

(A) Y2H assay using all 12 JAZ proteins fused to Gal4 BD (BD-JAZ) and the four AD-YAB fusions. Yeast cells cotransformed with the indicated combinations were selected in medium lacking Leu and Trp (-2) (Supplemental Figure 1A) and grown for 5 d in medium lacking Ade, His, Leu, and Trp without (-4) or with 3-aminotriazole (-4AT). Numbers on top of the panel represent JAZ proteins, from JAZ1 (1) to JAZ12 (12).

(B) Immunoblot using anti-GFP antibody of protein extracts from transgenic plants expressing JAZ1-GFP, JAZ3-GFP, JAZ4-GFP, and JAZ9-GFP proteins after PD by recombinant MBP-FIL, MBP-YAB2, MBP-YAB3, and MBP-YAB5 expressed in *E. coli*. MBP protein was used as negative control. A Coomassie blue-stained gel is shown below the immunoblot as representative of the amount of recombinant MBP-fused protein used in each sample. The first lane of the blot corresponds to 40 μL of the input protein extract used for PD. Asterisks mark the bands corresponding to the indicated protein when several bands are present.

(C) Immunoblot using anti-GR antibody of protein extracts from *35S:FIL-GR* transgenic plants after PD by recombinant MBP-JAZ proteins expressed in *E. coli*. Seven days postgermination *35S:FIL-GR* seedlings were treated with 20 μM DEX for 2 d to induce FIL-GR accumulation. MBP protein was used as negative control. *Col-0* protein extracts were used to discard unspecific antibody cross reactions. A Coomassie blue-stained gel is shown below the immunoblot as representative of the amount of recombinant MBP-fused proteins used in each sample. Forty microliters of the protein extracts used for PD is shown as input. Asterisks mark the band corresponding to the indicated protein when several bands are present.

(D) Immunoblot using anti-GFP antibody of protein extracts from *35S:JAZ3-GFP* (top panel) and *35S:JAZ3ΔJas-GFP* (bottom panel) transgenic plants pulled down by recombinant MBP-FIL proteins expressed in *E. coli*. MBP protein was used as a negative control and MBP-JAZ3 was used as a positive interaction control. The lowest panel corresponds to a Coomassie blue-stained gel showing the amount of recombinant MBP-fused protein used in each

YAB domain is associated with DNA binding (Kanaya et al., 2002; Stahle et al., 2009; Shamimuzzaman and Vodkin, 2013; Franco-Zorrilla et al., 2014). Phylogenetic analysis distinguishes five subfamilies of YABs in the angiosperms, represented by the FILAMENTOUS FLOWER (FIL)/YAB1, YAB3, YAB2, YAB5, CRABS CLAW, and INNER NO OUTER clades (Bowman, 2000; Yamada et al., 2004; Lee et al., 2005). In eudicots such as Arabidopsis, the so-called vegetative YABs, FIL, YAB2, YAB3, and YAB5, are expressed in the abaxial domains of developing leaf and floral organ primordia (Siegfried et al., 1999; Sarojam et al., 2010). YABs fit into a highly complex and redundant network of TFs and small RNAs that promote organ polarity. The precise position of YABs within these networks is not certain, but based on YAB loss-of-function phenotypes, it has been proposed that YABs integrate adaxial-abaxial patterning with a program of lamina growth (Sarojam et al., 2010). In addition to promoting lamina growth, vegetative YABBYs also prevent shoot apical meristem, such as *WUSHEL*, *CLAVATA3*, and *KNOX* factors, from being expressed in the developing leaf primordia (Kumaran et al., 2002; Stahle et al., 2009; Sarojam et al., 2010).

Recent studies have begun to address how YABs function at the molecular level. Interactions between the vegetative YABs and the corepressors LEUNIG (LUG) and the closely related LEUNIG_HOMOLOG (LUH) suggest that YABs act in repression complexes (Stahle et al., 2009). However, on the basis of transcriptomics analysis, a more recent study has proposed that YABs are likely to be bifunctional, acting either as activators or, when associated with LUG and LUH, as repressors (Bonaccorso et al., 2012).

Previous studies showed that JA can induce anthocyanin accumulation (Franceschi and Grimes, 1991; Feys et al., 1994; Shan et al., 2009). In this study, we demonstrate that YAB TFs are required for JA-dependent anthocyanin accumulation through direct activation of *MYB75* (*PAP1*). JAZ3 directly interacts with YAB transcription factors, attenuating their transactivation function and consequently repressing anthocyanin biosynthesis. We also show that YABs are involved in other JA-mediated responses such as chlorophyll loss and susceptibility to *Pseudomonas syringae* DC3000 infection. We propose that, upon degradation of JAZ proteins in response to JA-Ile, YABs are released to activate downstream signal cascades leading to different JA responses that take place in specific tissues of Arabidopsis plants.

RESULTS

JAZ3 Physically Interacts with FIL and YAB3

JAZ proteins serve as signaling hubs mediating the crosstalk of JAs with multiple pathways involved in plant responses to biotic and abiotic stresses, plant growth, and metabolic reprogramming (Pauwels and Goossens, 2011; Kazan and Manners, 2012). To determine how the JAZ proteins regulate diverse genetic pathways, we used JAZ3 as bait to identify interacting proteins. Screening an Arabidopsis cDNA library in the GAL4 yeast two-hybrid (Y2H) system identified the TF FIL/YAB1, confirming previously published results by the Arabidopsis Interactome Mapping Consortium (Arabidopsis Interactome Mapping Consortium, 2011).

FIL/YAB1 is a member of a small family of TFs that includes YAB2, YAB3, and YAB5. Therefore, we tested the interaction between all 12 JAZ proteins and the four FIL/YABs in Y2H assays. As shown in Figure 1A, BD-JAZ3 interacted with AD-FIL and AD-YAB3. However, the interaction with YAB3 was weaker than that with FIL and only occurred under standard assay conditions, but not under more stringent conditions (5 mM 3-aminotriazole; Supplemental Figures 1A and 1B). Besides JAZ3, FIL also showed weaker interactions with JAZ1, JAZ4, and JAZ9 in the Y2H assays, which were not reproducible in the presence of 3-aminotriazole (Supplemental Figure 1A).

These interactions were confirmed using in vitro pull-down (PD) assays. We expressed maltose binding protein (MBP) fused to FIL, YAB2, YAB3, or YAB5 in *Escherichia coli* and purified the proteins with amylose resins. Each activated resin was incubated with protein extracts from transgenic plants expressing JAZ1, JAZ3, or JAZ9 fused to GFP (Chini et al., 2007) and separated by SDS-PAGE before immunoblotting with anti-GFP antibody (Figure 1B). Similarly, MBP-fused JAZ1, JAZ3, JAZ4, and JAZ9 were incubated with 35S:FIL-GR protein extracts induced with dexamethasone (DEX) and pulled-down proteins identified by immunoblotting with anti-GR antibody (Figure 1B). As shown in Figures 1B and 1C, only JAZ3 was able to interact with FIL in both assays. The interaction of JAZ3 with YAB3 was also confirmed (Figure 1B), and JAZ1 showed only a marginal interaction with FIL. Therefore, we concluded that only JAZ3 shows a reliable interaction with FIL and YAB3 in both Y2H and PD assays.

The C-terminal Jas domain of JAZ proteins is responsible for the interaction with COI1 and all JAZ-interacting TFs identified to date (Lorenzo et al., 2004; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Pauwels and Goossens, 2011; Qi et al., 2011; Song et al., 2011, 2013; Zhu et al., 2011; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Fonseca et al., 2014). Therefore, we examined whether a truncated version of JAZ3 lacking the Jas domain (JAZ3ΔJas) was still capable of interacting with FIL in PD assays. Remarkably, JAZ3ΔJas retained its full ability to interact with FIL (Figure 1D), indicating that FIL does not require the Jas domain for interaction, in contrast to all JAZ targets described so far.

Figure 1. (continued).

sample. The first lane of the blot correspond to 40 μL of the input protein extract used for PD. Asterisk marks the band corresponding to the indicated protein when several bands appear in the picture.

(E) Mapping the FIL binding domain of JAZ3. Y2H assays using FIL fused to the GAL4 Binding Domain (BD-FIL) and full-length JAZ3 or truncated derivatives fused to the GAL4 activation domain (AD-JAZ3). Numbers represent amino acid positions relative to the full-length protein. Yeast cells cotransformed with the indicated combinations were selected in medium lacking Leu and Trp (-2) and grown for 5 d in medium lacking Ade, His, Leu, and Trp without or with 5 mM 3-aminotriazole (-4, -4AT) to select for interactions. AD represents the empty pGADT7 vector.

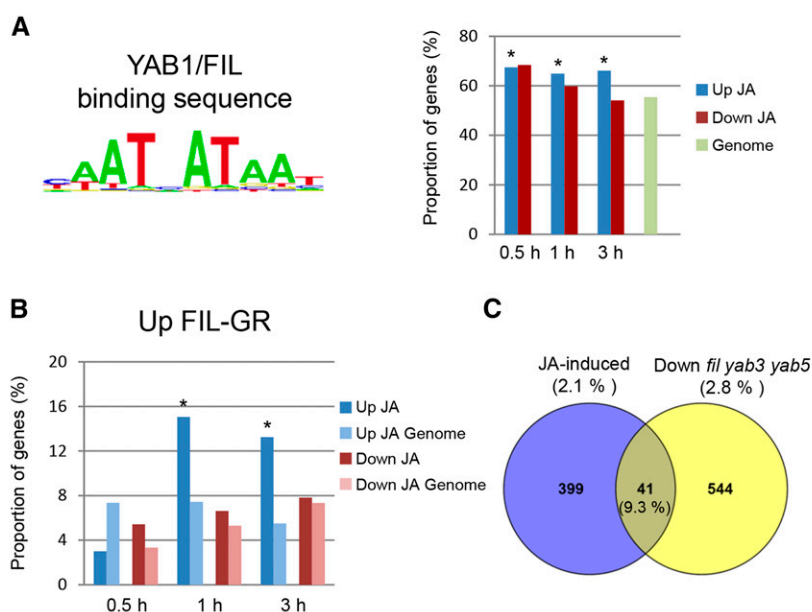

Figure 2. YAB Binding Sites Are Enriched among JA-Inducible Promoters.

(A) Left: DNA binding motif of YAB1, as described by Franco-Zorrilla et al. (2014). Right: Percentages of JA-inducible genes (0.5-, 1-, and 3-h treatments), whose promoters (1 kb upstream) contain YAB1 binding sites. Blue bars represent the percentage of JA upregulated genes (Up JA) and red bars the percentage of JA downregulated genes (Down JA) at each time point. The green bar (Genome) represents the percentage of promoters in the complete gene set represented in the array that contain the same YAB binding sites. Asterisks indicate statistically significant differences (P value < 0.01, hypergeometric test) between the indicated percentages and the corresponding genome set.

(B) Enrichment of JA-regulated genes in a transcriptomic data set corresponding to genes upregulated in response to the activation of the FIL-GR translational fusion with dexamethasone (8 h postactivation). Asterisks indicate significant differences (P value < 0.01, hypergeometric test) between the percentage of genes up- or downregulated by JA (Up by JA or Down by JA) in the FIL-GR data set compared with the proportion of JA-induced or JA-repressed genes in the whole genome (Up by JA in Genome or Down by JA in the Genome). Gene lists corresponding to FIL-GR were obtained from Bonaccorso et al. (2012).

(C) Venn diagram representing the overlap between genes upregulated 3 h after JA treatment and genes downregulated in triple *fil yab3 yab5* mutants (Sarojam et al., 2010). The proportion of genes differentially expressed in each data set relative to the complete gene set is indicated (Up JA, 2.1%; Down triple, 2.8%), as well as the proportion of overlapping genes (9.3%; random expected percentage: 2.1% $\times$ 2.8% = 0.06%).

These results were confirmed in Y2H assays using BD-FIL and truncated versions of JAZ3 fused to the AD of GAL4 (Figure 1E). Fragments JAZ3ΔJas and JAZ3ΔN interacted with FIL, whereas JAZ3ΔZIM and Jas fragments did not. Taken together, the PD and Y2H results using fragments of JAZ3 indicate that, in contrast to all previous JAZ targets, the FIL-JAZ3 interaction is not mediated by the Jas domain, but most likely involves the ZIM domain.

YAB Binding Sites Are Enriched among JA-Inducible Promoters

We recently described the DNA binding specificities of the main families/subfamilies of plant transcription factors, including YAB1 and YAB5 (Franco-Zorrilla et al., 2014). The identification of YAB binding sequences in the promoter regions of particular subsets of genes would help in the identification of targets of these TFs in the biological process under study. To evaluate the biological relevance of YABs in the regulation of JA responses, we studied whether YAB DNA binding sequences were present in the promoters of JA-regulated genes.

We first focused on early transcriptional responses to JA obtained from publicly available transcriptomic data (Goda et al., 2008). We identified genes up- and downregulated by JA at three time points (0.5, 1, and 3 h after JA treatment) under stringent criteria (fold change >2 or ≤2 and false discovery rate < 0.05) and scanned their promoter regions for FIL/YAB1 DNA binding sequences. We observed significant overrepresentation of FIL binding elements in the promoters of genes upregulated in response to JA at any time point, compared with the representation of FIL binding sites in the entire genome (Figure 2A). Slight, but not significant, overrepresentation was observed in the promoters of downregulated genes. These data point to a role of FIL/YAB1 in the transcriptional activation in response to JA.

As a complementary analysis, we studied the involvement of FIL in JA-related gene expression using genotypes affected in FIL activity. In particular, we analyzed publicly available transcriptomic data derived from transgenic plants overexpressing a translational fusion of FIL to the ligand binding domain of the rat glucocorticoid receptor (*35S:FIL-GR*), used to identify direct targets of FIL (Bonaccorso et al., 2012). We selected genes upregulated by DEX in this genotype (using the same criteria as above) and evaluated in silico their regulation by JA using the BAR Expression Browser tool (Toufighi et al., 2005). We observed a significant enrichment (P < 0.01) of early JA-inducible genes

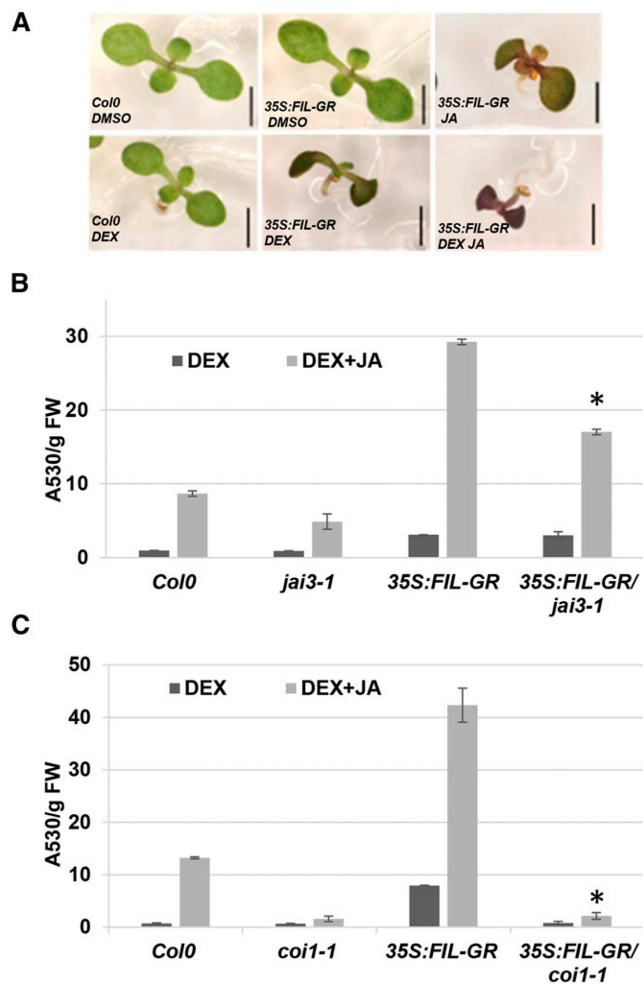

Figure 3. Ectopic Expression of FIL Results in Increased Anthocyanin Levels.

(A) Anthocyanin content of 7-d-old *35S:FIL-GR* transgenic Arabidopsis seedlings grown on MS medium supplemented with 20 μM DEX plus/minus 50 μM JA. Ethanol was the solvent used for JA stock and was added in control treatments. DMSO is the solvent used for DEX stock and was added in control treatment. Bar = 2 mm.

(B) Anthocyanin content of 10-d-old *35S:FIL-GR* transgenic Arabidopsis seedlings either in *Col-0* or mutant *jai3-1* backgrounds. Seedlings were grown on Johnson medium for 5 d and then supplemented with 20 μM DEX plus/minus 50 μM JA for five more days. Error bars represent SD. Asterisks indicate statistically significant differences between *35S:FIL-GR* in the *jai3-1* background compared with *35S:FIL-GR* in the presence of JA (P value < 0.01, *t* test). FW, fresh weight.

(C) Anthocyanin content of 10-d-old *35S:FIL-GR* transgenic Arabidopsis seedlings either in *Col-0* or mutant *coi1-1* backgrounds presented as in **(B)**. Arabidopsis *jai3-1* and *coi1-1* JA-insensitive mutants were used as controls. Error bars represent SD. Asterisks indicate statistically significant differences between *35S:FIL-GR* in the *coi1-1* background compared with *35S:FIL-GR* in the *Col-0* background in the presence of JA (P value < 0.01, *t* test).

(1 and 3 h) in the data sets corresponding to genes positively regulated upon translational activation of FIL, compared with the representation of JA-induced genes in the entire genome (Figure 2B; Supplemental Data Set 1). These data point again toward a positive role of FIL in the activation of JA-related responses. Finally, using data sets from a *fil yab3 yab5* triple loss-of-function mutant (Sarojam et al., 2010), we observed a large overlap between JA-induced genes and those downregulated in the *fil yab3 yab5* mutant (9.3% genes overlapping, 0.06% expected by chance; Figure 2C; Supplemental Data Set 1).

Taken together, these data point to a role for YAB transcription factors in the transcriptional activation of JA-related responses.

Ectopic Expression of *FIL* Results in Increased Anthocyanin Levels in Response to JA

Ectopic expression of *FIL* in Arabidopsis produces small downwardly curling (epinastic) cotyledons and leaves that are either dark green or reddish, likely due to the accumulation of anthocyanins (Siegfried et al., 1999; Bonaccorso et al., 2012; Zhang et al., 2013).

To test whether *FIL* has a role in JA-mediated anthocyanin accumulation, we analyzed anthocyanin levels in transgenic Arabidopsis lines expressing *FIL* fused to the ligand binding domain of the rat glucocorticoid receptor (*35S:FIL-GR*; Bonaccorso et al., 2012). In the absence of the synthetic glucocorticoid DEX, these lines were phenotypically normal, but when treated with DEX, they showed elevated levels of anthocyanins and produced epinastic leaves resembling JA-treated seedlings (Figure 3A; Supplemental Figure 2). Interestingly, application of DEX together with JA produced more extreme phenotypes compared with DEX or JA treatment alone, resulting in smaller dark-purple seedlings (Figure 3A). Quantification of anthocyanin content of treated *35S:FIL-GR* lines confirmed that the reddish phenotype is due to a significant increase of anthocyanin levels upon DEX treatment and that simultaneous application of DEX and JA (or its mimic COR) produced a 6- to 9-fold increase in anthocyanin levels, compared with DEX treatment (Figures 3B and 3C; Supplemental Figure 2). These results point to FIL functioning as a transcriptional activator in JA-dependent anthocyanin accumulation.

Based on the Y2H and PD assays described above, it is likely that YAB activity is regulated through interactions with the JAZ3 repressor. To confirm this possibility, we investigated whether defects in JA signaling suppress anthocyanin accumulation in *35S:FIL-GR* lines following exposure to both DEX and JA. Accordingly, we found that the dominant-negative *jai3-1* mutation caused a significant reduction in anthocyanin levels (Figure 3B), whereas anthocyanin accumulation was completely abolished in the *coi1-1* mutant background, where JAZ repressors are stabilized (Figure 3C). These findings further support FIL being an activator of JA-induced anthocyanin accumulation and point to the existence of a FIL-GR-JAZ3 repressed complex in planta.

YABs Are Required for JA-Mediated Responses in Aerial Tissues

To establish whether *FIL* and other *YAB*s are required for activation of the JA pathway, we analyzed typical JA-mediated phenotypes, such as anthocyanin accumulation, inhibition of chlorophyll production, and hypocotyl and root growth inhibition, in different *yab* mutant backgrounds. As shown in Figure 4A, *fil yab3* double mutants accumulate lower levels of anthocyanin than the wild type when grown in the presence of 50 μM JA, whereas JA-induced

anthocyanin accumulation was almost completely suppressed in quadruple *fil yab2 yab3 yab5* mutants. These results suggest that YAB2, YAB3, and YAB5 function redundantly with FIL in mediating JA-induced anthocyanin production with YAB3 and FIL being the major players in this response.

Analysis of chlorophyll content revealed that both *fil yab3* double and *fil yab2 yab3 yab5* quadruple mutants were as insensitive to JA-mediated inhibition of chlorophyll biosynthesis as the JA-insensitive *coi1-16* and *jai3-1* mutants (Figure 4B). Moreover, conditional overexpression of *FIL* led to a higher chlorophyll reduction upon DEX/JA treatment compared with *Col-0* control (Figure 4C) and closely paralleled the reduction seen in the JA-hypersensitive *35S:MYC2* lines. These results suggest that YAB TFs are also required for JA signaling leading to the inhibition of chlorophyll levels.

It has been established recently that JA plays an important role in hypocotyl elongation under different light conditions (Robson et al., 2010; Chen et al., 2013; Kim et al., 2013). To further explore the role of YABs in JA signaling, we also assessed whether *yab* mutants were affected in JA-dependent hypocotyl length inhibition. *35S:FIL-GR* transgenic lines showed an increase in hypocotyl length upon DEX treatment, although JA treatment had the same inhibitory effect as observed in *Col-0* plants (Supplemental Figure 3A). Furthermore, hypocotyl length measurement in *fil yab3* double and *fil yab2 yab3 yab5* quadruple mutants revealed a hypocotyl growth defect in those mutants (Supplemental Figure 3B), pointing to a role of YAB TFs in hypocotyl elongation. Consistent with their lack of expression in roots, none of the *yab* mutant combinations tested were affected in their response to JA-mediated root growth inhibition (Supplemental Figure 4).

Altogether, results from these phenotypic analyses are consistent with a role of YABs as positive regulators of different aspects of the JA function in Arabidopsis seedlings.

YAB TFs Play a Role in Plant Defense Responses

Some strains of *Pseudomonas* such as *P. syringae* pv *tomato* (*Pto*) DC3000 produce Coronatine, a bacterial phytotoxin that functionally mimics JA-Ile. COR activates the JA pathway, which counteracts salicylic acid (SA)-dependent defenses against the bacteria, leading to increased plant susceptibility to bacterial infections. Thus, we examined resistance of *yab* mutant combinations and the *35S:FIL-GR* line to *Pto* DC3000. *35S:FIL-GR* transgenic plants were induced by DEX and simultaneously infected with *P. syringae* DC3000 and bacterial growth scored at 2 d postinfection. This analysis revealed that *35S:FIL-GR* plants were extremely susceptible to DC3000 infection compared with *Col-0*, with bacterial growth being two logs higher in the transgenic plants (Figure 5A). By contrast, infection of *fil/+ yab3* and *fil/+ yab2 yab3 yab5* mutants displayed reduced bacterial growth compared with *Col-0* plants with the *fil/+ yab2 yab3 yab5* mutant being similar to the JA-insensitive mutant *coi1*-1 (Figure 5B). Disease symptoms (chlorosis and necrosis) fully correlated with bacterial growth scores (Supplemental Figure 5).

FIL Is a Direct Activator of *MYB75* and Is Repressed by JAZ3

Previous work suggested that *MYB75* (*PAP1*), a key regulator of anthocyanin biosynthesis, is a direct target of FIL (Bonaccorso

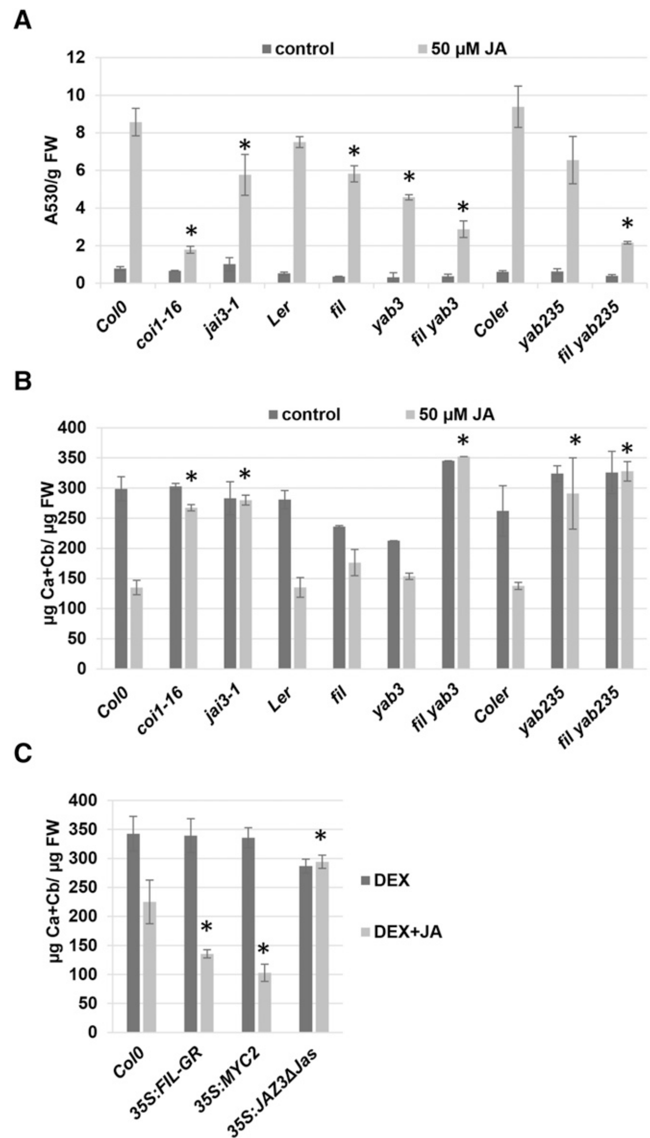

Figure 4. YABs Are Required for JA-Mediated Responses in Aerial Tissues.

(A) Anthocyanin content of 10-d-old wild-type and *yab* mutant Arabidopsis seedlings grown in Johnson medium with or without 50 µM JA. Error bars represent SD. Asterisks indicate statistical significant differences between the corresponding samples and their corresponding wild type (L*er* for *fil8 yab3* mutants or *Coler* for *fil8 yab2 yab3 yab5*) (P value < 0.01, *t* test). FW, fresh weight.
(B) Chlorophyll content of 10-d-old wild-type or *yab* mutant Arabidopsis seedlings grown in Johnson medium with or without 50 µM JA. JA-insensitive *coi1-16* and *jai3-1* mutants were used as controls. Error bars represent SD. Asterisks indicate statistically significant differences between samples and their corresponding wild type (L*er* for *fil8 yab3* mutants or *Coler* for *fil8 yab2 yab3 yab5*) (P value < 0.01, *t* test).
(C) Chlorophyll content of 10-d-old *35S:FIL-GR* transgenic Arabidopsis seedlings grown on Johnson medium with 20 µM DEX plus/minus 50 µM JA. Arabidopsis *35S:MYC2* and *35S:JAZ3*Δ*Jas* were used as JA-hypersensitive and -hyposensitive controls, respectively. FW, fresh weight. Error bars represent SD. Asterisks indicate statistically significant differences between the corresponding samples and *Col-0* (P value < 0.01, *t* test).

et al., 2012). Given this, we reasoned that the enhanced anthocyanin accumulation seen in *35S:FIL-GR* lines exposed to DEX and JA is likely due to increased *MYB75* expression. To confirm this, we introduced the *35S:FIL-GR* transgene into the *myb75-1* mutant background as well as the corresponding wild-type background (*Nossen* [*Nos*]). Exposing *35S:FIL-GR myb75-1* lines to DEX or DEX/JA failed to produce anthocyanin accumulation (Figures 6A and 6B). While this observation is consistent with JA enhancing the activity of FIL (and, therefore, expression of *MYB75*), it is also possible that JA enhances MYB75 activity through JAZ degradation (Qi et al., 2011). Thus, to distinguish between these possibilities, we compared *MYB75* expression in DEX- or JA-treated *35S:FIL-GR* plants to those receiving a combined DEX/JA treatment. RT-qPCR analysis showed that there was an ~5-fold increase in *MYB75* expression, suggesting that JA does indeed enhance the activity of FIL, which in turn activates *MYB75* expression (Figure 6C).

We next examined the regulation of a *MYB75* reporter by FIL and JAZ3 in transient expression assays in *Nicotiana benthamiana* leaves. For the reporter construct, we placed the *MYB75* promoter region (1.18 kb; Supplemental Figure 6A) upstream of luciferase and used the *35S:FIL* and *35S:JAZ3ΔJas* as effector constructs (Figure 7A). Coexpression of *pMYB75:Luc* with *35S:FIL* resulted in an increase of LUC activity, consistent with FIL functioning as a direct activator of the *MYB75* promoter (Figure 7B). In our transient assay, the *MYB75* promoter was highly responsive to COR, most likely through recognition of the endogenous *N. benthamiana* COI1 receptor (Figure 7C). By contrast, coexpression of *pMYB75:Luc* with the *35S:JAZ3ΔJas* construct reduced reporter activity even when cells were exposed to COR, indicating that JAZ3ΔJas represses *MYB75* expression (Figures 7B and 7C). Moreover, coexpression of the reporter with a combination of both *35S:FIL* and *35S:JAZ3ΔJas* led to a reduction in the transactivation capacity of FIL both in basal conditions and in the presence of COR. In summary, these results support the idea that FIL is a direct transcriptional activator of *MYB75* and that JA enhances FIL activity most likely through the degradation of JAZ3 protein that is associated with FIL.

Scanning of the *MYB75* promoter region revealed several putative FIL DNA binding elements (FBEs) according to Franco-Zorrilla et al. (2014) (Supplemental Figure 6A). To test whether FIL directly binds to the *MYB75* promoter, we performed electrophoretic mobility shift assays (EMSAs) using MBP-FIL protein purified from *E. coli* and a 50-bp DNA fragment covering the FBE sequence closer to the transcriptional start site (FBE1; Supplemental Figure 6). Unlabeled FBE1 was used as specific competitor of the binding, whereas a 50-bp probe containing a G-box present in the *MYB75* promoter was used as unspecific competitor. As shown in Figure 7D, MBP-FIL was able to specifically bind to the probe containing the FBE1. A retarded band appeared only when MBP-FIL was used, but not when using a different MBP-fused protein (MBP-JAZ3). This binding was competed by the specific cold FBE1 probe, but not by the unspecific G-box probe. A lower band (higher mobility) also appeared when using MBP-FIL. However, this band was not competed by the cold probe and therefore may correspond to a truncated FIL fragment with loose specificity.

The assays described above strongly support that *MYB75* is a direct target of the FIL TF. If this is the case, we hypothesized that

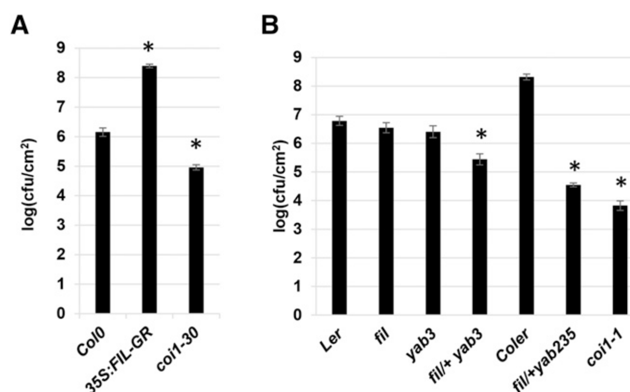

Figure 5. YABs Participate in Plant Defense Responses against *P. syringae* Infection.

(A) Growth of *Pto* DC3000 on wild-type Arabidopsis *Col-0*, *35S:FIL-GR*, and *coi1-30* plants 2 d after spray inoculation. Plants were pretreated with 20 μM DEX for 16 h prior to infection. Asterisks indicate statistically significant differences with the control *Col-0* (P value < 0.01, *t* test).

(B) Growth of *Pto* DC3000 on wild-type Arabidopsis and different *yab* mutant backgrounds. Bacterial counts are expressed as log (colony-forming units [cfu]/cm^2). Error bars indicate SE. The results are representative of two independent experiments. *fil/+ yab3* and *fil/+ yab235* are heterozygous for the *fil8* allele and were used instead *of fil yab3* and *fil yab235* homozygous plants because the extreme phenotype of these plants made it challenging to perform the pathotest assays. Asterisks indicate statistical significant differences with their corresponding control L*er* or *Coler* (P value < 0.01, *t* test).

the lack of anthocyanin accumulation observed in the *yab* mutants could be restored by constitutively expressing *MYB75/PAP1* in the mutant background. Accordingly, we found that introducing the dominant *pap1-D* mutation (activation-tagging line overexpressing MYB75/PAP1; Borevitz et al., 2000) into the *fil8 yab3* mutant increased anthocyanin accumulation even in the absence of JA (Figure 7E). This result further supports that FIL/YAB3 are direct activators of *MYB75/PAP1* expression; therefore, the defect in anthocyanin accumulation in *fil8 yab3* is due to the lack of *MYB75/PAP1* expression in this mutant background. In the presence of JA, *pap1-D* completely restored JA-dependent anthocyanin accumulation in *fil8 yab3*. This finding is consistent with the previously reported regulation of MYB75 by JAZ1 (Qi et al., 2011). Therefore, these results suggest that regulation of MYB75 activity depends both on activation of *MYB75* expression by FIL/YAB3 and derepression of MYB75 by JA-mediated degradation of JAZ1.

DISCUSSION

In the absence of JA-Ile, JAZ proteins bind to and repress the activity of transcription factors that promote JA responses in plants (Katsir et al., 2008; Chung et al., 2009; Fonseca et al., 2009a). Thus, to better understand the pathways regulated by JA, it is important to identify TFs that are bound by JAZ proteins. While previous studies have identified numerous TFs belonging to the bHLH, MYB, and EIN3/EIL families (Gimenez-Ibanez and Solano,

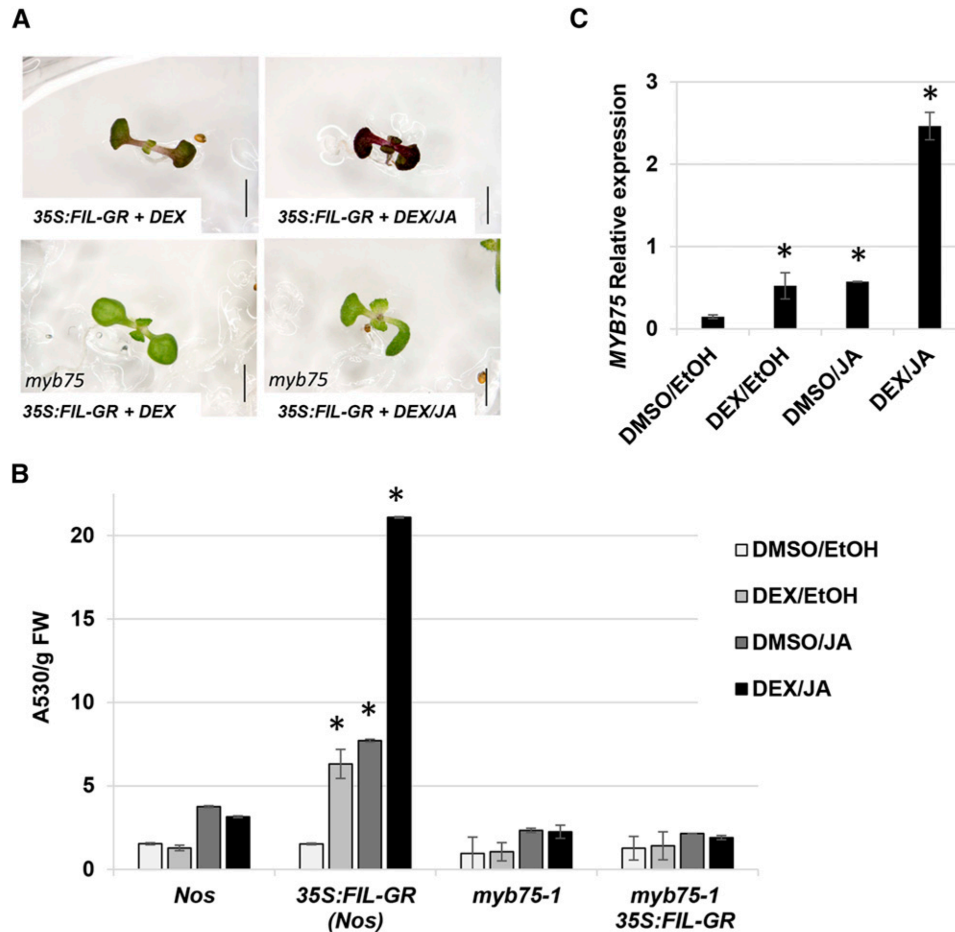

Figure 6. Anthocyanin Accumulation in *35S:FIL-GR* Is Dependent on *MYB75*.

(A) Eight-day-old *35S:FIL-GR* transgenic Arabidopsis seedlings either in wild-type (*Nos*) or *myb75-1* backgrounds grown on MS medium supplemented with 20 or 20 μM DEX plus 50 μM JA. Bars = 2 mm.

(B) Anthocyanin content of 8-d-old *35S:FIL-GR* transgenic Arabidopsis seedlings either in wild-type (*Nos*) or *myb75-1* backgrounds. Plants were grown for 8 d in MS medium supplemented with 20 μM DEX plus/minus 50 μM JA. DMSO and ethanol were used as solvents for DEX and JA, respectively, and added in control treatments. FW, fresh weight. Error bars represent SE. Asterisks indicate statistically significant differences between the corresponding samples and their reference wild type (*Nos*) (P value < 0.01, *t* test).

(C) RT-qPCR analysis of *MYB75* expression relative to *ACTIN* in 10-d-old *35S:FIL-GR* seedlings following a 4-h exposure to a mock (DMSO/EtOH), DEX (DEX/EtOH), JA (DMSO/JA), or both DEX and JA (DEX/JA) treatment. Asterisks indicate statistically significant differences between the corresponding samples and their control (DMSO/EtOH) (P value < 0.01, *t* test).

2013), this study identifies the YABs as a novel class of JAZ-interacting TFs. With the exception of DELLA repressor proteins, which interact through the JAZ N-terminal domain (Hou et al., 2010; Yang et al., 2012), most TFs interact with the JAZ proteins through their C-terminal domain. Our domain mapping assays suggest that YABs may be atypical in that they interact with the JAZ proteins via their N-terminal domain, although the precise amino acids involved in this interaction have not yet been mapped. This finding was further confirmed in our transactivation assays, where the responsiveness of the *MYB75* promoter:reporter to FIL activity was reduced in the presence of a JAZ protein lacking the C-terminal domain (JAZ3ΔJAS; Figure 7). Interestingly, repression still occurs even in the presence of the JA analog COR. As JAZΔJAS is not degraded by JA/COR application, it keeps repressing FIL activity even in the presence of JA/COR.

Our analysis implicates YABs in a variety of JA-regulated processes, including anthocyanin accumulation, chlorophyll degradation, and plant-pathogen interactions. Consistent with YABs regulating JA responses, we found the recently identified FIL DNA binding motif (Franco-Zorrilla et al., 2014) overrepresented in the promoters of JA-regulated genes, particularly in genes induced after 3 h of treatment with JA (Figure 2A). Although it has previously been proposed that YABs function in repressive complexes (Stahle et al., 2009), more recent work has suggested that YABs are bifunctional and thus capable of activating downstream targets (Bonaccorso et al., 2012). Moreover, the observed enrichment of

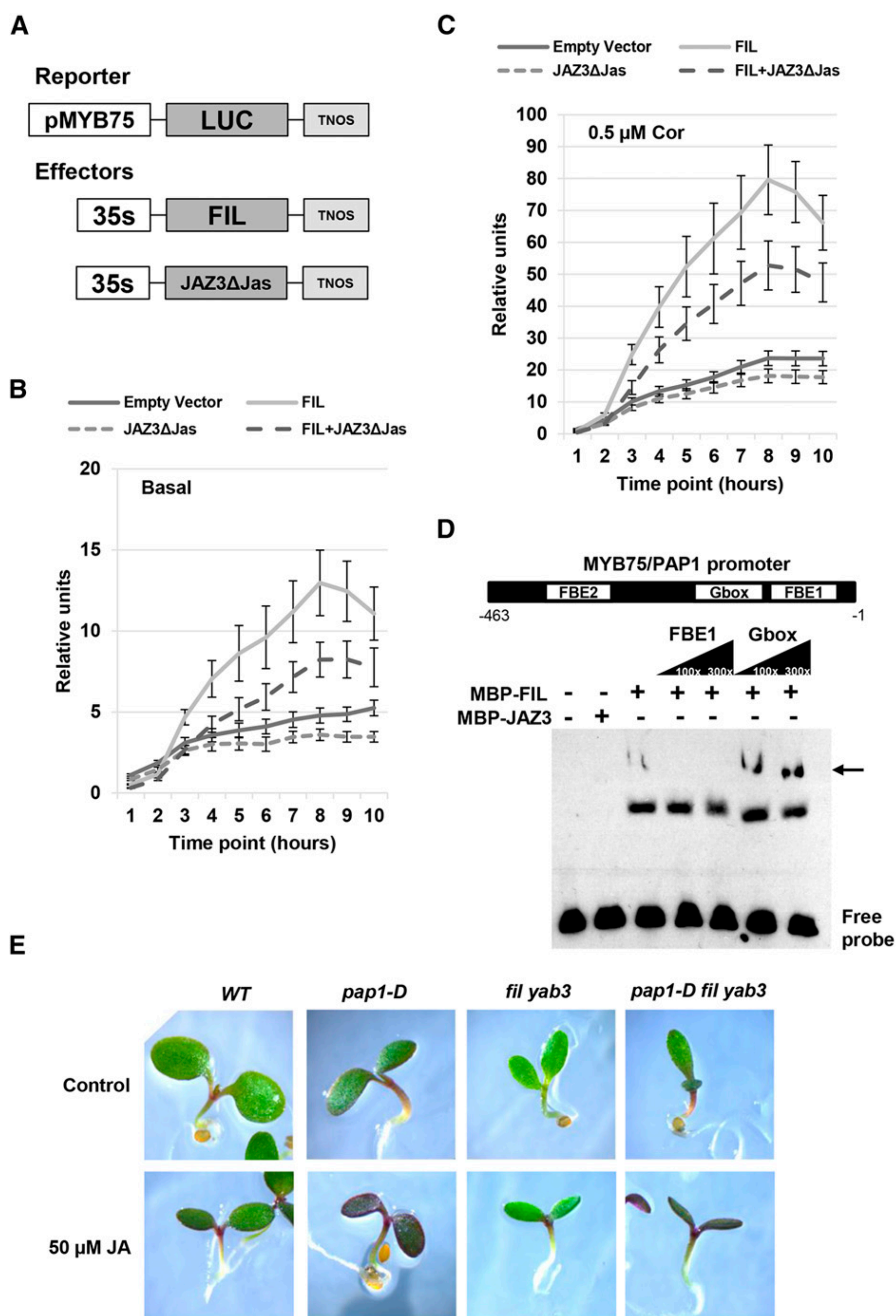

Figure 7. FIL Is a Transcriptional Activator of MYB75 and JAZ3 Is Able to Repress Its Activity.

(A) Schematic representation of the constructs used in transient expression experiments in *N. benthamiana*. The *MYB75* promoter fused to the firefly *LUC* gene was used as a reporter. *FIL* and *JAZ3ΔJas* genes expressed under the CaMV *35S* promoter were used as effectors.

(B) Transactivation assay using *35S:FIL* and *35S:JAZ3ΔJas* as effectors in basal conditions (no treatment). Error bars represent SD of 12 replicates.

(C) Transactivation assay using *35S:FIL* and *35S:JAZ3ΔJas* as effectors in inductive (0.5 μM COR) conditions. Error bars represent SD of 12 replicates.

(D) EMSA experiment showing that MBP-FIL fusion protein binds to the DNA probe of the *MYB757PAP1* promoter containing the putative FIL binding element 1 (FBE1). Biotin-labeled FBE1 probe was incubated with MBP-FIL protein, and the free and bound probe were separated in an acrylamide gel. As indicated, unlabeled probes (FBE1 and G-box) were used as specific and unspecific competitors, respectively. The MBP-FIL-DNA complex is indicated with an arrow. The lower retarded band (higher mobility) likely corresponds to a retarded band by a FIL truncated derivative that lacks sequence specificity.

(E) Anthocyanin content of 5-d-old *Col-0*, *pap1-D*, *fil8 yab3*, and *pap1-D fil8 yab3* Arabidopsis seedlings grown on MS medium plus/minus 50 μM JA. The wild type, *pap1-D*, *fil8 yab3*, and *pap1-D fil8 yab3* are all *Col-0* × *Ler*.

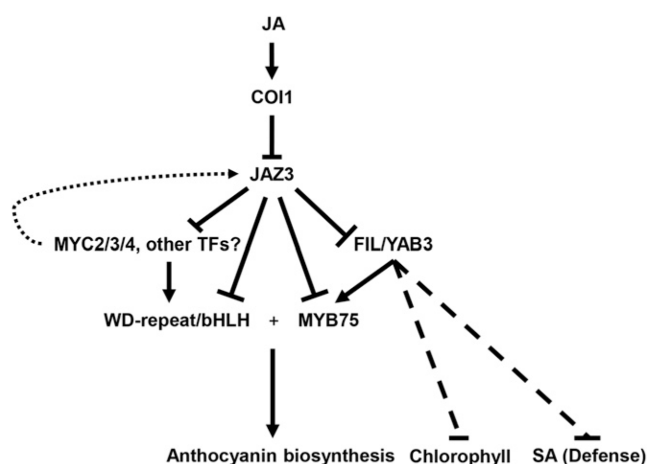

Figure 8. Model for FIL Function in JA Signaling.

JA induces the degradation of JAZ proteins via the SCF^{COI1} complex leading to the activation of FIL/YAB3 and other TFs (including MYC2, MYC3, MYC4, MYB75, and others). Activation of FIL/YAB3 results in the induction of *MYB75* leading to the synergistic formation of active WD-repeat/bHLH/MYB75 complexes and the accumulation of anthocyanin. Moreover, JA-dependent activation of FIL/YAB3 also leads to a chlorophyll breakdown and antagonism of the SA-mediated defense response, most likely through indirect mechanisms (broken arrows). A negative feedback loop arises from JA-induced activation of JAZ expression (dotted arrow). Solid arrows indicate direct physical interactions/regulation.

JA-regulated genes in data sets containing genes that are positively regulated by FIL provides further support for the conclusion that FIL promotes JA responses through transcriptional activation (Figures 2B and 2C; Supplemental Data Set 1). This is also consistent with the binding of FIL to the FBE1 in the *MYB75* promoter and the requirement of FIL for *MYB75* expression.

Although many JA-responsive genes have FIL DNA binding motifs in their promoters, it is clear from our analysis that only a small fraction of these genes are actually elevated following conditional overexpression of FIL in the absence of JA (Supplemental Data Set 1). This can be explained by the formation of FIL-JAZ3 protein complexes, which presumably bind to and inactivate a significant proportion of ectopically expressed FIL protein in the absence of JA, which is consistent with the hypothesis that JAZ3 represses FIL activity. In addition to FIL, our yeast and PD assays showed that YAB3 is also capable of interacting with the JAZ3 protein, suggesting that YAB-JAZ3 interactions are extensive. The JA response defects of *fil yab3* double mutants are similar to those observed in the *fil yab2 yab3 yab5* quadruple mutant (Figure 4), suggesting that FIL and YAB3 are the major players and act redundantly in JA responses.

The most compelling evidence for in planta FIL-JAZ3 interaction comes from analysis of *MYB75* regulation. This gene is an integral component of the WD-repeat/bHLH/MYB complex that regulates the expression of anthocyanin biosynthetic genes (Gonzalez et al., 2008) and is rapidly upregulated following exposure to exogenously applied JA (Goda et al., 2008; Loreti et al., 2008; Shan et al., 2009; Qi et al., 2011). In addition to being JA responsive, *MYB75* is activated by DEX or DEX/cycloheximide in 35S:FIL-GR plants, demonstrating that *MYB75* is positively regulated by FIL, most likely in a direct manner (Bonaccorso et al., 2012). This presumably accounts for the dark green/reddish appearance of Arabidopsis plants engineered to express FIL at high levels, a phenotype that is also apparent in lines expressing YAB3, but less so with YAB2 and YAB5 (Siegfried et al., 1999; our unpublished data). In this study, we found clear evidence that FIL can drive higher levels of *MYB75* expression in the presence of JA or the JA analog COR, not only in planta but also in transactivation assays (Figures 6 and 7). Moreover, we found that FIL can directly bind the promoter of *MYB75*, specifically recognizing a FBE close to the transcriptional start site. While it is possible that the enhanced anthocyanin accumulation seen in transgenic lines following combined DEX/JA treatment could conceivably arise from the release of MYB75 from MYB75-JAZ complexes (Qi et al., 2011), our data indicate that it also occurs via direct transcriptional activation of *MYB75* following the release of FIL from FIL-JAZ3 complexes (Figure 7). This can be inferred from the transactivation assays, where enhanced activity of FIL presumably reflects COR-induced degradation of *N. benthamiana* JAZ proteins that are likely to associate with this protein. Finding that anthocyanin accumulation is partially or completely suppressed in the *jai3-1* and *coi1-1* mutant backgrounds provides additional support for FIL activity being modulated by JA (Figure 3). Restoration of anthocyanin production in the *fil8 yab3* mutant background by expression of *MYB75* (using the *pap1-D* mutant) also supports the role of FIL/YAB3 in the regulation of MYB75 expression. Moreover, the increase in anthocyanin accumulation in response to JA in the *fil8 yab3 pap1-D* mutant indicates that activation of MYB75 requires both induction of *MYB75* expression by FIL/YAB3 and derepression of MYB75 by degradation of JAZ1, as described by Qi et al. (2011).

The observed lack of anthocyanin accumulation in the cotyledons of *fil yab3* double and *fil yab2 yab3 yab5* quadruple mutant seedlings following exposure to JA (Figure 4A; Supplemental Figure 7) clearly shows that JA-induced anthocyanin accumulation in this tissue is dependent on YAB activity. Residual anthocyanin accumulation in the *fil yab3* double and *fil yab2 yab3 yab5* quadruple mutant seedlings is localized in hypocotyls (Supplemental Figure 7) and presumably reflects *MYB75* activity under the control of non-YAB TFs or the activity of *MYB90*, *MYB113*, and *MYB114* (Qi et al., 2011).

Given the pattern of YAB and JAZ protein accumulation, we propose that the YAB-JAZ complexes predominantly arise within the abaxial side of cotyledons and developing lateral organs (Siegfried et al., 1999; Goldshmidt et al., 2008). Under conditions of biotic or abiotic stress, JA accumulation in lateral organs triggers JAZ3 degradation, allowing FIL/YAB3 to activate *MYB75* expression and derepressing MYB75 from MYB75/JAZ1 complexes, leading to the synergistic formation of active WD-repeat/bHLH/MYB75 complexes and the accumulation of anthocyanin in these tissues (Figure 8). Like *MYB75*, other genes encoding components of the WD-repeat/bHLH/MYB complex are induced by JA (Qi et al., 2011), leading us to speculate that their activation may similarly be dependent on the activity of TFs that interact with JAZ proteins (MYC2/3/4 and others). According to this model, JAZ proteins fulfill two roles: First, they ensure that FIL/YAB3 and possibly other WD-repeat/bHLH/MYB targeting TFs are only active in the presence of JA; second, they rapidly limit activity of

the WD-repeat/bHLH/MYB complexes when JA levels begin to decline. In the latter case, this negative feedback loop arises from JA-induced activation of JAZ expression (Chini et al., 2007; Thines et al., 2007).

In addition to anthocyanin accumulation, JA also inhibits root growth, causes chlorophyll breakdown (Franceschi and Grimes, 1991; Feys et al., 1994; Shan et al., 2009), represses hypocotyl elongation (Robson et al., 2010; Chen et al., 2013; Kim et al., 2013), and inhibits SA-dependent defense responses (Gimenez-Ibañez and Solano, 2013). Not unexpectedly, JA still inhibits root growth in *yab* mutants (Supplemental Figure 4), reflecting the fact that YABs are not active in root tissue. However, altering YAB activity did have an effect on JA-mediated chlorophyll breakdown (Figure 4), suggesting that YABs may contribute to this aspect of JA activity.

In this study, we also uncovered a function for FIL/YAB3 as promoters of hypocotyl growth (Supplemental Figure 3). It has been established that JA plays an important role in hypocotyl elongation (Chen et al., 2013). However, FIL does not seem to alter JA signaling in this particular scenario, since FIL overexpressing plants are as sensitive as the wild type to JA-dependent hypocotyl growth inhibition.

YABs are traditionally thought of as being growth promoting, as their expression is associated with ectopic blade outgrowths seen in *kan1 kan2* mutants, and *fil yab3* mutant leaves are much smaller than those of the wild type (Siegfried et al., 1999; Eshed et al., 2004). However, somewhat paradoxically, plants with elevated levels of YAB activity often have small downward-curling leaves (Sawa et al., 1999; Siegfried et al., 1999). Similarly, the role of JA in restricting hypocotyl, petiole, and leaf growth (Barto and Cipollini, 2005; Yan et al., 2007; Kim et al., 2013) raises the possibility that these phenotypes, at least in leaves and petioles, might be due to increased YAB activity. Future experiments will need to assess this possibility more thoroughly.

In addition to leaf expansion, FIL also regulates leaf polarity and flower development (Chen et al., 1999; Sawa et al., 1999; Siegfried et al., 1999). However, the lack of JA perception (*coi1* mutant) does not have these phenotypic effects, suggesting that JA (through JAZ3) only regulates a set of the FIL/YAB functions.

We also found evidence for YABs being involved in the regulation of defense responses against the biotrophic pathogen *P. syringae*. Some *P. syringae* strains have evolved a sophisticated strategy for manipulating hormonal homeostasis by producing coronatine (COR), a mimic of the bioactive jasmonate hormone JA-Ile (Fonseca et al., 2009b). COR contributes to disease symptomatology by inducing chlorotic lesions (Kloek et al., 2001; Brooks et al., 2004; Uppalapati et al., 2007), facilitates entry of the bacteria into the plant host by stimulating the opening of stomata (Melotto et al., 2006, 2008b), and promotes bacterial growth by inhibiting SA-dependent defenses required for *P. syringae* resistance because of its activation of the antagonistic JA pathway (Cui et al., 2005; Laurie-Berry et al., 2006). Enhanced susceptibility to *P. syringae* DC3000 (a *P. syringae* strain producing COR) was observed following FIL induction, whereas increased resistance was observed in *fil yab3* double mutants (Figure 5; Supplemental Figure 5). Based on this, we propose that FIL induction triggers a JA-dependent inhibition of SA-dependent defense responses, whereas the loss of YAB activity enhances resistance to bacteria due to the inability of bacterially produced COR to suppress the SA-dependent defense responses of the plant.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana Col-0, Landsberg *erecta* (*Ler*), *Columbia erecta* (*Coler*), and *Nos* are the genetic backgrounds of wild-type and transgenic lines used throughout the work. Seedlings were either grown on Johnson or 0.5× Murashige and Skoog (MS) medium at 21°C under a 16-h-light/8-h-dark cycle. Knockout lines *myc2/jin1-2* and *jai3-1* have been previously described (Lorenzo et al., 2004; Chini et al., 2007), and *coi1-1* and *coi1-16* were provided by J. Turner. *pap1-D* mutant was provided by Daoxin Xie. Lines segregating for *fil-8*, *yab3-2*, *fil-8 yab3-2*, *yab2-1 yab3-2 yab5-1*, and *fil-8 yab2-1 yab3-2 yab5-1* have been described previously (Stahle et al., 2009). The *myb75*-1 mutant has been previously described (Bhargava et al., 2010). The *35S:MYC2-GFP* and 35S:JAZ3ΔC-GFP lines were previously described (Lorenzo et al., 2004; Chini et al., 2007), as was the *35S:FIL-GR* line (Bonaccorso et al., 2012).

35S:FIL-GR in *jai3-1* and *coi1-1* backgrounds were generated by crossing the corresponding parental single homozygous lines. F2 segregating progenies of these crosses were selected on 50 mg/L kanamycin for *35S:FIL-GR* and on 50 μM JA for *jai3-1* and *coi1-1* backgrounds.

Yeast Two-Hybrid Assays

Full-length FIL, YAB2, YAB3, and YAB5 coding sequences carrying a stop codon were amplified with Expand High Fidelity polymerase (Roche) using Gateway-compatible primers (Supplemental Table 1). PCR products were cloned into pDONR207 with the Gateway BP II kit (Invitrogen) and the sequence was verified. FIL, YAB2, YAB3, and YAB5 constructs were used in Gateway (Invitrogen) LR reactions, in combination with the destination yeast expression vectors pGADT7gateway (Gal4 AD) and pGBKT7gateway (Gal4 BD), and were then checked by sequencing. JAZ1 to JAZ12 construct in pGADT7gateway and pGBKT7gateway and JAZ3 truncated derivatives were previously described (Chini et al., 2007, 2009).

To assess protein interactions, the corresponding plasmids were cotransformed into *Saccharomyces cerevisiae* AH109 cells following standard heat shock protocols (Chini et al., 2009). Successfully transformed colonies were identified on yeast synthetic dropout lacking Leu and Trp. At 3 d after transformation, yeast colonies were plated in synthetic dropout lacking Ade, His, Leu, and Trp with or without 5 mM 3-aminotriazole to test protein interaction and incubated at 28°C for 2 to 4 d. Empty vectors pGADT7gateway or pGBKT7gateway were also cotransformed as negative controls.

Protein Extracts and PD Assays

MBP-FIL and MBP-YAB fusion proteins were generated by transferring pDONR207 constructs into the pDEST-TH1 vector (Hammarstrom et al., 2002; (Fonseca and Solano, 2013). Twelve-day-old Arabidopsis wild-type seedlings and lines expressing *35S:JAZ3-GFP* or *35S:JAZ3ΔJas-GFP* (Chini et al., 2007) were ground in liquid nitrogen and homogenized in extraction buffer containing 50 mM Tris-HCl, pH 7.4, 80 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 50 mM MG132 (Sigma-Aldrich), and complete protease inhibitor (Roche). After centrifugation (16,000*g* at 4°C), the supernatant was collected. For in vivo pull-down experiments, 6 mg of resin-bound MBP fusion protein was added to 1 mg of total protein extract and incubated for 1 h at 4°C with rotation. After washing, samples were denaturalized, loaded on 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with the corresponding antibody (anti-GFP-horseradish peroxidase antibody 1:1000 [Milteny Biotec] or anti-GR antibody 1:1000 [ThermoFisher]).

Analysis of Gene Expression Profiles and Identification of *cis*-Motifs

Microarray data corresponding to the JA time-course experiment (Goda et al., 2008) were downloaded from the Gene Expression Omnibus (GSE39384) and analyzed according to Godoy et al. (2011). Sequence AATNATAA, corresponding to the YAB1 binding motif identified by Franco-Zorrilla et al. (2014), was scanned at the promoter regions (1 kb upstream of the transcription start site) of JA-regulated genes using PatMatch (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) to obtain the proportions of gene promoters containing the YAB1 binding motif. Overrepresentation of promoters containing the YAB1 binding sequence was evaluated in relation to the complete set of genes represented in the ATH1 array, following a hypergeometric distribution.

Transcriptomic data sets corresponding to FIL targets (*35S:FIL-GR*) and triple *fil yab3 yab5* mutant were obtained from Bonaccorso et al. (2012) and Sarojam et al. (2010), respectively. For the exploratory analysis of FIL targets in response to JA, genes up- and downregulated upon 4 and 8 h of DEX treatment (Bonaccorso et al., 2012) were analyzed with the e-Northern tool in BAR (http://bar.utoronto.ca/). Using these data, genes with a fold change in expression in treated (JA) versus control plants of >2 or < -2 were considered as being upregulated and downregulated, respectively. Proportions of query genes up- and downregulated in response to JA were compared with the corresponding proportions of the complete set of genes represented in the ATH1 arrays and differences statistically evaluated following a hypergeometric distribution.

Anthocyanin and Chlorophyll Quantification

Seedlings were grown for 10 d in MS medium with or without 50 μM JA. The aerial part of 6 to 12 seedlings was pooled for each replicate. Three independent replicates were collected from different plates. Anthocyanin quantification was performed as described by Swain and Hillis (1959). Anthocyanin content is expressed as A530/g FW, where A530 is the absorbance at 530 nm and FW the fresh weight of the sample. Values represent mean ± SD, and Student's *t* test was applied. The experiment was repeated three times with similar results.

Six to 10 seedlings from the same plates were pooled for chlorophyll measurements. Acetone 80% (v/v) was used for extraction, and absorbance at 645 and 663 nm was measured in a spectrophotometer. Chlorophyll content is calculated as [(Ca = 12.25 A663 – 2.79 A646) + (Cb = 21.50 A646 – 5.10 A663)] / μg FW, where Ca means chlorophyll *a* and Cb means chlorophyll *b*.

Luciferase Assay

Transcriptional activity of FIL was measured using the promoter of *MYB75* (1.18 kb; Supplemental Table 1) cloned into pGWB435 carrying the Luciferase reporter gene. Leaves of *Nicotiana benthamiana* were transiently infiltrated with *Agrobacterium tumefaciens* strains bearing *pMYB75:LUC* and combinations of *35S:FIL* and *35S:JAZ3ΔJas-GFP* constructs plus the silencing suppressor p19. Twenty-four hours after agroinfiltration, 1-cm discs were collected from the leaves with the aid of a cylindrical borer and carefully transferred, the abaxial side upwards, to 96-well microplates filled with 175 mL of water and 25 mL of D-luciferin substrate (0.1 mg/mL; Sigma-Aldrich #L9504). One disc was used per well and at least 16 disc replicates per sample. Levels of luciferase activity were measured every hour, for a total of 48 h, using the LB 960 Microplate Luminometer (Berthold), which operates MikroWin 2000 software.

EMSA

For the EMSAs, DNA was labeled with biotin using Pierce's Lightshift Chemiluminescent EMSA kit. DNA binding and EMSA were performed as previously described (Solano et al., 1998). Sequences of the *MYB75* promoter and DNA probes are described in Supplemental Figure 6. The DNA probes were obtained by PCR using oligos FBE1 Fw (5′-TAATACGACTCACTATAGGGtcacatactcacactctcta-3′), FBE1 Rev (5′-aaggaagagggtatatatagtatgg-3′), FBE2 Fw (5′-TAATACGACTCACTATAGGGacttctccatcttgtaatcg-3′), FBE2 Rev (5′-cgggtatatatgttttcaga-3′), Gbox Fw (5′-TAATACGACTCACTATAGGGtgtggatatcaaacatgcac-3′), Gbox Rev (5′-agagagtgtgagtatgtgag-3′), and 5′Biotin T7promoter Fw (5′-TAATACGACTCACTATAGGG-3′) for labeling.

RT-qPCR Analysis

Ten-day-old *35S:FIL-GR* plants were exposed to 4 h mock (1:1000 dilution DMSO/ethanol), DEX (20 μM DEX/1:1000 ethanol), JA (50 μM JA/1:1000 DMSO), or DEX/JA (20 μM DEX/50 μM JA) before being harvested and RNA extracted. One microgram of total RNA was used for cDNA synthesis, and RT-qPCR analysis was conducted according to Bonaccorso et al. (2012). Three biological replicates were used for each treatment and expression was calculated relative to *ACTIN*.

Bacterial Assays on Arabidopsis

Pto DC3000 growth assays in Arabidopsis were performed by spray inoculation. Briefly, overnight bacterial cultures were pelleted and resuspended in sterile 10 Mm $MgCl_2$. Three- to four-week-old plants were sprayed with a bacterial suspension containing 10^8 colony-forming units/mL bacteria (OD_{600} = 0.2) with 0.04% Silwet L-77. Leaf discs were harvested after 2 d and ground in 10 mM $MgCl_2$. Population counts were performed at 2 d after infiltration. In both cases, serial dilutions of leaf extracts were plated on Luria-Bertani agar with appropriate antibiotics. Each data point represents the average of four replicates, each containing two leaf discs from different plants. Error bars indicate SE. These experiments were repeated three times with similar results, and representative results are shown. Photographs represent disease symptoms 3 d after inoculation on analyzed genotypes.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: FIL/YAB1 (AT2G45190), YAB2 (AT1G08465), YAB3 (AT4G00180), YAB5 (AT2G26580), MYB75 (AT1G56650), JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17860), JAZ4 (AT1g48500), JAZ5 (At1g17380), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 (At1g70700), JAZ10 (At5g13220), JAZ11(At3g43440), and JAZ12 (At5g20900).

Supplemental Data

Supplemental Figure 1. Yeast two-hybrid assay showing FIL-JAZ3 interaction in the presence of 3-aminotriazole.

Supplemental Figure 2. Anthocyanin content of 7-d-old *35S:FIL-GR* transgenic Arabidopsis seedlings grown on MS medium supplemented with 10 μM DEX plus/minus 1 μM coronatine (COR; a mimic of JA-Ile).

Supplemental Figure 3. Hypocotyl growth of *35s:FIL-GR* and *yab* mutants in the presence/absence of JA.

Supplemental Figure 4. Root growth inhibition of 7-d-old wild type and *yab* mutants.

Supplemental Figure 5. Disease symptoms in leaves of wild type, *yab* mutants, and *35S:FIL-GR* lines.

Supplemental Figure 6. Nucleotide sequence and graphic representation of *MYB75/PAP1* promoter.

Supplemental Figure 7. Anthocyanin accumulation on 5-d-old Arabidopsis *fil yab3* and *fil yab2 yab3 yab5* mutants compared with their respective wild types (L*er* for upper panel and *Coler* for bottom panel) grown in MS medium with 50 μM JA.

Supplemental Table 1. Oligonucleotides used for cloning.

Supplemental Data Set 1. Gene data sets related to Figure 2.

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AUTHOR CONTRIBUTIONS

M.B. and R.S. designed the research. M.B, J.F.G., S.G.-I., G.F.-B., and J.M.F.-Z. performed the research. M.B, J.F.G., S.G.-I., G.F.-B., J.M.F.-Z., and R.S analyzed the data. M.B, J.F.G., S.G.-I., G.F.-B., J.M.F.-Z., and R.S. read and edited the article. M.B., J.F.G., J.M.F.-Z., and R.S, wrote the article.

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