Arabidopsis MYC Transcription Factors Are the Target of Hormonal Salicylic Acid/Jasmonic Acid Cross Talk in Response to Pieris brassicae Egg Extract*

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Arabidopsis (Arabidopsis thaliana) plants recognize insect eggs and activate the salicylic acid (SA) pathway. As a consequence, expression of defense genes regulated by the jasmonic acid (JA) pathway is suppressed and larval performance is enhanced. Cross talk between defense signaling pathways is common in plant-pathogen interactions, but the molecular mechanism mediating this phenomenon is poorly understood. Here, we demonstrate that egg-induced SA/JA antagonism works independently of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors, which controls the ERF branch of the JA pathway. In addition, treatment with egg extract did not enhance expression or stability of JASMONATE ASSOCIATED MYC2-LIKEs, which are negative regulators of the JA pathway. Investigating the stability of MYC, MYC2, and MYC4, the most abundant MYC DNA-binding transcription factors that additively control jasmonate-related defense responses, we found that egg extract treatment strongly diminished MYC protein levels in an SA-dependent manner. Furthermore, we identified WRKYs as a novel and essential factor controlling SA/JA cross talk. These data indicate that insect eggs target the MYC branch of the JA pathway and cover an unexpected modulation of SA/JA antagonism depending on the biological context in which the JA pathway is activated.

Plants face a multitude of enemies with different virulence or feeding strategies. During evolution, plants have developed distinct defense mechanisms to specifically respond to these attacks. In animal–microbial pathogens and insects have evolved countermeasures to interfere with plant defenses, each in an arms race that culminated in a diversification of plant recognition systems on one side and vast repertoires of pathogen effectors on the other side (Dangl and Jones, 2001). The plant hormones salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) are key regulators of signal transduction pathways that are activated after recognition of the invader and that lead to expression of defense genes. Biotroph pathogens actively trigger the SA pathway, necrotrophs a combination of ET and JA pathways, whereas feeding insects mostly induce the JA pathway (Pieterse et al., 2012). However, these signaling pathways are tightly interconnected to allow a fine control of resource utilization for growth and rapid adaptation to a changing environment. Evidence has accumulated over recent years that plant enemies take advantage of this signal cross talk to evade or suppress defenses (Pieterse et al., 2012).

SA plays a central role in disease resistance, both locally and systemically. It accumulates strongly in leaves upon perception of microbe-associated molecular patterns and controls the genetic program responsible for the synthesis of pathogenesis-related proteins or antimicrobial compounds. SA also is required for protecting undamaged parts of the plant against further infections, a process called systemic acquired resistance (Vlot et al., 2009). One essential component of the SA pathway is the transcriptional coactivator NON EXPRESSOR OF PR GENES1 (NPR1). Upon SA accumulation and change of cellular redox state, NPR1 monomerizes and translocates to the nucleus, where it interacts with TGA transcription factors that bind to the promoter of SA-responsive genes (Fu and Dong, 2013). Among those, WRKY transcription factors influence positively and negatively the expression of downstream defense genes (Eulgem and Somssich, 2007).

The JA pathway is involved in different processes, including plant growth, development, and responses to biotic and abiotic stresses (Howe and Jander, 2008; Browse, 2009). JA levels rise in response to wounding or herbivory, triggering large changes in gene expression (Reymond et al., 2000, 2004; de Vos et al., 2005; Devoto et al., 2005; Eilting et al., 2008). Signaling events responsible for this transcriptional reprogramming are relatively well understood. Upon stress, JA is synthesized from membrane-derived fatty acids through
several steps, including oxygenation by lipoxygenases (LOXs), cyclization, and \( \beta \)-oxidation. JA is then conjugated to Ile to form JA-Ile, the bioactive molecule that is perceived by a receptor complex containing CORONATINE-INSENSITIVE1 (COI1) and JASMONE ZIM-domain (JAZ) repressors (Chini et al., 2007; Thines et al., 2007; Fonseca et al., 2009; Yan et al., 2007). COI1 is part of an SCF-type E3 ubiquitin ligase complex that mediates degradation of JAZ proteins by the proteasome. In unstimulated plants, JAZ repressors inhibit the activity of transcription factors by interacting with the adaptor protein NINJA and the corepressor TOPLESS (Pauwels et al., 2010). JA-Ile accumulation leads to degradation of JAZs, allowing transcription of JA-responsive genes, including the marker VEGETATIVE STORAGE PROTEIN2 (VSP2). Known direct targets of JAZ repressors are closely related bHLH factors, MYC2, MYC3, and MYC4 (Fernández-Calvo et al., 2011). These three MYCs interact with MYB proteins to regulate defense against insect herbivory by binding to a G-box motif found in the promoter of glucosinolate biosynthesis genes (Schweizer et al., 2013). Another subgroup of bHLH factors includes JASMONATE ASSOCIATED MYC2-LIKES (JAMS), which are negative regulators of the JA pathway. JAMs bind to MYC target sequences and act as transcription repressors, allowing fine regulation of defense gene expression (Song et al., 2013; Sasaki-Sekimoto et al., 2013).

The JA pathway consists of two distinct branches that respond to different aggressors. In response to wounding or herbivorous insects, the MYC branch activates some MYC transcription factors and JA-responsive genes, including VSP2. Necrotrophs utilize the ERF branch, which is controlled by APETAL2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors, including RPS2, and OCTADECANOID RESPONSE 1 (ORA1). The ERF branch uses the combination of JA and ET pathways to induce the marker gene PLANT DEFENSIN1.2 (PDF1.2) (Pieterse et al., 2012). Upon perception by its receptors, ET activates two primary transcription factors, ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1), which upregulate activity of the essential regulator EIN3 (Pieterse et al., 2009). EIN3 and EIL1 regulate the expression of most ET-responsive genes, including ERF1 and possibly ORA59. It has recently been shown that JAZs interact with EIN3 and EIL1 and repress their transcriptional activity, explaining the synergism in JA/ET gene regulation (Zhu et al., 2011).

Antagonism between SA, ET, and JA pathways has been demonstrated in many plant species (Pieterse et al., 2012). It is suggested to help the plant prioritize defense allocation toward the appropriate attacker by fine-tuning the induction of specific defense programs when these hormones accumulate (Reymond and Farmer, 1998; Spoel and Dong, 2008). However, attackers can also exploit this antagonism for their own benefit. For example, activation of the JA pathway by the biotroph pathogen Pseudomonas syringae pv tomato results in the inhibition of SA-dependent defenses through production of the virulence factor coronatine (COR), which is a JA-Ile analog (Brooks et al., 2005). Another bacterial effector, HopX1 from P. syringae pv tabaci, degrades JAZs and thus promotes virulence by inhibiting SA defenses through activation of the JA pathway (Gimenez-Ibanez et al., 2014). Relatively little information is available on the molecular mechanisms of JA/SA cross talk, but it was recently found that COR induces the expression of NAC transcription factors that inhibit SA biosynthesis (Zheng et al., 2012). In contrast, antagonism of the SA signaling pathway on the JA pathway has been extensively studied (Caarls et al., 2015). SA suppressed JA-dependent defense responses in tomato (Solanum lycopersicum; Doares et al., 1995), tobacco (Nicotiana tabacum; Niki et al., 1998), and Arabidopsis (Arabidopsis thaliana; Spoel et al., 2003). Consequently, stimulation of the SA pathway diminished plant response to a necrotrophic fungus (Spoel et al., 2007) and a generalist herbivore (Cipollini et al., 2004). In Arabidopsis, treatments with SA for pathogens that enhance SA levels inhibited expression of herbicide-induced VSP2 and necrotroph-induced PDF1.2 (Koornneef et al., 2008; Leon-Reyes et al., 2010). Pharmacological experiments with SA and methyl jasmonate (MeJA) treatments have shown that high doses of MeJA result in a long-lasting (up to 96 h) inhibitory effect on TeJA-induced PDF1.2 expression (Koornneef et al., 2008). This negative cross talk was conserved in several accessions (Koornneef et al., 2008), occurred downstream of JA biosynthesis (Leon-Reyes et al., 2010b), and required cytosolic activity of MYC1 (Sopel et al., 2003). Additional experiments demonstrated that ET can override NPR1 dependency of SA-JA antagonism, indicating that biotic induction of ET modulates the interaction between SA and JA pathways (Leon-Reyes et al., 2010b). Two recent studies discovered that SA treatment promotes ORA59 protein degradation (Van der Does et al., 2013) and inhibit ORA59 expression (Zander et al., 2014), providing the first clues on how gene expression from the ERF branch of the JA pathway is suppressed.

Eggs from herbivorous insects are frequently deposited on plant leaves and represent a threat as they give rise to feeding larvae. Evidence has accumulated that plants can recognize and respond to oviposition by inducing direct and indirect defenses (Hilker and Fatouros, 2015; Reymond, 2013). In Arabidopsis, insect eggs trigger cellular and molecular changes that are very similar to those that are caused by infection with biotroph pathogens. Indeed, eggs from the specialist lepidopteran herbivore Pieris brassicae induce a hypersensitive-like response, callose and reactive oxygen species accumulation, and the induction of pathogenesis-related genes (Little et al., 2007; Gouhier-Durand et al., 2013). Expression of PATHOGENESIS-RELATED1 (PR1) is strongly up-regulated at the site of oviposition in response to P. brassicae eggs but also in response to egg extracts from other insect species, including the generalist Spodoptera littoralis (Bruessow et al., 2010). Findings that SA accumulates to high levels following P. brassicae oviposition and that mutants in ENHANCED DISEASE SUSCEPTIBILITY1, SALICYLIC
**RESULTS**

**Egg-Induced Suppression of the JA Pathway Is Independent of ORA59 But Requires NPR1 and EIN2**

We previously found that treatment of Arabidopsis plants with *P. brassicae* and *S. littoralis* egg extracts inhibited the expression of JA-responsive genes that are induced by feeding larvae, including *LOX3, MAP*, and *MYROSINASE-ASSOCIATED* genes (Bruessow et al., 2010). This was accompanied by a better overall performance of the generalist herbivore *S. littoralis* but not of the specialist *P. brassicae*, which is adapted to Arabidopsis defense metabolites. Since both genes were lost in *sid2-1*, we concluded that egg-induced JA accumulation suppressed JA-dependent defenses (Bruessow et al., 2010). To test if this negative JA cross talk involves the ERF branch of the JA pathway, we treated 4-week-old Arabidopsis ecotype Columbia, *Col-0* and *ora59* plants with egg extract for 5 d and subsequently challenged them with *S. littoralis* larvae for 48 h (analysis of gene expression) or 10 d (larval performance assay). Egg extract suppressed insect-induced expression of *VSP2* in both wild-type and *ora59* plants (Fig. 1A). In addition, larvae gained significantly more weight on plants treated with egg extract compared with control plants, irrespective of the genotype (Fig. 1B). Thus, these results demonstrate that ORA59 is not involved in egg-induced SA/JA cross talk.

To further investigate which molecular players mediate suppression of the JA pathway by *P. brassicae* eggs, we established a simplified protocol where 12-d-old Arabidopsis were treated with egg extract for 24 h and subsequently wounded with forceps to mimic herbivory and activate the JA pathway (Fig. 2A). Similar to older plants challenged with larvae, treatment with egg extract strongly suppressed wound-induced VSP2 expression (Fig. 2B). This suppression was also observed for *LOX3* and *MAP* (Supplemental Fig. S1). In contrast, consecutive egg extract and wound treatments did not suppress *PDF1.2* expression, confirming that egg-induced SA/JA cross talk does not target the ERF branch but more likely the MYC branch of JA pathway (Fig. 2C). To verify that treatment with egg extract can substitute for the less controllable natural oviposition, plants were placed in a cage with *P. brassicae* females until one egg batch per leaf was laid, and 24 h later leaves were wounded for 3 h and collected for gene expression analysis. In a parallel experiment, oviposited plants were challenged with *S. littoralis* larvae for 10 d and larval performance assessed. Natural oviposition suppressed wound-induced *VSP2* expression and stimulated *S. littoralis* larval growth to a
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Figure 2. Egg extract-induced SA/JA cross talk is abolished in the npr1-1 ein2-1 mutant. A, Experimental scheme used with 12-d-old seedlings. One microliter of egg extract (EE) was applied underneath the first true leaf, the leaf was wounded twice with forceps and harvested after 3 h (EE + W). Controls consisted of plants only wounded (W) or plants only treated with egg extract (EE). B and C, Expression of VSP2 and PDF1.2 was measured by real-time PCR. Twelve-day-old seedlings were subjected to the treatments described in A. Values are the mean of three biological replicates and normalized to the reference gene SAND. Different letters within each genotype indicate significant differences at P < 0.05 (Tukey's highly significant difference test). D, Freshly hatched S. littoralis larvae were placed on 4-week-old Col-0 or npr1-1 ein2-1 plants, and larval weight was measured after 10 d as feed on EE-treated plants and control plants challenged with insects (C). Black bars indicate plants treated with egg extract (EE) for 1 d before insect challenge. The number of larvae used in each experiment is shown within the bars. Error bars indicate significant differences between control and treated plants (Student’s t test; ***P < 0.001; n.s., not significant). These experiments were repeated twice with similar results.
extract-treated and wounded plants, underlying the known JA-Ile-dependent degradation of JAZs by SCF<sup>COI1</sup> complex. Similarly, GUS activity was lower after wounding or after consecutive egg extract and wound treatment (Supplemental Fig. S3). Thus, suppression of JA-responsive genes in response to egg-induced SA/JA cross talk is not explained by enhanced JAZ expression or stabilized JAZs.

With the recent discovery that the MYC branch of the JA pathway is negatively regulated by JAMs, we next hypothesized that egg-induced SA could enhance the activity of these transcriptional repressors. To address this hypothesis, we made use of the quadruple JAM mutant <i>bhlh3 bhlh13 bhlh14 bhlh17</i>, which was shown to exhibit a significant increase in JA-regulated gene expression and resistance to herbivory (Song et al., 2013). <i>VSP2</i> was strongly induced by wounding in the mutant, but this induction was significantly diminished with egg extract pretreatment, ruling out a role for these factors in SA/JA cross talk (Fig. 3C).

**Egg-Induced SA Modulates MYC2, MYC3, and MYC4 Accumulation**

Since SA targets ORA59 protein stability in the ERF branch of the JA pathway (Van der Does et al., 2013), we then investigated whether a similar process occurs in the MYC branch. Twelve-day-old plants were treated with egg extract, wounding, or a combination of both, and accumulation of MYC2 was monitored by western blot with anti-MYC2 antibody. Treatment with egg extract had a marked effect on MYC2 protein accumulation, which was severely diminished compared with control levels (Fig. 4A). MYC2 levels were increased after wounding compared with control plants, but, strikingly, were again almost undetectable in the consecutive egg extract and wound treatment. To test if this egg-triggered inhibition of MYC2 accumulation was dependent on SA, we injected <i>sid2-1</i> plants to the same cross-talk conditions. In clear contrast with wild-type plants, egg extract treatment alone or in combination with wounding was unable to lower MYC2 levels in this SA-deficient mutant (Fig. 4A). This is consistent with our previous report showing that egg-induced inhibition of JA-responsive defense gene expression and enhanced larval performance were abolished in <i>sid2-1</i> (Bruessow et al., 2010). Thus, egg-induced SA/JA cross talk operates through MYC2 protein stability, and this process is analogous to the negative effect of SA on ORA59 accumulation. In addition, use of the proteasome inhibitor MG132 prevented egg-induced MYC2 degradation, implying a role for the 26S proteasome in the control of MYC2 stability (Fig. 4B).

Since inhibition of wound-induced <i>VSP2</i> expression by egg extract treatment was abolished in <i>npr1-1 ein2-1</i> (Fig. 2), we postulated that this absence of SA/JA antagonism may be explained by a failure to degrade MYC2. On the contrary, MYC2 protein levels were still highly reduced in response to egg extract treatment in <i>npr1-1 ein2-1</i> plants, with or without wounding (Fig. 4C).

To further substantiate our findings on MYC2, we measured the effect of egg extract on MYC3 and MYC4 stability. Because no antibodies against these proteins are available, we used 35S:<i>MYC3-GFP</i> and 35S:<i>MYC4-GFP</i> Arabidopsis lines. Similar to MYC2, levels of MYC3-GFP and MYC4-GFP were significantly reduced in plants treated with egg extract, and this effect was conserved in consecutive egg extract and wound treatments (Supplemental Fig. S4).

Finally, to test if egg-induced SA modulates MYC protein levels by inhibition of transcriptional activity, we measured MYC2, MYC3, and MYC4 expression in cross-talk conditions. MYC2 expression was similar in control and egg extract-treated plants, whereas it was induced by wounding and reduced by a consecutive
egg extract and wound treatment; like MYC3, in contrast, MYC3 and MYC4 showed similar expression levels in all conditions (Supplemental Fig. S5). It thus appears that there is no strict correlation between MYC expression and protein levels, suggesting that egg-induced SA regulates MYC protein levels through another process.

**WRKY75 Regulates Egg-Induced SA/JA Cross Talk**

We previously noticed that WRKY75 was highly induced by *P. brassicae* oviposition (Little et al., 2007). Since several WRKYs have been reported to modulate SA/JA antagonism (Caarls et al., 2015), this prompted us to test the involvement of this factor in response to egg extract treatment. The Arabidopsis T-DNA insertion mutant *wrky75-25* (Encinas-Villarejo et al., 2009) and a 35S:WRKY75 overexpressor line (Van Aken et al., 2013) were treated with *P. brassicae* egg extract and subsequently wounded. Controls consisted of no treatment or wounding alone. Expression of VSP2 was strongly up-regulated by wounding in 35S:WRKY75, but, contrary to Col-0, this induction was not suppressed by egg extract pretreatment. Indeed, wounding or egg extract treatment followed by wounding induced VSP2 to similar levels (Fig. 5A). In *wrky75-25*, expression of both genes was not induced by wounding and was not different between all treatments (Fig. 5A). In line with these results, enhanced *S. littoralis* larval performance after egg extract pretreatment was not observed in 35S:WRKY75. In addition, *wrky75-25* mutant allowed much more growth of *S. littoralis* compared with Col-0, and this effect was even more pronounced in egg extract-treated plants (Fig. 5B).

Since wound-induced VSP2 expression was not inhibited by egg extract treatment in 35S:WRKY75 plants, we then tested whether overexpression of WRKY75 was interfering with egg-triggered MYC2 degradation. However, MYC2 protein levels were still strongly reduced in response to egg extract treatment or in response to consecutive egg extract and wound treatments in 35S:WRKY75 plants (Fig. 5C).

Absence of SA/JA cross talk in 35S:WRKY75 could be due to impaired egg-induced SA signaling. We analyzed the expression of genes that are strongly up-regulated by oviposition (Little et al., 2007). The SA marker gene *PR1*, a chitinase gene (*CHIT*), and a trypsin inhibitor gene (*TI*) were all markedly up-regulated by egg extract treatment or by a consecutive egg extract and wound treatment. This up-regulation was similar in Col-0, 35S:WRKY75, and *wrky75-25*, indicating that WRKY75 does not block the SA pathway but rather acts at another level (Supplemental Fig. S6).

**DISCUSSION**

Cross talk between defense signaling pathways has been the subject of intense research in recent years. It is thought to represent a way to optimize resource allocation to properly fight pathogens or insect attackers. In
contrast, plant enemies also can hijack cross talk to their own benefit. We recently discovered that insect eggs are recognized by Arabidopsis. Egg-induced SA accumulation antagonized expression of JA-related genes, which resulted in enhanced larval performance of a generalist herbivore (Bruessow et al., 2010). In this work, we decided to investigate the molecular mechanisms by which insect eggs target the JA pathway.

ORA59 is a key transcription factor that controls expression of defense genes belonging to the ERF branch of the JA pathway, including the marker gene PDF1.2. We find here that egg-induced SA/JA cross talk still occurs in ora59 mutant. In addition, induction of PDF1.2 after wounding was not subjected to SA/JA antagonism by insect eggs. The ERF branch is involved in resistance to necrotroph pathogens and requires simultaneous activation of ET and JA signaling pathways (Lorenzo et al., 2003). The distinct MYC branch of the JA pathway is triggered by wounding in herbivory and regulates VSP2 expression (Pieterse et al., 2012). Indeed, JA is not able to induce VSP2 in the myc2 myc3 myc4 triple mutant (Fernández-Calvo et al., 2011). Thus, our results strongly indicate that insect eggs target the MYC branch and not the ERF branch of the JA pathway. It is intriguing that egg-induced SA accumulation leads to a specific SA/JA cross talk that only affects one branch of the JA pathway. In experiments where inducers of the SA pathway were either the biotrophic pathogen Hyaloperonospora parasitica or exogenous application of SA, it was shown that MeJA up-regulation of PDF1.2 was suppressed (Koornneef et al., 2008). Thus, the biological context in which SA is induced is likely to influence the outcome of SA/JA cross talk and further investigation on additional factors responsible for this specificity will be necessary.

Several hypotheses were then tested to explain how egg-induced SA accumulation antagonizes defense gene expression controlled by the MYC branch of the JA pathway. We found that SA did not increase the expression of the nondegradable splice variant JAZ10.4 and did not stabilize JAZ1 and JAZ10 protein levels. JAZ10.4 variant was previously shown to bind to MYC2 but not to COI1, and Arabidopsis plants overexpressing JAZ10.4 exhibited JA-insensitive phenotypes, indicative of a reduced JA signaling pathway (Chung and Howe, 2009). JAZ10.4 and JAZ8, another
transcriptional repressor that is not degraded by the JA-Ile-SCF<sup>core</sup> complex (Shyu et al., 2012), are thought to constitute a negative feedback loop that prevents exacerbated activation of JA responses. In line with experiments using exogenous application of SA (Van der Does et al., 2013), we conclude that egg-induced SA/JA cross talk is not regulated by increased expression of JAZ10.4 or stabilization of JAZ repressors.

Recently, a subclass of bHLH factors closely related to MYC2, MYC3, and MYC4 was shown to antagonize MYC activity. bHLH3, bHLH13, bHLH14, and bHLH17 are repressors that bind to cis-regulatory elements of MYC target genes and inhibit transcription (Fonseca et al., 2014; Song et al., 2013; Sasaki-Sekimoto et al., 2013). We tested whether these factors could participate in the suppression of JA responses mediated by egg-induced SA. VSP2 expression was still inhibited by a combined egg extract and wound treatment in the quadruple bhlh3 bhlh13 bhlh14 bhlh17 mutant, ruling out the involvement of this family of repressors in egg-induced SA/JA cross talk. It would be interesting to test their role in the ERF branch of the JA pathway, but altogether our data and those of Van der Does et al. (2013) point to separate mechanisms to modulate JA-dependent transcriptional responses. On one hand, stabilized JAZs and bHLH repressors counteract the activation of JA responses to fine-tune defense and development. On the other hand, accumulation of ET in response to biotrophs and insect eggs interferes with JA defense gene expression via other routes.

Assessing MYC protein stability, we discovered that treatment with egg extract strongly stabilized MYC2, MYC3, and MYC4 levels. This effect was due to increased protein stability since MYC proteins were not degraded in the absence of egg extract treatment. The fact that all three MYCs were degraded by egg extract treatment is likely explained by their shared redundant activity. They are known to form homo- and heterodimers and additively control the expression of defense genes and resistance to herbivores (Switzer et al., 2013; Fernández-Calvo et al., 2011). Interestingly, a study recently reported that JAZ9 is degraded by exogenous SA application, implying a molecular mechanism for how SA/JA cross talk targets the ERF branch of the JA pathway (Van der Does et al., 2013). It is striking that two seemingly different SA/JA cross talks target different transcription factors. How such specificity is achieved will require further work, but it is tempting to speculate that some components are shared.

We found that MYC2, MYC3, and MYC4 expression was similar in control and egg-extract-treated plants. However, contrary to MYC3 and MYC4, MYC2 was induced by wounding, and this induction was reduced when plants were pretreated with egg extract. Interestingly, the promoter of MYC2 contains three G-box elements (Promomer; http://bbc.botany.utoronto.ca). G-box is a known binding site of MYC2, MYC3, and MYC4 (Fernández-Calvo et al., 2011; Godoy et al., 2011). MYC2 thus seems to be part of a positive feedback loop and is subjected to SA/JA cross talk. We cannot rule out that suppression of MYC2 expression contributes overall to the antagonistic effect of egg-induced SA accumulation, but our data rather suggest that MYC protein degradation is an essential component of this phenomenon.

Regulated proteolysis is an important component of plant signaling pathways (Sadanandom et al., 2012). Besides JAZs that are targeted for degradation upon activation of the JA pathway by the SCF<sup>core</sup> complex, MYCs were also shown to be unstable proteins. For example, the circadian clock regulates MYC2 protein level. MYC2 accumulates during the day and is degraded during the night (Shin et al., 2012). MYC2, MYC3, and MYC4 are stabilized by light, via the action of red and blue light photoreceptors, and also by JA treatment (Chico et al., 2014). Phosphorylation-dependent proteolysis of MYC2 was reported to be essential for its transcriptional activity (Zhai et al., 2013). In these cases, MYC protein degradation was mediated by the proteasome. Recently, it was shown that MYC2 is polyubiquitinated by PLANT U-BOX PROTEIN10 (PUB10), which belongs to the U-box family of E3 ligases (Jung et al., 2015). Thus there is growing evidence that the JA pathway is tightly coordinated with other environmental inputs and that part of this regulation operates at the translational level. Here, we provide evidence that egg-induced SA/JA cross talk also targets MYC protein stability through the proteasome. Further research will be necessary to identify factors that control MYC and ORA59 degradation in the context of SA-mediated JA antagonism.

NPR1 is required for SA-mediated suppression of the ERF branch of the JA pathway, although this effect can be modulated by ET (Spöl et al., 2003; Leon-Reyes et al., 2009). For egg-induced SA/JA cross talk, we previously found that suppression of JA-responsive genes and enhanced larval performance was conserved in npr1-1 ein2-1 (Bruessow et al., 2010) and ein2-1 (Bruessow, 2011) single mutants. Interestingly, we show here that egg-induced SA/JA cross talk was abolished in the npr1-1 ein2-1 double mutant, implying that NPR1 and ET are collectively necessary to suppress the MYC branch. Antagonism between ET and JA pathways has been reported previously. For example, ET perception negatively affected expression of wound-regulated genes (Rojo et al., 1999), and ein2-1 plants were more resistant to herbivory (Bodenhausen and Reymond, 2007). At the molecular level, EIN3 and EIL, which are key transcription factors of the ET pathway, interacted with and repressed MYC2 transcriptional activity (Song et al., 2014). Here, we show that PDF1.2 expression was induced by egg extract treatment and that this induction was abolished in npr1-1 ein2-1, suggesting that the ET pathway is activated by insect eggs. However, the finding that MYC2 protein degradation in response to egg extract treatment still occurred in npr1-1 ein2-1 strongly suggests that NPR1 and the ET pathway modulate JA defenses independently of MYC factors and not through EIN3/EIL1-mediated inhibition of MYC activity.
In this study, we identified WRKY75 as a novel regulator of egg-induced SA/JA cross talk. Overexpression of WRKY75 abolished egg extract-mediated suppression of VSP2 expression and did not lead to enhanced larval performance. In contrast, MYC2 was still degraded by egg extract treatment in 35S:WRKY75, similar to experiments with npr1-1 ein2-1. Since wound-activation of VSP2 was strongly diminished in wrky75-25 and maintained after egg extract treatment in 35S:WRKY75, our results are consistent with a positive role of WRKY75 in the control of the JA-regulated genes independently of MYC2. WRKY75 was previously shown to play a role in plant defense against biotrophs and necrotrophs (Encinas-Villarejo et al., 2009; Windram et al., 2012; Chen et al., 2013).

Noteworthy, WRKY75 overexpression was accompanied by elevated expression of JA-responsive genes, including PDF1.2, VSP1, and LOX2, and enhanced resistance against the necrotroph Sclerotinia sclerotiorum (Chen et al., 2013). In support of these findings, a WRKY DNA binding motif (W-box) was enriched in genes that were responsive to SA/JA cross talk (Van der Does et al., 2013).

Interestingly, we found that induction of WRKY75 by egg extract treatment was more pronounced in npr1-1 ein2-1 than in Col-0 (Supplemental Fig. S7), suggesting a link between NPR1/ET and WRKY75. We propose a model (Fig. 6) where insect eggs inhibit JA-dependent defense gene expression in two independent ways. First, SA accumulation leads to proteasome-dependent MYC degradation. Second, a combination action of ET and the ET pathway negatively regulates WRKY75 activity. This model explains why a simple loss of cross talk was observed in npr1-1 ein2-1 and 35S:WRKY75 independent of MYC2 protein degradation. Indeed, we postulate that a release of WRKY75 inhibition by NPR1/ET or overexpression of WRKY75 compensates for the reduced expression of JA-regulated genes, which results from MYC degradation. Noteworthy, egg-induced SA accumulation leads to reduced larval performance and is believed to be a strategy evolved by insects to attenuate JA-dependent defenses (Bruessow et al., 2010). Oviposition-induced WRKY75 expression may have evolved by the plant to counteract egg-induced cross talk. In turn, that NPR1/ET negatively control WRKY75 expression could represent a strategy to reinforce SA/JA cross talk, in an ongoing arms race between insects and plants. How NPR1, ET signaling, and WRKY75 are connected at the molecular level will represent an interesting challenge for future work.

Besides NPR1 and ET signaling, several proteins have been reported to modulate SA/JA cross talk in Arabidopsis. MAP KINASE4 is a negative regulator of the SA pathway and a positive regulator of the JA pathway (Petersen et al., 2000). Multiple TGA transcription factor mutants tga235 and tga2356 are blocked in SA-mediated inhibition of JA (Zander et al., 2010; Leon-Reyes et al., 2010b). For WRKY transcription factors, wrky50 and wrky51 mutants do not show SA suppression of JA-mediated PDF1.2 expression (Gao et al., 2011).

Overexpression of WRKY70 enhanced the expression of SA-related genes and suppressed JA-responsive gene expression (Li et al., 2004); however, a wrky70 mutant was not impaired in SA/JA cross talk (Leon-Reyes et al., 2010b). Finally, JA and SA induced WRKY62 downstream of NPR1. WRKY62 overexpression attenuated JA-responsive gene expression, whereas a wrky62 mutant had increased JA-responsive gene expression (Mao et al., 2007). WRKY75 thus adds to the list of regulators of SA/JA cross talk. Whether these factors have a direct or indirect role in controlling the SA/JA antagonism, whether they specifically mediate suppression of the ERF or MYC branch of the JA pathway, and by which mechanism they exert their function are, however, still largely unknown.

Manipulation of plant defense signaling pathways by attackers is the subject of intense research. In recent years, evidence has accumulated that different insects
use SA/JA cross talk to specifically inhibit plant defenses that are detrimental for their own development. In Arabidopsis, the silverleaf whitefly Bemisia tabaci was shown to activate the SA pathway and repress the expression of JA-dependent defenses, to which it is highly sensitive (Zarate et al., 2007). In Nicotiana attenuata, oral secretions from the bat armyworm Spodoptera exigua triggered SA accumulation, which resulted in lower JA levels, although larval performance was not evaluated (Diezel et al., 2009). In tomato, the mealybug Phenacoccus solenopsis activated the SA pathway, which in turn down-regulated the expression of JA-responsive genes. This effect was lost in SA-deficient plants and led to enhanced nymphal performance (Zhang et al., 2015). As a stunning indirect cross-talk manipulation, the Colorado potato beetle (Leptinotarsa decemlineata) was reported to exploit orally secreted bacteria. Symbionts belonging to three genera activated SA accumulation and expression of SA-regulated genes while suppressing JA responses. Antibiotic-treated larvae did not cause such response and gained less weight on tomato plants (Chung et al., 2013). Our finding that insect egg-induced activation of the SA pathway leads to diminished MYC protein levels and therefore suppresses JA-dependent defense gene expression provides a molecular mechanism that may be conserved between all these examples. It will be interesting in the future to explore whether manipulation of SA/JA cross talk by insects is widespread in nature and whether some plants have evolved strategies to counteract this phenomenon.

MATERIALS AND METHODS

Plant and Insect Growth Conditions

Arabidopsis (Arabidopsis thaliana) wild type (Col-0), mutants, and transgenic lines were grown in soil in a growth chamber (22°C, 10/14 h, high-6% CO₂, 100 μmol m⁻² s⁻¹, 60% relative humidity) as described previously (Reymond et al., 2007). Genotypes of npr1-1 ein2-1, wrky75-25, and 35S:WRKY75 were confirmed (Supplemental Fig. S8). Spodoptera littoralis (Egyptian cotton worm) eggs were obtained from Syngenta (Stein AG). Pieris brassicae (large white butterfly) eggs were reared in a greenhouse as described previously (Schlaeppi et al., 2003). Hundreds of P. brassicae eggs were regularly collected on cabbage leaves and after oviposition. They were gently washed with a pestle in an Eppendorf tube without addition of any liquid. After centrifugation (15,000 g, 3 min), the supernatant ("egg extract") was stored at −20°C. To standardize egg extract applications, all egg extracts were combined to constitute a bulk sample that was used for the treatments.

Cross-Talk Experiments

For experiments with 4-week-old plants, two spots of 2 μL of egg extract were applied onto each leaf. This amount corresponds to two egg batches of 20 eggs each. In total, two leaves each from 20 plants were treated. After 5 d, seedlings were removed with a paintbrush, and two freshly hatched S. littoralis larvae were placed onto each plant. Plants were placed in transparent plastic boxes in a growth room as described previously (Bodenhausen and Reymond, 2007). After 2 d of feeding, treated leaves from four plants were harvested for RNA analysis, and the remaining plants were left with larvae for another 8 d. At the end of the experiment, larvae were collected and weighed on a precision balance (Mx3000P, Mettler-Toledo). Controls consisted of untreated and/or uninjected plants.

For experiments with natural oviposition, 4-week-old plants were placed in a cage containing P. brassicae females for a maximum of 8 h. As soon as one egg batch was deposited on one leaf per plant, leaves were removed and transferred in a growth chamber for 5 d. Then, oviposited leaves were wounded twice with a forceps. Three hours later, treated leaves were collected for RNA analysis. For insect bioassay, each oviposited plant was challenged with two freshly hatched S. littoralis larvae for 10 d, after which larval weight was measured.

For experiments with 12-d-old seedlings, 1 μL of egg extract was deposited under the first true leaf from 12 seedlings. After 24 h, treated leaves were wounded twice with a forceps. Three hours later, whole shoots were cut from the root system with scissors and collected for RNA or immunoblot analyses. Other samples consisted of seedlings only treated with egg extract, only wounded, or untreated.

Treatment with MG132

Arabidopsis seedlings were germinated and grown on sterile mesh in Murashige and Skoog plates at 22°C under a 16-h-light/8-h-dark cycle in a phytotron. Twelve-day-old seedlings were transferred on the mesh to new plates containing 1 mL of liquid Murashige and Skoog. Seedlings were treated with 1 μL of egg extract, and the medium was supplemented with 50 μM MG132 (Sigma-Aldrich) for 1 d. Treatment with dimethyl sulfoxide was used as control. Afterward, seedlings were wounded with forceps for 3 h, and leaves were collected for immunoblot analyses.

Quantitative RT 1-Time PCR

Gene expression analysis was performed according to a previously published protocol (Dierer et al., 2014). Total RNA was isolated from Arabidopsis (Arabidopsis thaliana) wild type (Col-0), mutants, and transgenic lines grown in liquid nitrogen, and total RNA was extracted using Eppendorf Plant Mini Kit and treated with DNasel I. cDNA was synthesized from RNA using M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR reactions were performed using the Applied Biosystems Fast 7500 Real-Time PCR System with the following program: 95°C for 3 min, followed by 40 cycles of 10 s at 95°C and 20 s at 60°C. Values were normalized to the housekeeping gene SAND. Gene-specific primers are listed in Supplemental Table S1 online.

Protein Gel Blotting Analysis

Frozen seedling samples were homogenized by vortexing 1 min in 100 μL of extraction buffer (50 mM Tris-HCl, pH 7.4, 80 mM NaCl, 0.1% [w/v] Tween 20, 10% [w/v] glycerol, 1 mM DTT, 1% [w/v] Complete Mini Protease inhibitor cocktail [Roche]) in a TissueLyser II (Qiagen) with small plastic beads, centrifuged at 4°C for 10 min (15,000 g). Supernatant was collected, and samples containing 80 μg of proteins in 5% loading buffer (125 mM Tris-HCl, pH 6.8, 5% [w/v] SDS, 25% [w/v] glycerol, 2% [v/v] β-mercaptoethanol, 3.3 mg bromophenol blue/5 mL buffer) were boiled at 100°C for 2 min, cooled on ice, and centrifuged. Proteins were run into SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was subsequently stained with Ponceau S and detected with ImageQuant LAS4000 mini Luminescent Image Analyzer (GE Healthcare).

Histochemical Staining

GUS staining was performed as described previously (Little et al., 2007).

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource (www.arabidopsis.org) under the following accession numbers: MYC2 (At1g32640), MYC3 (At5g66760), MYC4 (At4g17880), JAZ1 (At1g19180), JAZ10 (At5g13220), PDF1.2 (At5g44220), ORA9 (At5g01610), NPR1 (At1g64280), EIN2 (At5g03280), VSP2 (At5g24770), WRKY75 (At5g13080), LOX3 (At1g17420), MAP (At5g4010), BHLH3 (At4g16340), BHLH3 (At4g14860).
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression of JA marker genes during egg extract-induced SA/JA cross talk.

Supplemental Figure S2. Oviposition-induced SA/JA cross talk is abolished in npr1-1 ein2-1 mutant.

Supplemental Figure S3. Egg extract treatment does not stabilize JAZ proteins.

Supplemental Figure S4. MYC3 and MYC4 proteins are degraded in response to egg extract treatment.

Supplemental Figure S5. Expression of MYC2, MYC3, and MYC4 in SA/JA cross talk conditions.

Supplemental Figure S6. Expression of oviposition-inducible genes.

Supplemental Figure S7. Expression of WRKY75 in SA/JA cross-talk conditions.

Supplemental Figure S8. Genotyping of npr1-1 ein2-1, 35S:WRKY75, and wrky75-25 lines.

Supplemental Table S1. List of primers for quantitative PCR analyses.

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LITERATURE CITED


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RETRACTION


The authors alerted the editors of Plant Physiology to irregularities in their publication and have taken the initiative to retract their article, with agreement from the Journal. Irregularities were uncovered in which proteins bands of Figure 4C were inappropriately duplicated and rotated as evidence for the wrong samples in Figure 4A. Further examination revealed that the wrong bands, which are thought to result from cross-reactions with the MYC2 antibody, were selected. The same error was repeated in Supplemental Figure S4. In addition, the original data for Figure 4, A and C, were not retained. The Reymond laboratory has since carried out additional experiments to find that the anti-MYC2 antibody that was used in the study is not specific. The claim that MYC2, MYC3, and MYC4 are degraded in response to egg extract treatment therefore is not supported by the data currently available, and the conclusion that the insect eggs suppress jasmonic acid-dependent immunity by targeted degradation of MYC transcription factors is not sound.

The authors regret any inconvenience or trouble that this action may cause and apologize sincerely to the readers, reviewers, and editors of Plant Physiology.