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A *Pseudomonas syringae* protein perturbs Arabidopsis hormone signaling by activating MAP KINASE 4

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SUMMARY

Pathogenic microbes often modulate phytohormone physiology in the host to their advantage. We previously showed that the *Pseudomonas syringae* effector protein AvrB perturbs hormone signaling exemplified by an up-regulation of jasmonic acid (JA) response gene expression and enhances plant susceptibility. Here we show that AvrB does so by interacting with the Arabidopsis mitogen activated protein kinase MAP KINASE 4 (MPK4) and HSP90 chaperone components. AvrB induces MPK4 activation and this is directly promoted by the HSP90 chaperone. A previously identified AvrB-interacting protein, RIN4, also is required for AvrB to perturb hormone signaling and induce plant susceptibility, likely by acting down-stream of MPK4. These findings uncover a novel pathway through which a bacterial effector protein stimulates host processes to the benefit of the *P. syringae* bacterium.

INTRODUCTION

Plant hormones play a vital role not only in growth, development, and responses to abiotic stresses, but also in plant immunity. Salicylic acid (SA), jasmonic acids (JA), and ethylene (ET) play a particularly important role in plant disease resistance to diverse pathogenic microbes (Spoel and Dong, 2008). The activation of JA and ethylene signaling in Arabidopsis is required for defenses against herbivores and necrotrophic microbial pathogens, such as *Botrytis cinerea* and *Alternaria brassicicola,* but often renders plants more susceptible to biotrophic and hemibiotrophic pathogens, such as *Hyaloperonospora parasitica* and *Pseudomonas syringae* (Robert-Seilaniantz et al., 2007). Conversely, SA primarily regulates disease resistance to biotrophic pathogens, but contributes to

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susceptibility to necrotrophic pathogens. The two pathways often antagonize each other, although synergism occurs under certain circumstances (Mur *et al.,* 2006). Recent data indicate that the SA and JA signaling pathways are subject to regulation by other phytohormones, forming a complex regulatory network (Spoel and Dong, 2008).

Not surprisingly, many plant pathogens perturb hormonal homeostasis or signaling to aid in the infection of plants (Robert-Seilaniantz *et al.*, 2007). Some do so by synthesizing phytohormones or compounds that functionally mimic phytohormones, while others secrete effector proteins into the host cell to modulate hormone biosynthesis or responses. *P. syringae* is known to use multiple strategies to alter plant hormone physiology. For example, some *P. syringae* strains produce a phytotoxin called coronatine, which structurally and functionally mimics JA-isoleucine, an active form of JA, to assist bacterial entry into plant tissues (Melotto *et al.*, 2006). In addition, this bacterium injects a large repertoire of effector proteins into the plant cells to enhance host susceptibility (Cunnac *et al.,* 2009). While many of these effectors are known to directly inhibit plant immune signaling pathways (Zhou and Chai, 2008), several effector proteins have been reported to alter hormone responses in plants. For example, transgenic expression of AvrRpt2 enhances auxin response gene expression (Chen *et al.*, 2007). Likewise, transgenic expression of AvrPtoB increases ABA content in plants (Torres-Zabala *et al.,* 2007). In addition, several *P. syringae* effectors are capable of inducing JA-response gene expression (He *et al.,* 2004). However, our knowledge of how these proteins alter plant hormone signaling is quite limited.

The *P. syringae* effector AvrB is known to trigger disease resistance in soybean plants carrying *Rpg1b* (Ashfield *et al.,* 2004) and Arabidopsis plants carrying *RPM1* (Grant *et al.,* 2005). In Arabidopsis, AvrB directly associates with the RPM1-interacting protein RIN4 and induces RIN4 phosphorylation in plants (Mackey *et al.,* 2002). Consistent with a role in inducing protein phosphorylation, the crystal structure of AvrB has features resembling protein kinases (Lee *et al.,* 2004; Desveaux *et al.*, 2007), although the biochemical function of AvrB remains to be elucidated.

In susceptible plants lacking the cognate resistance genes, AvrB assists *P. syringae* colonization in the host. In soybean plants lacking the corresponding resistance gene *Rpg1b,* AvrB enhances *P. syringae* virulence through an unknown mechanism (Ashfield *et al.,* 1995). On Arabidopsis plants, *avrB* induces the transcription of a JA response gene in Arabidopsis (He *et al.,* 2004). Expression of AvrB as a transgene in Arabidopsis plants lacking *RPM1* enhances plant susceptibility to a nonpathogenic strain of *P. syringae* bacteria (Shang *et al.*, 2006). Both AvrB-induced JA response gene expression and susceptibility in Arabidopsis requires COI1, a JA receptor (Yan *et al.*, 2009), indicating that AvrB enhances plant susceptibility by perturbing hormone signaling. Our previous work also showed that the *AvrB* transgene-induced susceptibility in Arabidopsis requires RAR1 (Shang *et al.,* 2006), a co-chaperone for HSP90. AvrB can interact with RAR1 *in vivo,* although it was not determined if this interaction occurs directly or indirectly. These results suggest that HSP90 and its client proteins are involved in the AvrB-induced JA signaling.

In this study, we show that MPK4 is regulated by HSP90. AvrB directly interacts with RAR1, and this enables AvrB to associate with HSP90 and MPK4 *in vivo,* consequently promoting MPK4 kinase activation. MPK4 directly interacts with RIN4 and phosphorylates RIN4 *in vitro*. Molecular and genetic evidence indicated that HSP90, MPK4 and RIN4 form a new pathway through which AvrB perturbs hormone signaling and induce plant susceptibility.

RESULTS

HSP90, but Not *TAO1* and *RPM1,* Are Required for AvrB-Induced Susceptibility

Because RAR1 and another protein, SGT1, are co-chaperones for HSP90 (Takahashi *et al.,* 2003; Hubert *et al.,* 2003), we reasoned that the HSP90 complex may be required for AvrB to enhance plant susceptibility. We therefore tested if the AvrB-induced plant susceptibility required HSP90. As we showed previously (Shang *et al.,* 2006), expression of the AvrB transgene in the *rpm1* mutant background enhanced the growth of the nonpathogenic *P. syringae hrcC* mutant strain in plants (Figure 1A). Co-infiltration of geldanamycin (GDA), a specific inhibitor of HSP90, did not affect the AvrB accumulation (Figure S1A), but abolished the *AvrB* transgene-induced bacterial growth in *AvrB-3xFLAG/rpm1* plants (Figure 1A). GDA does not have deleterious effects on the *P. syringae* bacterium at the concentration used for the experiment (Figure S1B), indicating that HSP90 is required for the AvrB-induced susceptibility to the bacterium. It was shown recently that, in addition to RPM1, another nucleotide-binding, leucine-rich-repeat (NB-LRR) resistance protein called TAO1 recognizes AvrB and triggers weak resistance responses in Arabidopsis (Eitas *et al.,* 2008). Because the stability of NB-LRR proteins often is regulated by HSP90 and RAR1 (Holt III *et al.,* 2005; Azevedo *et al.,* 2006), the observed AvrB activity could be caused indirectly by the function of TAO1. We therefore generated *AvrB-3xFLAG/tao1/rpm1* plants by crossing the *tao1-11* mutant (Eitas *et al.,* 2008) to the *AvrB-3xFLAG/rpm1* transgenic line. The *AvrB* transgene enhanced susceptibility to *P. syringae* in both *AvrB-3xFLAG/tao1/rpm1* and *AvrB-3xFLAG/TAO1/rpm1* plants (Figure 1B), indicating that neither TAO1 nor RPM1 is required for the AvrB-induced plant susceptibility.

JA Responses, but Not JA Biosynthesis, Is Induced by AvrB Transgene

We earlier showed that AvrB induces the expression of *RAP2.6,* a JA response gene (He *et al.,* 2004). To further determine the perturbation of JA signaling by AvrB, we examined expression of two additional JA response genes *PDF1.2* and *THI2.1* (Robert-Seilaniantz et al., 2007) in plants expressing the *AvrB* transgene. Estradiol-induced expression of the *AvrB* transgene led to 2–6 fold induction of *PDF1.2* (Figures 1C and 1D) and 15–30 fold induction of *THI2.1* transcripts (Figures S1C and S1D) in *AvrB*/*rpm1* plants compared to *rpm1* plants. Further more, expression of *AvrB* resulted in ~5 fold increase of anthocyanin (Figure S1E), a phenotype associated with elevated JA signaling (Shan *et al*., 2009). To determine if the elevated JA responses in *AvrB* transgenic plants was caused by increased JA accumulation or signaling, we measured JA content in plants expressing *AvrB*. Figure S1F shows that the expression of *AvrB* does not alter JA content in the plants. Together these results demonstrate that the AvrB-induces JA responses, but not JA biosynthesis.

The HSP90 Chaperone, but Not TAO1, Is Required for AvrB-Induced JA Responses

To determine the involvement of the HSP90 chaperone in the perturbation of hormone signaling, we focused on JA response genes and examined the expression of *PDF1.2* and *THI2.1* in *AvrB-3xFLAG/rpm1* transgenic plants upon GDA-treatment. The GDA-treatment completely abolished the AvrB-induced *PDF1.2* and *THI2.1* expression (Figures 1C and S1C). Likewise, the AvrB-induced *THI2.1* and anthocyanin accumulation are also abolished in *AvrB/rar1-29/rpm1* plants (Figures S1D and S1E), indicating an essential role for RAR1. In contrast, *PDF1.2* was induced normally by the *AvrB* transgene in the *tao1/rpm1* background, indicating that the effect of AvrB on JA responses is independent of *TAO1* and *RPM1* (Figure 1D).

Because the AvrB protein delivered from the *P. syringae* bacterium is expected to exist at a very low level in the host cell, the observed *PDF1.2* induction by the *AvrB* transgene might have resulted from the over accumulation of AvrB. To unequivocally determine if the

HSP90 complex is required for AvrB to induce *PDF1.2* expression during bacterial infection, we inoculated *rpm1* plants with DC3682 (*avrB*) along with GDA. While DC3682 (*avrB*) strongly induced *PDF1.2* expression 6 and 9 hours post inoculation in buffer-treated plants, it failed to induce *PDF1.2* expression in the presence of GDA (Figure 1E). Similarly, we examined *PDF1.2* expression in *rar1-29/rpm1* upon the inoculation of DC3682 (*avrB*). The *avrB*-induced *PDF1.2* expression was completely blocked in the *rar1-29/rpm1* mutant background (Figure 1F), further confirming a role of RAR1 in AvrB-induced JA signaling (Shang *et al.,* 2006). Together these results demonstrate that the effect of AvrB on JA responses requires the HSP90 chaperone components, but not TAO1.

AvrB Interacts with HSP90 through RAR1

We previously showed that AvrB can interact with RAR1 *in vivo* in a co-immunoprecipitation (co-IP) assay (Shang *et al*., 2006). Because RAR1 is a zinc-containing protein, the relatively high concentration of EDTA in our buffer system may lead to a release of free cysteine residues potentially impacting the specificity in protein-protein interactions. We therefore performed the co-IP experiment in the absence of EDTA. Figure S2 shows that the AvrB-RAR1 interaction occurs in the absence of EDTA, further supporting the specificity of interaction.

We next used an *in vitro* pull-down assay to determine if AvrB directly interacts with individual components of the HSP90 complex. AvrB directly interacted with RAR1, but not HSP90 or GST (Figure 2A). A weak interaction was also detected between AvrB and SGT1b under longer exposure. The RAR1 protein is consisted of an N terminal CHORD1 domain and a C terminal CHORD2 domain connected by a CCCH domain (Shirasu, 2009). The two CHORD domains are known to interact with HSP90 and SGT1, respectively. GST pull-down assays showed that the N terminal CHORD1 domain is sufficient to interact with AvrB (Figure 2B).

Co-IP experiment showed that HSP90 was capable of interacting with AvrB in plants (Figure 2C). Importantly, the AvrB-HSP90 interaction was largely abolished in the *rar1-29* background (Figure 2D), indicating that the interaction is specific. Together with previous findings on AvrB-RAR1 interaction (Shang *et al.,* 2006), these results indicate that AvrB is associated with the HSP90 complex, primarily through a direct interaction with RAR1.

AvrB Interacts with and Induces Phosphorylation of MPK4

Transgenic expression of AvrB suppresses Pathogen/Microbe-Associated Molecular Pattern (PAMP/MAMP)-induced responses (Shang *et al.,* 2006), which are known to involve the activation of the MAP kinases MPK4, MPK3 and MPK6 (Bittel and Robatzek, 2007). MPK6 and MPK3 are thought to positively regulate disease resistance to *P. syringae*, whereas MPK4 negatively impact resistance to *P. syringae* (Petersen *et al*., 2000), likely through its regulation of multiple hormone pathways. We therefore determined if expression of an *AvrB-3xFLAG* transgene affected MPK phosphorylation. Specific antibodies against MPK4 and MPK6 were used to immunoprecipitate MPK4 and MPK6 proteins from control plants (*rpm1*) or plants expressing the *AvrB* transgene (*AvrB-3xFLAG/rpm1*), and then the level of dual phosphorylation on MPK4 and MPK6 was determined by immunoblot using anti-phospho-ERK1 antibodies. The *AvrB* transgene specifically enhanced the phosphorylation of MPK4, but not MPK6 (Figures 3A and S3A). The AvrB-stimulated phosphorylation of MPK4 correlated with greater MPK4 kinase activity as indicated by stronger phosphorylation of myelin basic protein (MBP; Figure S3B). We further tested if AvrB delivered from the bacterium similarly induced MPK4 phosphorylation in plants. While Arabidopsis *rpm1* plants inoculated with the strain carrying en empty vector showed a MPK4 phosphorylation slightly above the background level (Figure S3C), plants

inoculated with DC3682 (*avrB*) exhibited strong MPK4 phosphorylation (Figures 3B and S3C). The DC3682 (pDSK)-induced MPK4 phosphorylation was significantly less than observed in a prior report (Qiu *et al.,* 2008), likely because of different time points were used as PAMP-triggered MPK activation is known to be transient and time-sensitive. Ong and Innes (2006) showed that AvrB enhances the growth of *P. syringae* on susceptible cultivars of soybean and that this virulence activity is blocked by specific amino acid substitutions at threonine 125 (Thr125Ala), arginine 266 (Arg266Gly), and aspartate 297 (Asp297Ala). We tested if these mutants were able to induce MPK4 phosphorylation. The *avrB*-induced MPK4 phosphorylation was diminished by the $avrB^{T125A}$, $avrB^{R266G}$ and $avrB^{D297A}$ substitutions (Figs. 3B and 3C), indicating that the induction of MPK4 phosphorylation is correlated with the virulence function of AvrB.

We next determined if *avrB* mutant forms that are unable to induce MPK4 phosphorylation are capable of inducing *PDF1.2* expression. While the bacterial strain carrying WT *avrB* induced *PDF1.2* expression by ~3 fold compared to the strain carrying an empty vector, the strains carrying $avrB^{T125A}$ and $avrB^{R266G}$ were completely unable to induce *PDF1.2* expression (Figure 4A), indicating that the virulence function of AvrB in soybean is correlated with its ability to induce both MPK4 phosphorylation and JA signaling in Arabidopsis.

To further determine a role of MPK4 in the AvrB-induced JA responses and susceptibility in plants, we crossed the *AvrB-3xFLAG/rpm1* transgenic line (Shang *et al.,* 2006) with the *mpk4* mutant (Petersen *et al.,* 2000) to generate *AvrB-3xFLAG/MPK4/rpm1* and *AvrB-3xFLAG/mpk4/rpm1* plants. Figure 4B shows that the *PDF1.2* expression was reduced to background level in *AvrB-3xFLAG/mpk4/rpm1* plants, suggesting that MPK4 is required for the AvrB-induced JA responses. Furthermore, the *AvrB* transgene enhanced the growth of a nonpathogenic *P. syringae* mutant *hrcC* in *AvrB-3xFLAG/MPK4/rpm1* but not *AvrB-3xFLAG/mpk4/rpm1* plants (Figure 4C). Together these results are consistent with a role of MPK4 in the AvrB-induced disease susceptibility and hormone signaling.

AvrB Interacts with MPK4

The involvement of MPK4 in AvrB-induced JA signaling and disease susceptibility prompted us to test protein-protein interaction between AvrB and MPK4. *In vitro* pull-down experiments showed that AvrB is capable of interacting with MPK4 (Figure 5A). The structure of AvrB suggests that AvrB may act as an enzyme related to protein kinases, with amino acid residue D297 as a potential active site. This residue was previously shown to be required for AvrB virulence function, but not its ability to interact with RIN4 (Ong and Innes, 2006). The $AvrB^{D297A}$ substitution also did not affect AvrB-MPK4 interaction, suggesting that this residue may be specifically required for the activation of MPK4 phosphorylation, but not protein-protein interaction.

Co-IP assays showed that transgenic AvrB can interact with MPK4 *in vivo* (Figures 5B and S4). Because RAR1 is required for AvrB function, we tested if RAR1 plays a role in AvrB-MPK4 interaction *in vivo*. The association of AvrB with MPK4 was largely impaired in *AvrB-3xFLAG/rar1-29/rpm1* plants (Figure 5B), indicating that the interaction is specific and that RAR1 is required for stable AvrB-MPK4 interaction in plants. Furthermore, the AvrB-induced phosphorylation, but not the basal phosphorylation of MPK4, was compromised in *AvrB-3xFLAG/rar1-29/rpm1* plants (Figure 5C). These results suggest that AvrB, upon the association with RAR1, interacts with and induces the phosphorylation of MPK4 *in vivo*.

HSP90 Positively Regulates MPK4 Activity

HSP90 is a molecular chaperone responsible for the maturation of a large number of signaling proteins, particularly protein kinases (Pearl and Prodromou, 2006). The requirement of RAR1 in AvrB-induced phosphorylation of MPK4 prompted us to test if HSP90 interacts with and regulates MPK4. A pull-down assay failed to detect a direct interaction between MPK4 and RAR1 *in vitro* (Figure 6A). However, MPK4 is capable of interacting with HSP90 and SGT1b protein *in vitro* (Figure 6B). To determine if MPK4 is capable of interacting with the HSP90 *in vivo,* we generated *NP-MPK4-3xFLAG* transgenic plants in which *MPK4-3xFLAG* was expressed under the control of *MPK4* native promoter. A co-IP assay showed that MPK4-3xFLAG protein indeed interacted with the endogenous HSP90 protein (Figure 6C).

We next determined if HSP90 plays a role in MPK4 kinase activity following normal induction by PAMPs. WT Arabidopsis plants were co-infiltrated with GDA and the *hrcC* mutant strain, which is considered to carry a collection of PAMPs, and the endogenous MPK4 protein was isolated by immunoprecipitation. An *in vitro* kinase assay demonstrated that the MPK4 activity was greatly reduced in GDA-treated plants (Figure 6D). We further tested the role of HSP90 in MPK4 activation by flg22, a well studied PAMP, by transiently expressing MPK4-3xFLAG in protoplasts. While the control protoplasts treated with flg22 showed strong MPK4 activity, protoplasts exposed to GDA showed a marked reduction of MPK4 activity. Furthermore, JA-induced *PDF1.2* expression was completely abolished by GDA treatment (Figure 6E). Together these results indicated that MPK4 is directly regulated by HSP90, with the latter playing a critical role in regulating MPK4 activity and JA signaling.

RIN4 Positively Regulates *PDF1.2* Expression Downstream of MPK4

Because AvrB is known to interact with RIN4 (Mackey *et al.,* 2002), and RIN4 negatively regulates disease resistance to *P. syringae* (Kim *et al.,* 2005), we asked if RIN4 is involved in AvrB-induced *PDF1.2* expression and plant susceptibility. Figure 7A shows that *avrB* was able to induce *PDF1.2* expression in *rps2/rpm1* mutant plants, but not *rps2/rpm1/rin4* mutant plants, indicating that RIN4 is required for AvrB to induce JA signaling. We next transformed the *AvrB* transgene in *rps2/rin4* mutant plants. The transgenic plants did not show enhanced growth of the *P. syringae hrcC* bacteria (Figure S5A). Together these results are consistent with a role of RIN4 in the AvrB-induced *PDF1.2* expression and plant susceptibility. To test if RIN4 normally plays a role in JA responses, we treated *rps2/rin4* mutant plants with MeJA and examined *PDF1.2* expression. Figure 7B shows that the JA-induced *PDF1.2* expression was significantly compromised in *rps2/rin4* mutant compared to the *rps2* control plants. To further determine a role of RIN4 in JA responses, we examined a transgenic line that overexpresses *RIN4* (Kim *et al.,* 2005). Figure 7C shows that plants overexpressing RIN4 constitutively expressed *PDF1.2*. Together these results indicate that RIN4 positively modulates JA responses.

Because both RIN4 and MPK4 interacted with AvrB and were required for the AvrB-induced JA responses, we tested if RIN4 and MPK4 interact. A GST pull-down assay showed that RIN4 and MPK4 indeed interacted *in vitro* (Figure 7D), and a co-IP assay showed that they interacted *in vivo* (Figure 7E). We next asked if MPK4 is capable of phosphorylating RIN4 *in vitro*. The MPK4-3xFLAG was expressed in protoplasts, induced by flg22, and MPK4-3xFLAG was isolated by anti-FLAG immunoprecipitation. The isolated MPK4 strongly phosphorylated recombinant RIN4 protein (Figures 7F and S5B), suggesting that RIN4 is a substrate for MPK4. In the *rps2/rin4* mutant plants, the DC3682 (*avrB*) bacteria induced MPK4 phosphorylation normally (Figure S5C), indicating that *RIN4*

is not required for the AvrB-induced MPK4 phosphorylation. Taken together, these results suggest that RIN4 acts downstream of MPK4.

DISCUSSION

The study presented here shows that AvrB enhances plant susceptibility by promoting the phosphorylation of MPK4, which subsequently perturbs hormone signaling. Genetic and biochemical studies showed that this process is assisted by the molecular chaperone HSP90 and its co-chaperone RAR1. The previously identified AvrB-interacting protein RIN4 appears to act down-stream of MPK4 to regulate JA responses. Thus, the results uncover an unexplored regulatory mechanism for MPK4 that is actively promoted by a bacterial effector protein to enhance plant susceptibility.

Our observation that the plasmamembrane localized AvrB protein (Nimchuk *et al*., 2000) interacts with MPK4 is consistent with previous reports on MPK4. MPK4 is activated by MAP kinase kinases MKK1 and MKK2 during normal signaling in plants (Qiu *et al.,* 2008; Gao *et al.,* 2008). MKK1-MPK4 and MKK2-MPK4 complexes are located in both plasmamembrane and nucleus (Gao *et al.,* 2008). It is important to note that the AvrB-induced MPK4 phosphorylation and JA responses occurs when AvrB is delivered by the *P. syringae* bacterium. AvrRpm1, another *P. syringae* effector known to interact with RIN4 (Mackey *et al.,* 2002), does not appear to induce MPK4 phosphorylation (Qiu *et al.,* 2008), suggesting that the induction of MPK4 phosphorylation is specific to AvrB. Several lines of evidence indicate that the AvrB-induced MPK4 phosphorylation is biologically significant. The loss of *MPK4* renders plants unable to express *PDF1.2* in response to JA (Petersen *et al.,* 2000), it also prevents transgenic *AvrB* from inducing *PDF1.2* expression and susceptibility to bacteria. Furthermore, AvrB mutants that are compromised in virulence function were unable to induce MPK4 phosphorylation. These mutants are also unable to induce JA responses.

Previous studies have indicated that MPK4 indirectly impact multiple hormone signaling pathways (Brodersen *et al*., 2006; Petersen *et al*., 2000). *mpk4* plants show elevated SA signaling but a lack of JA-induced expression of *PDF1.2* and are defective in the expression of a subset of ET pathway genes. The mechanism by which MPK4 regulates these pathways is not well understood. MPK4 has also been shown to sequester the transcription factor WRKY33 in the nucleus (Qiu *et al*., 2008). Treatment of plants with *P. syringae* or flg22 leads to a release of WRKY33 which then activate the transcription of a small number of genes including *PAD3*, which encodes a cytochrome P450 monoxygenase required for the biosynthesis of camalexin (Zhou *et al*., 1999). However, camalexin is not required for *P. syringae* resistance (Glazebrook and Ausubel, 1994), and WRKY33 does not appear to account for the hormone-response genes regulated by MPK4 (Qiu *et al.,* 2008). *mpk4* mutants over-accumulate *EDS1* and *PAD4* transcripts (https://www.genevestigator.com/gv; Zimmermann *et al*., 2004), which likely explains the overaccumulation of EDS1 protein in *mpk4* (Brodersen *et al*., 2006). The JA-induced *PDF1.2* expression can be restored by introducing *eds1* mutation into *mpk4* plants, suggesting that MPK4 modulates hormone signaling through EDS1 and PAD4. However, the interpretation is complicated by complex cross-talks among SA, JA and ethylene pathways. Indeed, EDS1 and PAD4 only accounted for some, but not all, MPK4-regulated ET response genes (Brodersen *et al*., 2006), suggesting that additional mechanisms remain to be found. It remains to be determined if the *AvrB*-induced *PDF1.2* expression and disease susceptibility involve *EDS1* and *PAD4*.

The AvrB-induced phosphorylation of MPK4 is consistent with the possibility that AvrB is related to protein kinases (Lee *et al.,* 2004; Desveaux *et al*., 2007). R266 of AvrB makes direct contact with nucleotide, whereas D297 is a potential active site of AvrB. Both

residues are required for MPK4 phsosphorylation. An intriguing possibility is that AvrB functionally mimics MKKs to phosphorylate MPK4. However, attempts to detect MPK4 phosphorylation by AvrB *in vitro* were unsuccessful. While a direct phosphorylation of MPK4 by AvrB cannot be ruled out, it is equally possible that AvrB may enhance the activity of upstream kinases, such as MKK1 and MKK2, to induce MPK4 phosphorylation.

HSP90 is a molecular chaperone responsible for the maturation and stability of a large number of signaling proteins in animals and fungi. In plants, the HSP90 complex is known to regulate resistance protein stability, but a role in regulating other signaling proteins has not been demonstrated. Our analysis demonstrated that Arabidopsis MPK4 interacts with HSP90, and MPK4 kinase activity is promoted by HSP90.

Our results showed that AvrB can directly interact with RAR1, MPK4, and RIN4, as indicated by our *in vitro* pull-down and co-IP assays. It is not clear if AvrB interacts with the three proteins simultaneously or sequentially. It remains to be determined if different domains in AvrB are involved in the interaction with different proteins. Nonetheless, the AvrB appears to interact with a CHORD domain in RAR1, and this interaction is required for AvrB-HSP90 and AvrB-MPK4 interactions *in vivo*. Furthermore, the AvrB-induced MPK4 phospohrylation also requires RAR1. Interestingly, RAR1 is known to enhance SGT1-HSP90 interaction (Boter *et al.,* 2007). It is possible that RAR1 plays an important role in assisting protein-protein interactions in the HSP90 chaperone complex to promote the maturation of HSP90-associated proteins. These results are consistent with our findings that RAR1 is required for AvrB to induce plant susceptibility (Shang *et al.,* 2006) and JA responses (this study). RAR1 does not appear to affect basal MPK4 phosphorylation. Instead, it specifically recruits AvrB to the HSP90 complex to regulate MPK4 phosphorylation.

RIN4 is known to be targeted by AvrB to trigger RPM1 resistance. Our results indicate that the AvrB-RIN4 interaction also contributes to AvrB-induced JA responses and plant susceptibility. RIN4 is required for JA-induced expression of *PDF1.2,* and RIN4 overexpression resulted in increased *PDF1.2* expression in the absence of JA, indicating that RIN4 positively modulates JA responses. AvrB has been reported to induce RIN4 phosphorylation (Mackey *et al.,* 2002). We were unable to detect RIN4 phosphorylation *in vivo* either in the presence or absence of AvrB for reasons unknown, which prevented us from testing whether MPK4 is required for AvrB-induced phosphorylation of RIN4. Nonetheless, our protein-protein interaction results indicated that RIN4, MPK4, and AvrB may exist in the same protein complex. It is formally possible that the AvrB-induced RIN4 phosphorylation is mediated by MPK4. This is supported by the *in vitro* phosphorylation of RIN4 by MPK4 in this study. Consistent with the idea that RIN4 acts downstream of MPK4, the *rin4* mutant does not affect AvrB induces MPK4 phosphorylation.

Taken together, our results indicate that RAR1, HSP90, MPK4, and RIN4 constitute a new pathway modulating JA signaling and, possibly, other hormone signaling. This pathway is directly targeted by AvrB to increase plant susceptibility to *P. syringae* bacteria. These findings are consistent with the proposal that RIN4 is a virulence target for AvrB to enhance plant susceptibility (Dangl and Jones, 2001) and that the resistance protein RPM1 has evolved to “guard” RIN4 to trigger disease resistance in response to AvrB.

Several recent reports showed that *P. syringae* effectors, including HopM1, HopU1, AvrPto, AvrPtoB, and HopAI1 (Nomura *et al.,* 2006, Fu *et al*., 2007, He *et al.,* 2006; Xiang *et al.,* 2008; Gohre *et al.* 2008; Gimenez-Ibanez *et al*., 2009, Zhang *et al.,* 2007), directly block immune responses by inhibiting or degrading host proteins required for plant immunity. The results presented here illustrate how a *P. syringae* effector protein promotes a host pathway

to induce inappropriate defenses that, in turn, make the plant more susceptible to *P. syringae*.

EXPERIMENTAL PROCEDURES

Arabidopsis Mutants and Transgenic Lines

The following Arabidopsis materials were used in this study: *rpm1* (formerly described as *rps3-1*; Bisgrove *et al.,* 1994), *rar1-29* (Shang *et al.,* 2006), *tao1-11* (Eitas *et al.,* 2008), *AvrB-3xFLAG/rpm1* (Shang *et al.,* 2006), *mpk4* (Petersen *et al.*, 2000), *rps2/rpm1*/*rin4* (kim *et al,.* 2005), *rps2*/*rin4* (kim *et al,.* 2005), and *Dex::RIN4* (kim *et al,.* 2005). All materials are in Col-0 background except for *mpk4,* which is in Ler background..

Construction of Transgenic Plants Expressing *MPK4-3xFLAG* under the Control of *MPK4* Native Promoter

The *MPK4* coding sequence was PCR-amplified from cDNA using primers 5'-AGACTCGAGATGTCGGCGGAGAGTTGTTTCG-3' and 5'-ATAACTAGTCACTGAGTCTTGAGGATTGAAC-3', and inserted between the *Xho*I and *Csp45*I sites of pUC19-35S-FLAG-RBS (Li *et al.* 2005) to generate pUC19-35S-MPK4-FLAG-RBS. A 0.9kb upstream sequence of *MPK4* was PCR-amplified using primers 5'-GAGGAATTCTCAATCGGTGCTAAGCTATAAC-3' and 5'-TGTGGTACCCGGAGCAAAATTCCTCACAAC-3'. The 35S promoter of pUC19-35S-MPK4-FLAG-RBS was replaced with the *MPK4* upstream fragment to generate pUC19-NP-MPK4-FLAG-RBS. Then the NP-MPK4-FLAG-RBS was transferred to pCAMBIA1300 with the *EcoR*I and *Sal*I sites. The construct was transformed into *Arabidopsis* plants (Col-0) by *Agrobacterium*-mediated transformation. Transgenic plants were selected on Murashige and Skoog plates containing 25 mg/L hygromycin.

Construction of *AvrB-3xFLAG/rar1-29/rpm1*, *AvrB-3xFLAG/mpk4/rpm1* and *AvrB-3xFLAG/tao1/rpm1*

An estradiol-inducible *AvrB* transgenic line *AvrB-3xFLAG/rpm1* (Shang *et al.*, 2006) was crossed to *mpk4* and *tao1-11* mutant lines to generate *AvrB-3xFLAG /mpk4/rpm1* and *AvrB-3xFLAG/tao1/rpm1* lines. The corresponding control lines *AvrB-3xFLAG /MPK4/rpm1* and *AvrB-3xFLAG/TAO1/rpm1* were siblings of the *AvrB-3xFLAG /mpk4/rpm1* and *AvrB-3xFLAG/tao1/rpm1* mutant lines. The presence of *AvrB-3xFLAG* and *sgt1b*, *mpk4* and *tao1* mutations was confirmed by PCR-based genotyping. The *mpk4* mutation was caused by a transposon insertion (Petersen *et al.,* 2000). The presence of WT *MPK4* sequence was verified by using primers 5'- TTGAAGTTCTCTCTCTGCGG-3' and 5'-GTATGTTCCTCCTCTTCGTC-3'. The presence of the transposon was verified by using primers 5'-TATGACTGGGCACAACAGAC-3' and 5'-TATGTCCTGATAGCGGTCCG-3'. The *tao1-11* mutant carries a T-DNA insertion. The presence or absence of the WT *TAO1* sequence was verified by using primers *5'*-CCCCTAAAGTTGGTTTTGAGC-3' and 5'-AAATCAGGAAGCTCCTTCAGG-3'. The presence of the *AvrB-3xFLAG* transgene was verified by using primers 5'-CAGTAAGTCGAATACGCCTGAA-3' and 5'-AAATCGGAAGATATTGCTTGTC-3'. F2 plants that were heterozygous for *mpk4* were inoculated with *P. syringae* DC3000 (*avrB*) at 10^8 CFU/ml, and plants failed to develop HR were identified as homozygous *rpm1* plants. Plants carrying *AvrB-3xFLAG* that were homozygous for *mpk4* were then identified in the F3 generation. To generate *AvrB-3xFLAG/rar1-29/rpm1* plants, the *AvrB-3xFLAG* was introduced into *rar1-29/rpm1* plants (Shang *et al.,* 2006) by transformation, and a line expressing a similar amount of AvrB-3xFLAG compared to the *AvrB-3xFLAG/rpm1* transgenic line was used for experiments.

Bacterial Strains

Bacterial strains used in this study include DC3682 and a *hrcC* mutant derived from DC3000 (He *et al.,* 2004, Yuan *et al.,* 1996). WT *avrB*, $avrB^{T125A}$, $avrB^{R266G}$ and $avrB^{D297A}$ mutant plasmids (Ong and Innes, 2006) were introduced into DC3682 in this study.

Plant Treatment

Five-week-old plants were sprayed once with 20 μM estradiol solution containing 0.01% silwet L-77 to induce the transgenic AvrB-3xFLAG expression. For bacterial delivery of AvrB, plants were infiltrated with DC3682 bacteria carrying *avrB* at 2×10^6 CFU/mL (for gene expression assay) or 2×10^8 CFU/mL (for MPKs activation assay). For JA-treatment, plants were infiltrated with 50 μM MeJA. To inhibit HSP90 activity, leaves were infiltrated with 2.5 μM GDA in 0.1% DMSO. 0.1% DMSO was used as control.

Bacterial Growth Assay

Five-week-old plants pre-induced with estradiol for 48 hr were infiltrated with *hrcC* mutant bacteria (5×10^5 CFU/ml), and bacterial populations were determined at the indicated times. Each data point consisted of at least six replicates. To inhibit HSP90 activity, GDA was mixed with the bacterial suspension prior to infiltration. To determine whether GDA alone affects bacteria growth, *P. syringae hrcC* bacteria was grown in luquid KB medium with GDA, and optical density at OD_{600} was measured hourly during the exponential phase.

Quantitative RT-PCR

The transcript levels of *PDF1.2* and *THI2.1* were determined by real-time RT-PCR using SYBR Premix Ex Taq™ kit (TaKaRa) following the manufacturer's instructions. Actin was used as a reference gene. Primers 5'-GGTGTCATGGTTGGTATGGGTC-3' and 5'-CCTCTGTGAGTAGAACTGGGTGC-3' were used for *PDF1.2,* 5'-TTGGGTAAACGCCATTCTCG-3' and 5'-ACATTGTTCCGACGCTCCAT-3' were used for *THI2.1,* and primers 5'-TGGTGGAAGCACAGAAGTTG-3' and 5'-GATCCATGTTTGGCTCCTTC-3' were used for actin.

Constructs for GST- and His- Fusion Proteins

To construct GST-fusion plasmids, coding sequences were PCR-amplified from cDNA and inserted into pGEX-6P-1 (GE Healthcare Life Science) between *BamH*I and *Sal* I (for GST-HSP90.1, GST-MPK4, and GST-RAR1), *EcoR*I and *Sma*I (for GST-SGT1b) or *EcoR*I and *Xho*I (for GST-CHORDI-CC and GST-CHORDI). To construct His-fusion plasmids, coding sequences were inserted into pET30a (company name) between *BamH*I and *Sal*I (for MPK4-His and HSP90-His), *Kpn*I and *EcoR*I (for SGT1b-His) or *Kpn*I and *Sal*I (for AvrB-His), or *Bam*HI and *Sal*I (for RIN4-His). PCR primers used for these constructs were: 5'-ATTGAATTCGCCAAGGAATTAGCAGAGAAAG-3' and 5'-ATGCCCGGGATACTCCCACTTCTTGAGCTCC-3' for GST-SGT1b, 5'-CTCGGATCCATGGAAGTAGGATCTGCAACG-3' and 5'-ATTGTCGACGACCGCCGGATCAGGGCTGCTG-3' for GST-RAR1, 5'-AGCGAATTCATGGAAGTAGGATCTGCAAC-3' and 5'-ATCCTCGAGTCATTGATTAATGTCTATCAC-3' for GST-CHORDI-CC, 5'-AGCGAATTCATGGAAGTAGGATCTGCAAC-3' and 5'-ATCCTCGAGTCAAACTGGTTTCTCAGTTGTG-3' for GST-CHORDI, 5'-TAGGATCCGTTGCGATGGCGGATGTTCAG-3' and 5'-TTAGTCGACTTCCTCCATCTTGCTCTC-3' for GST-HSP90.1 and HSP90.1-His, 5'-ATAGGATCCATGTCGGCGGAGAGTTGTTTCG-3' and 5'-ATAGTCGACCACTGAGTCTTGAGGATTGAAC-3' for GST-MPK4 and MPK4-His, 5'-

AGAGGTACCATGGCCAAGGAATTAGCAGAG-3' and 5'-ATGGAATTCATACTCCCACTTCTTGAGCTCC-3' for SGT1b-His, 5'-ATCGGTACCATGGGCTGCGTCTCGTC-3' and 5'-ATAGTCGACAAAGCAATCAGAATCTAGC-3' for AvrB-His, 5'-GACGGATCCATGGCACGTTCGAATGTACC-3' and 5'-GAGGTCGACTTTTCCTCCAAAGCCAAAGC-3' for RIN4-His.

GST Pull-Down and His Pull-Down Assays

For GST pull-down assays, bacterial cells expressing GST-fusion protein were lysed in a buffer containing: 25 mM Tris, PH7.5, 150 mM NaCl, 3 mM DTT. Approximately 20 mg bacterial lysate was incubated with 30 μL glutathione agarose beads in a centrifuge tube for 30 min, and washed once with GST wash buffer: 25 mM Tris, PH7.5, 150 mM NaCl, 3 mM DTT and 0.2% triton x-100. 20 mg bacterial lysate containing His-tagged protein was then added to the agarose beads and incubated for 1 hr and washed three times with the wash buffer. The bound protein was eluted with 100 μl 15 mM reduced GSH (25mM Tris, PH9.0). Approximately 1% of input and one fifth of eluted protein were subjected to immunoblot analysis.

For His pull-down assays, approximately 20 mg bacterial lysate containing His-tagged protein was incubated with 30 μL Ni-NTA agarose (QIAGEN) in a centrifuge tube for 30 min, washed once with His wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0). The agarose beads were then incubated with 20 mg bacterial lysate containing GST-tagged protein for 1 hr, and washed three times with the His wash buffer. The bound protein was eluted with 100 μl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH 9.0). Approximately 1% of input and one fifth of eluted protein100 μl were subjected to immunoblot analysis.

Protoplast Preparation and Transfection

Protoplast preparation and transfection were essentially as described (Li *et al.*, 2005), except that the transfected protoplasts were incubated in W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5mM KCl, and 2 mM MES pH 5.7) instead of 0.4 M mannitol.

Immunoprecipitation

To isolate MPK4 and MPK6 proteins for MPK phosphorylation assays, five-week old plants were treated with estradiol for 36 hr to induce AvrB-3xFLAG. Protein was extracted with IP buffer (50 mM HEPES, pH7.5, 50 mM NaCl, 10 mM EDTA, 0.1% Triton, 1mM DTT, 2 mM NaF, and protease inhibitor cocktail; Roche). Approximately 2 mg total protein lysate was incubated for 1 hr with 3 μg anti-MPK4 or anti-MPK6 antibodies at 4°C followed by incubation for 1 hr with 20 μl fast flow protein A agarose beads (Upstate). After washing the beads four times with the IP buffer, the proteins were eluted by boiling in 60 μl Laemmli loading buffer. For MPK4 activity assays, plants were infiltrated with 2×10^8 CFU/ml *hrcC* mutant bacteria along with GDA and incubated for 6 hr prior to protein extraction and immunoprecipitation. Protein A agarose beads containing MPK4 were used for MPK activity assays.

FLAG-tagged proteins were isolated by immunoprecipitation as described (Shang *et al.,* 2006). To detect AvrB-3xFLAG-HSP90 interaction in plants, *AvrB-3xFLAG/rpm1* transgenic plants were sprayed with estradiol 48 hr prior to protein extraction. To detect MPK4-3xFLAG-HSP90 interaction in plants, protein was extracted from *NP-MPK4-3xFLAG* transgenic plants for immunoprecipitation. To isolate MPK4-3xFLAG protein from protoplasts, protoplasts were transfected with the 35S::MPK4-3xFLAG

plasmid and then induced with 100 nM flg22 15 min prior to immunoprecipitation. Where indicated, GDA or 0.1% DMSO was added 3 hr prior to flg22 treatment.

To isolate HA-tag proteins, approximately 200 μg total protein extract was incubated at 4°C for 1 hr with 2 μg mouse anti-HA monoclonal antibody (TianGen) followed by incubation with fast flow protein A agarose beads (Upstate) for 2 hours. The beads were washed six times with IP buffer, and the bound protein was eluted by boiling in 60 μl Laemmli loading buffer.

MPK Activity Assay

The activity of MPK4 and MPK4-3xFLAG protein was determined by using myelin basic protein as a substrate. 4 μl protein A agarose beads containing IgG-MPK4 complex or 2 μL MPK4-3xFLAG eluted from anti-FLAG immunoprecipitation were incubated with 1 μg MBP in 20 μl reaction buffer containing: 50 mM HEPES, PH7.4, 10mM $MgCl_2$, 1mM DTT, 50 uM ATP and 1 μCi γ-^{32}P ATP. Reactions were allowed to proceed for 30 min at 30°C and terminated by adding Laemmli buffer and boiling for 5 min. The phosphorylation on MBP was determined by electrophoresis and autoradiography.

Immunoblot Analysis

Primary antibodies used for immunoblots included rabbit anti-MPK4 and anti-MPK6 antibodies (Sigma), rabbit anti-HSP90 (Chen *et al.*, 2009), rabbit anti-RAR1 antibodies (Shang *et al.,* 2006), mouse anti-HA monoclonal antibody (TianGen), mouse anti-FLAG monoclonal antibody (Sigma), and rabbit anti-phospho-ERK1 antibodies (Cell signaling). Secondary antibodies included HRP-conjugated mouse anti-rabbit light chain monoclonal antibody (for total and phosphorylated MPK4 and MPK6; CHEMICON), HRP-conjugated goat anti-rabbit antibodies (for HSP90 and RAR1; Sigma), HRP-conjugated goat anti-mouse antibodies (for HA-tagged proteins; Sigma). In co-immunoprecipitation experiments, approximately 1% of input and one third of eluted protein were subjected to immunoblot analysis.

Protein samples were electrophoresed through a 10% SDS/PAGE gel. Protein was electrotransferred to an Immobilon P membrane (Millipore). Immunodetection was performed with a 1:5,000 dilution of anti-RAR1, anti-MPK4, anti-MPK6, anti-HSP90, anti-HA, and anti-FLAG antibodies or a 1:2,500 dilution of anti-phospho-ERK1 or anti-RAR1 antibodies. The blot was then hybridized with HRP-conjugated secondary antibody and visualized with ECL or ECL Plus Western blotting detection reagents (GE Healthcare, Amersham™), following manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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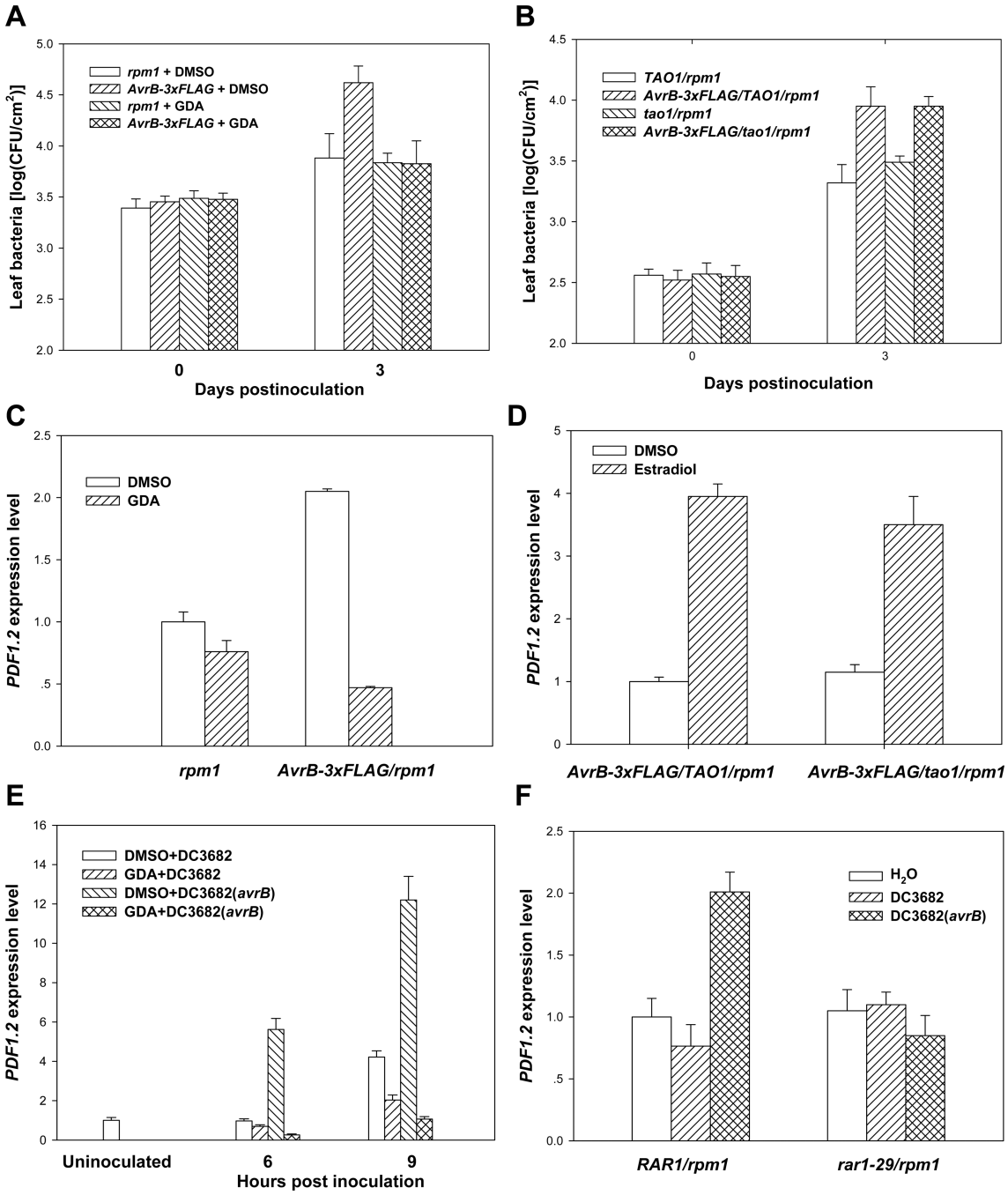

Figure 1. HSP90 Chaperone Components, but Not TAO1, Are Required for AvrB-Induced Susceptibility and *PDF1.2* Expression

(**A**) GDA inhibits *AvrB-3xFLAG* transgene-induced susceptibility. (**B**) TAO1 is not required for AvrB-induced susceptibility. Plants of the indicated genotypes were pre-treated with estradiol, infiltrated with *hrcC* mutant bacteria in the presence of GDA or buffer (DMSO), and bacterial populations within leaves determined at the indicated times (**A** and **B**). (**C**) GDA abolishes *PDF1.2* induction by the *AvrB-3xFLAG* transgene. (**D**) TAO1 is not required for *PDF1.2* induction by the *AvrB-3xFLAG* transgene. (**E**) GDA inhibits *PDF1.2* induction by bacterially delivered AvrB. (**F**) RAR1 is required for *PDF1.2* induction by bacterially delivered AvrB. Arabidopsis plants of the indicated genotype were infiltrated

with estradiol for 24 hours (**C** and **D**) or the indicated bacterial strains (**E** and **F**) for 6 and 9 hours (**E**) or 6 hours (**F**), and RNA isolated for quantitative RT PCR. For experiments in **C** and **E**, GDA or buffer (DMSO) was co-infiltrated into the leaves. The *PDF1.2* expression was determined by real-time RT-PCR. Data in are representative of three independent experiments with similar results.

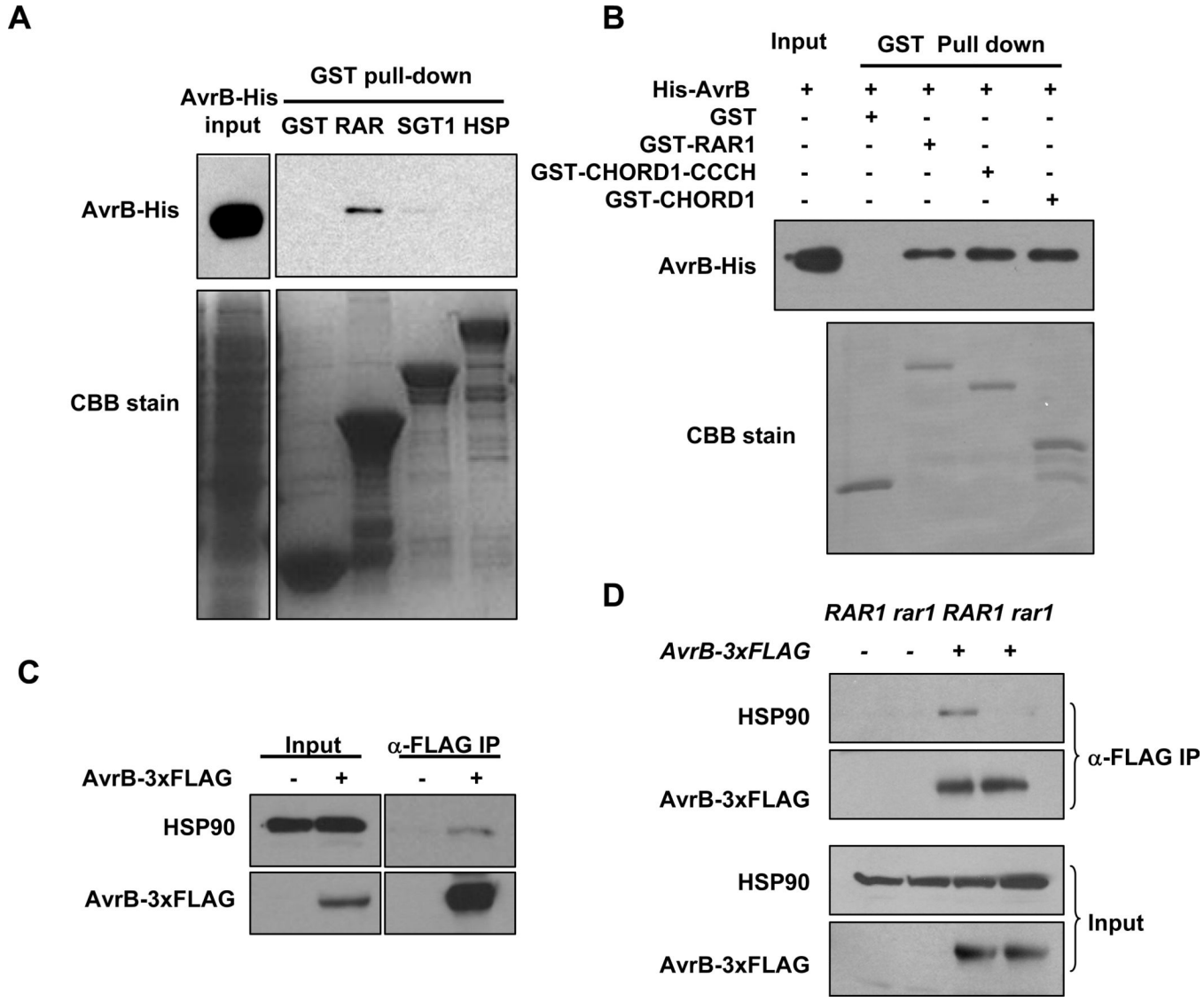

Figure 2. AvrB Interacts with the HSP90 Chaperone

(**A**) AvrB directly interacts with RAR1. An equal amount of AvrB-His recombinant protein was incubated with bacterial lysates containing GST or GST-tagged RAR1, SGT1b, or HSP90. The presence of AvrB-His in the GST pull-down was detected by immunoblot using an anti-His antibody. Coomassie Brilliant Blue (CBB) staining shows the amounts of GST-fusion proteins. (**B**) CHORD1 domain is sufficient for interaction with AvrB. GST-tagged full-length and truncated RAR1 were incubated with equal amount of His-AvrB in a GST pull-down assay, the presence of His-AvrB in the protein complex was detected by anti-His immunoblot. (**C**) AvrB interacts with HSP90 in plants. Arabidopsis *rpm1* plants with (+) or without (−) the *AvrB-3xFLAG* transgene were induced with estradiol, and protein extract was immunoprecipitated with an agarose-conjugated anti-FLAG antibody. Amounts of HSP90 proteins in the immune complex were determined by immunoblot using anti-HSP90 antibodies. (**D**) RAR1 is required for AvrB-HSP90 interaction *in vivo*. Co-immunoprecipitation assay for AvrB-HSP90 interaction was conducted as in (**C**) using Arabidopsis *rpm1* (*RAR1*) or *rpm1/rar1-29* (*rar1*) plants containing (+) or lacking (−) the *AvrB-3xFLAG* transgene.

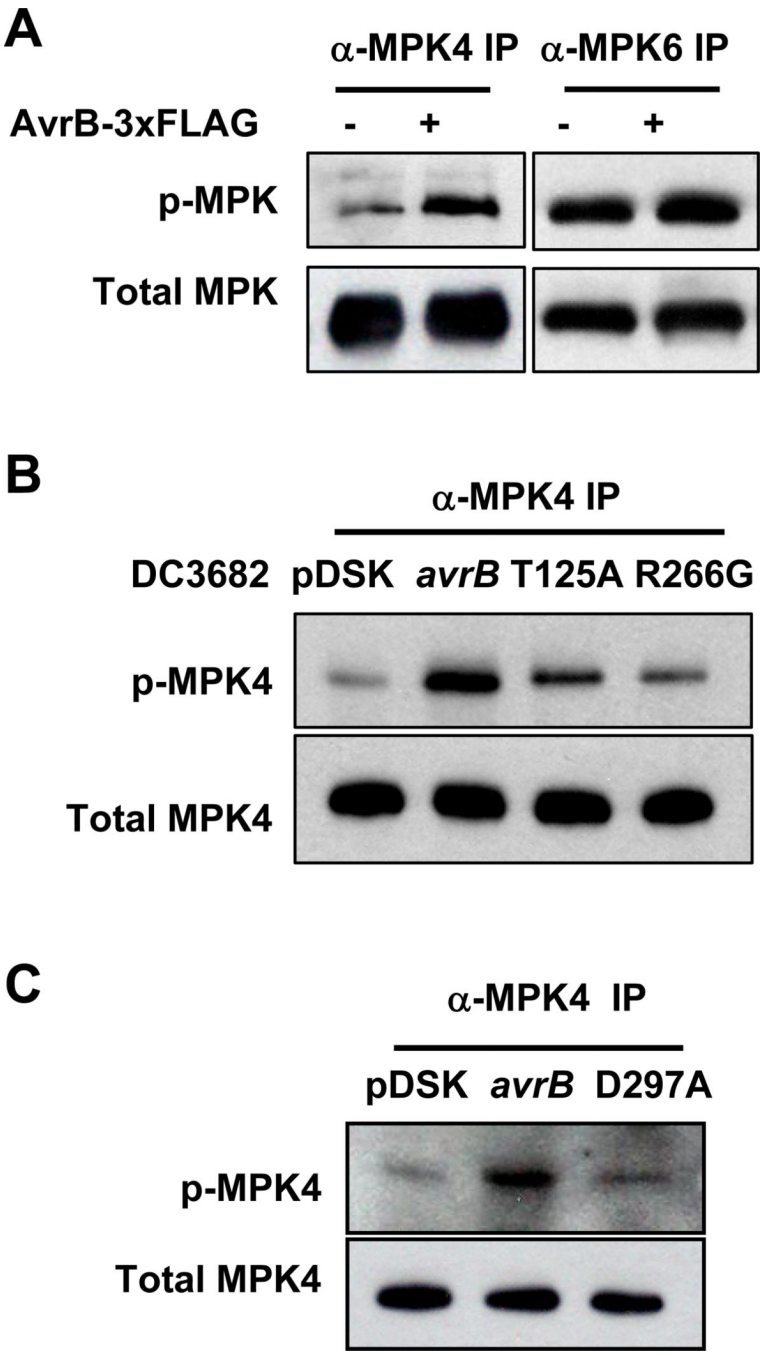

Figure 3. AvrB Directly Interacts with and Induces Phosphorylation of MPK4

(**A**) AvrB-3xFLAG induces MPK4 phosphorylation. Specific anti-MPK antibodies were used to immunoprecipitate MPK4 and MPK6 proteins from estradiol-treated *rpm1* plants with (+) or without (−) the estradiol-inducible *AvrB-3xFLAG* transgene, and the level of dual phosphorylation on MPK4 and MPK6 was determined by immunoblot using anti-phospho-ERK1 antibodies. Equal loading of immunoprecipitated proteins was confirmed by immunoblot using anti-MPK4 and anti-MPK6 antibodies. (**B**) and (**C**) Bacterially-delivered AvrB induces MPK4 phosphorylation. Arabidopsis *rpm1* plants were inoculated with 10^8 CFU/ml DC3682 carrying an empty vector (pDSK), WT *avrB*, *avrB*^{T125A}, *avrB*^{R266G} or *avrB*^{D297A} mutant plasmids for 6 hrs, and the phosphorylation state of the

immunoprecipitated MPK4 was determined by immunoblot using anti-phospho-ERK1 antibodies.

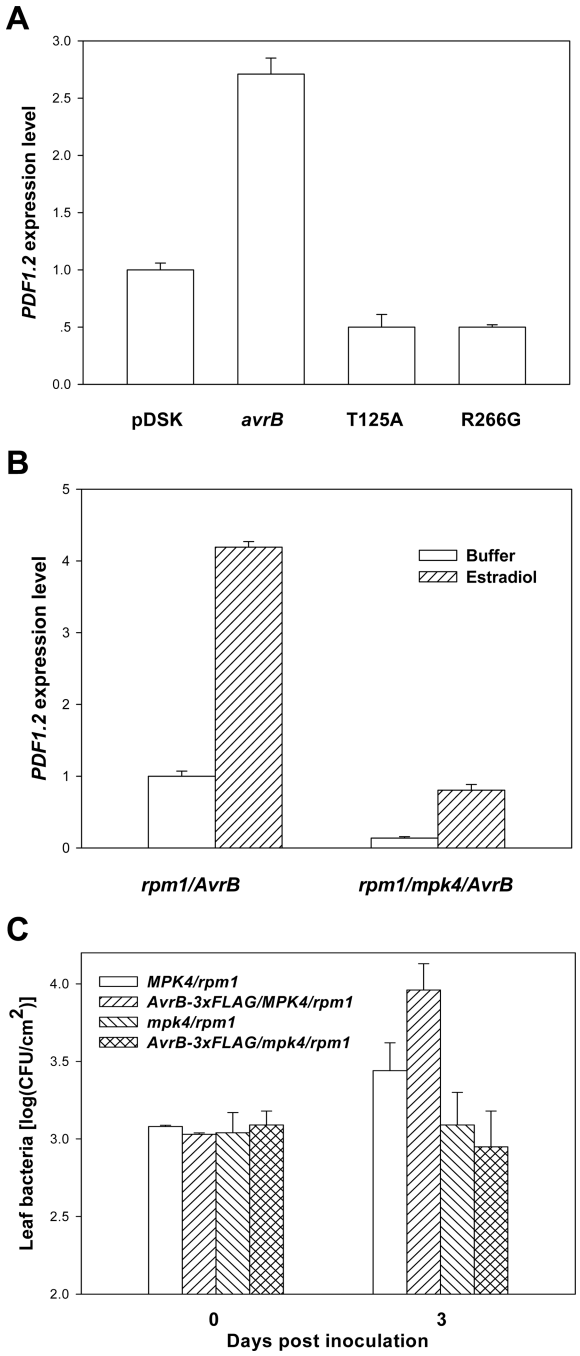

Figure 4. MPK4 Is Required for AvrB-Induced *PDF1.2* Expression and Susceptibility

(**A**) The *avrB*^{T125A} and *avrB*^{R266G} mutations abolish JA-signaling activity. Arabidopsis *rpm1* plants were inoculated with DC3682 carrying an empty vector (pDSK), WT *avrB,* *avrB*^{T125A}, or *avrB*^{R266G}. RNA was isolated 6 hr post inoculation, and the expression of *PDF1.2* was determined by real-time RT-PCR. (**B**) MPK4 is required for *PDF1.2* induction by the *AvrB-3xFLAG* transgene. Plants of the indicated genotypes were treated with estradiol for 24 hrs, and RNA was isolated for real-time RT-PCR. (**C**) The *AvrB-3xFLAG* transgene does not induce susceptibility in the *mpk4* mutant. Plants of the indicated genotypes were pre-treated with estradiol, inoculated with the *P. syringae hrcC* mutant

strain, and the bacterial population in the leaf was determined at the indicated times. The data shown are representative of two independent experiments with similar results.

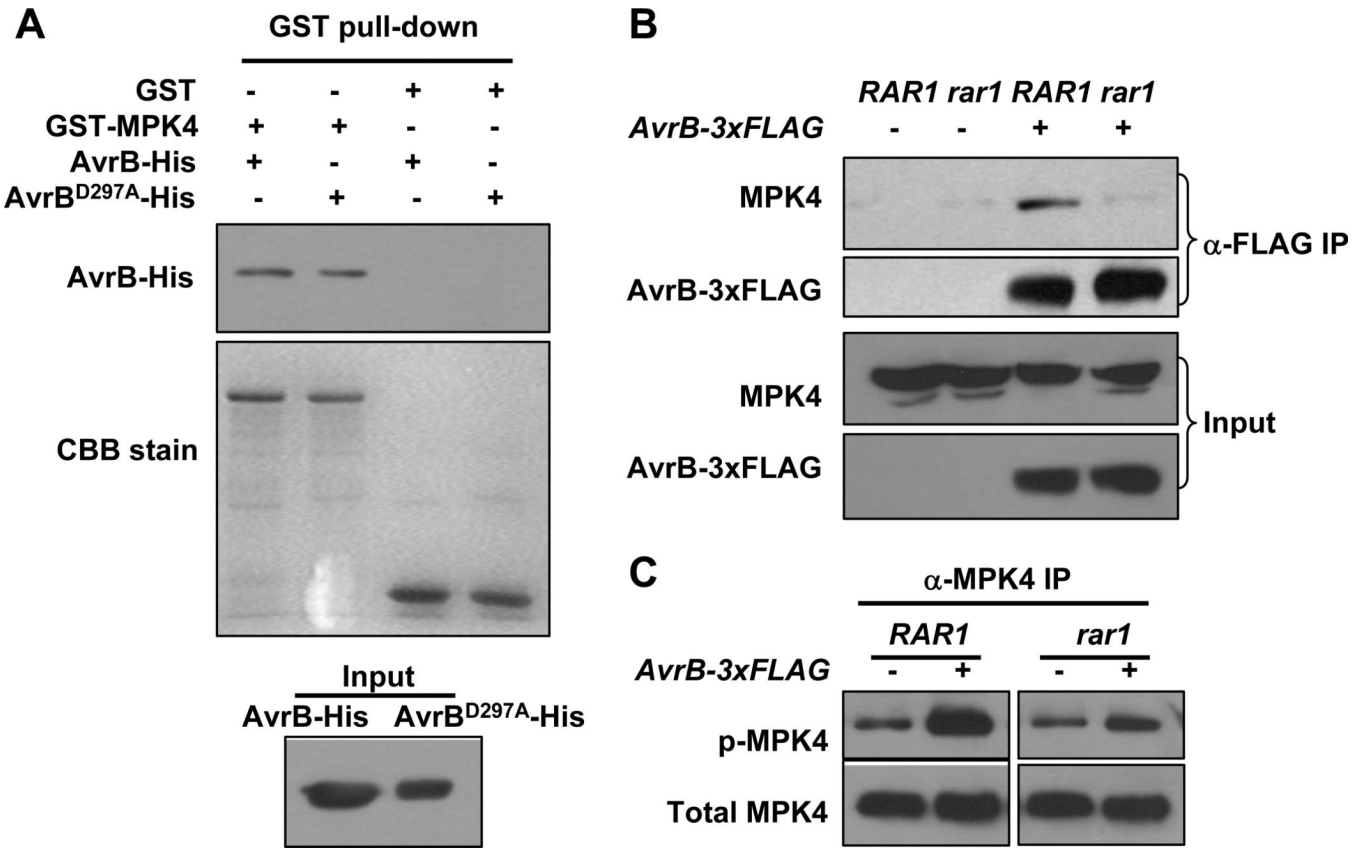

Figure 5. AvrB Interacts with MPK4

(**A**) AvrB interacts with MPK4 *in vitro*. Equal amounts of AvrB-His or AvrB^{D297A}-His were incubated with GST or GST-MPK4, and the presence of AvrB after GST pull-down was detected by immunoblot using anti-His antibody. (**B**) AvrB-3xFLAG interacts with MPK4 in plants in a RAR1-dependent manner. *RAR1/rpm1* (*RAR1*) or *rar1-29/rpm1* (*rar1*) plants with or without the *AvrB-3xFLAG* transgene were induced with estradiol, and protein extracts were immunoprecipitated with agarose-conjugated anti-FLAG antibody. The presence of MPK4 in the immune complex was detected by immunoblot using anti-MPK4 antibodies. (**C**) RAR1 is required for AvrB-3xFLAG-induced MPK4 phosphorylation. Plants of the indicated genotypes were induced with estradiol, and the phosphorylation state of immunoprecipitated MPK4 was determined by immunoblot using anti-phospho-ERK1 antibodies.

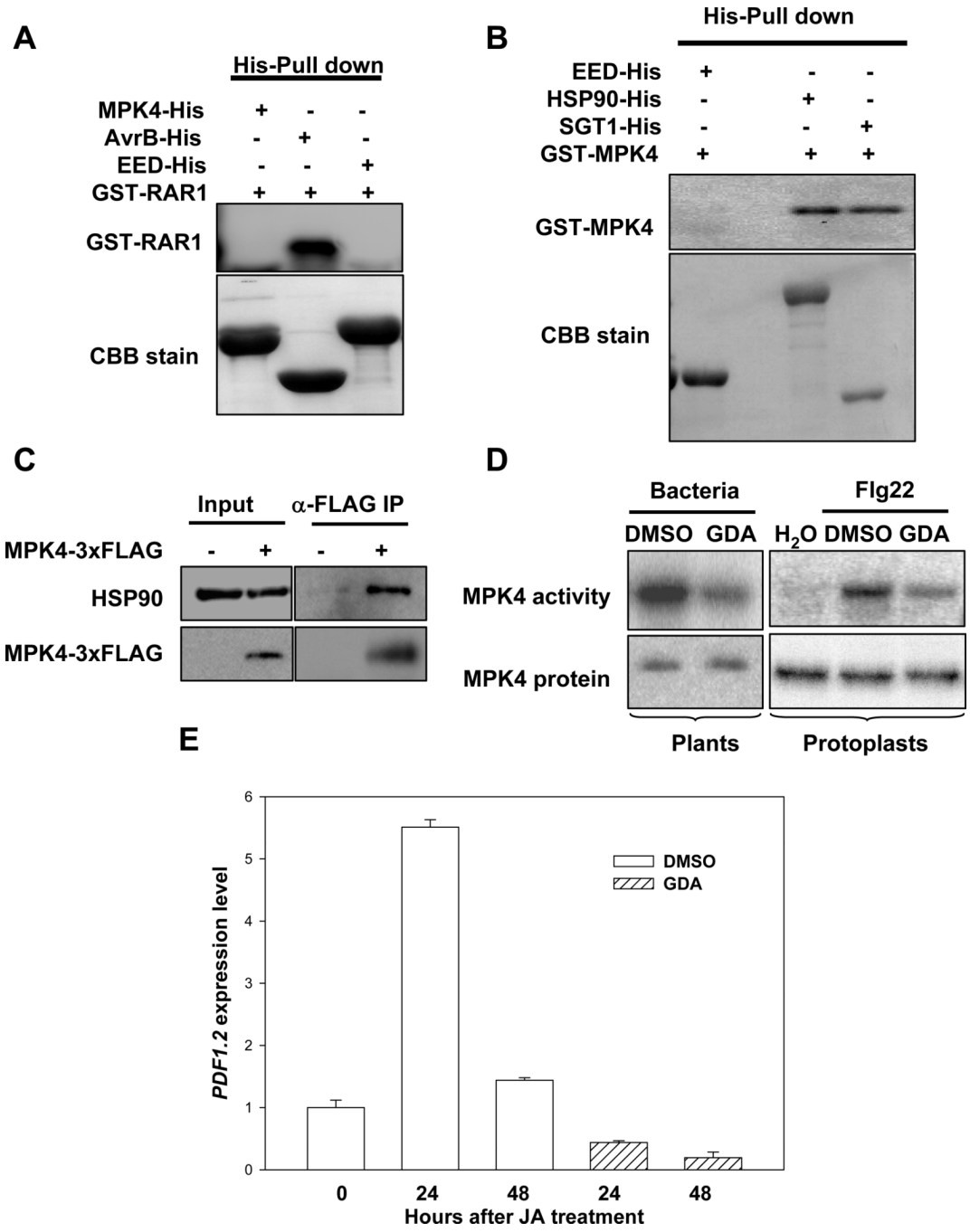

Figure 6. HSP90 Promotes MPK4 Activation

(**A**) MPK4 does not interact with RAR1 *in vitro*. AvrB-His and GST-RAR1 were expressed in *E. coli* and purified proteins used in a pull-down assay. The presence of GST-RAR1 was detected by immunoblot using anti-RAR1 antibodies. The AvrB-His protein was used as a positive control. A His-tagged human WD-repeat protein EED (Han *et al.,* 2007) was included as a negative control. (**B**) MPK4 interacts with SGT1b and HSP90 *in vitro*. GST-MPK4, SGT1b-His, and HSP90-His proteins were expressed in *E. coli*. The presence of GST-MPK4 was detected by immunoblot using anti-MPK4 antibodies. (**C**) MPK4 interacts with HSP90 in plants. Protein extracts from transgenic plants carrying *NP-MPK4-3xFLAG*

were immunoprecipitated with an agarose-conjugated anti-FLAG antibody, and the presence of HSP90 was determined by immunoblot using anti-HSP90 antibodies. The presence of MPK4-3xFLAG was determined by immunoblot using anti-MPK4 antibodies. (**D**) GDA inhibits PAMP-induced MPK4 activation. WT Arabidopsis plants (Col-0) were infiltrated with *hrcC* bacteria in the presence of GDA or buffer (DMSO). Kinase activity of the immunoprecipitated MPK4 protein was determined using an *in vitro* kinase assay employing myelin basic protein (MBP) as a substrate. To assess MPK4 activation in protoplasts, MPK4-3xFLAG was expressed in protoplasts prepared from WT plants, treated with GDA or buffer (DMSO), and induced with flg22. The MPK activity of the immunoprecipitated MPK4-3xFLAG was determined using the *in vitro* kinase assay with MBP as a substrate. (**E**) GDA inhibits MeJA-induced *PDF1.2* expression. WT plants were pretreated with GDA or buffer (DMSO) prior to the application of MeJA. *PDF1.2* expression was determined using real-time RT-PCR.

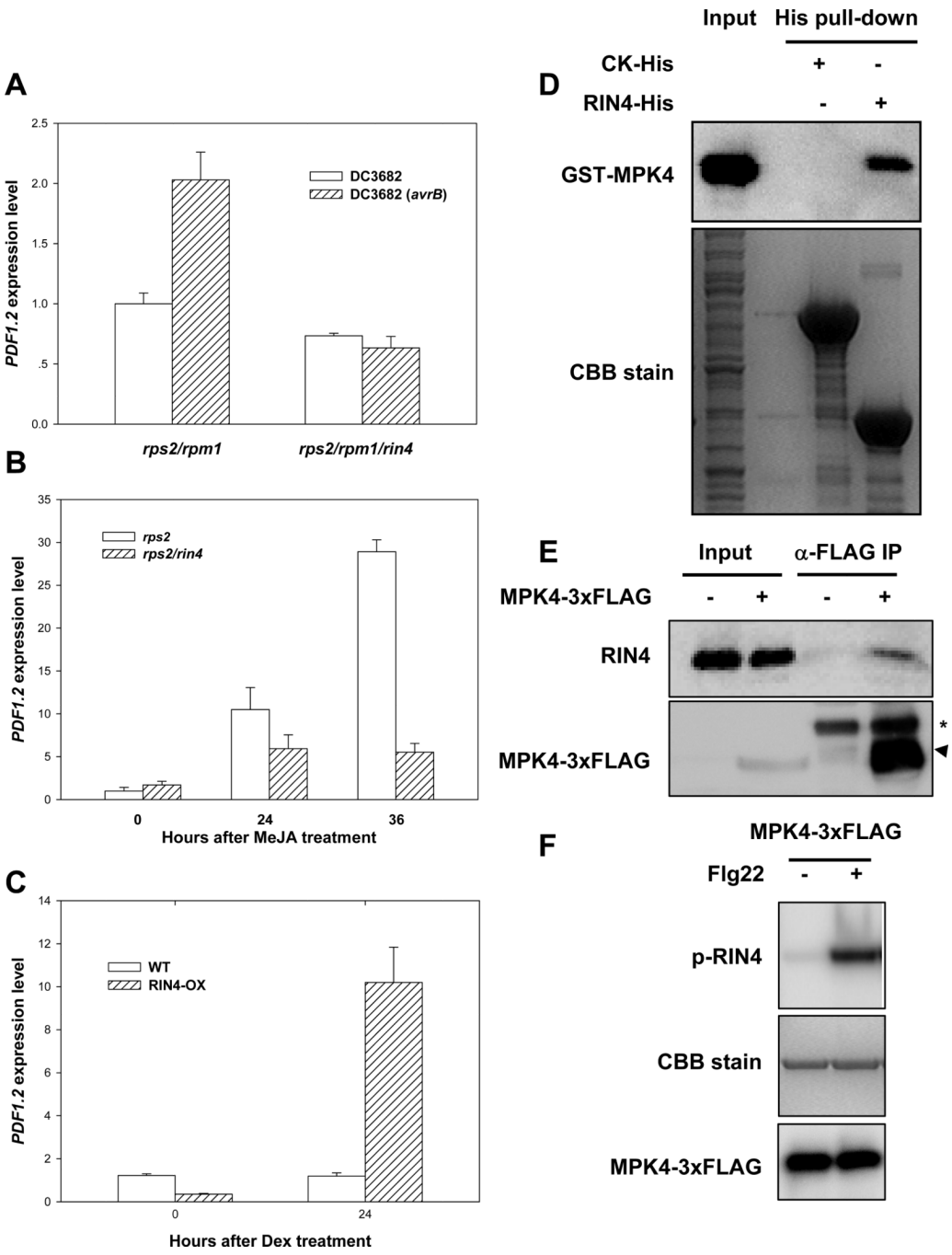

Figure 7. RIN4 Mediates *PDF1.2* Induction Down-Stream of MPK4

(**A**) RIN4 is required for *PDF1.2* induction by bacterially delivered AvrB. Plants of the indicated genotypes were infiltrated with the indicated bacterial strains and RNA was isolated 6 hr later for gene expression analysis. (**B**) RIN4 is required for JA-induced *PDF1.2* expression. Plants of the indicated genotypes were treated with MeJA at the indicated times before RNA isolaton. (**C**) Overexpression of RIN4 constitutively activates *PDF1.2* expression. WT or RIN4 transgenic (RIN4-ox) plants were treated with dexmethosome as described (Kim *et al.,* 2005) for the indicated times before RNA was isolated for *PDF1.2* expression analysis. (**D**) RIN4 interacts with MPK4 *in vitro*. Recombinant GST-MPK4

protein was incubated with bacterial lysates containing RIN4-His or CK-His (negative control as in Fig. 7A). (**E**) RIN4 interacts with MPK4 in plants. Protein extract from transgenic plants carrying *NP-MPK4-3xFLAG* was immunoprecipitated with an agarose-conjugated anti-FLAG antibody, and the presence of RIN4 in the immune complex was determined by immunoblot using anti-RIN4 antibodies. The presence of MPK4-3xFLAG was determined by immunoblot using anti-FLAG antibodies. Arrow head indicates MPK4-3xFLAG, whereas asterisk indicates IgG heavy chain from the anti-FLAG antibody used in immunoprecipitation. (**F**) MPK4 phosphorylates RIN4 *in vitro*. MPK4-3xFLAG was stimulated with (+) or without (−) flg22 in protoplasts, immunoprecipitated with anti-FLAG antibody, and the isolated MPK4-3xFLAG protein was incubated with recombinant RIN4 protein in an *in vitro* kinase assay. RIN4 phosphorylation (p-RIN4) was detected by autoradiography. CBB stain indicates amount of RIN4 protein in the gel.