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Running Title: Role of DRB protein in bacterial resistance

Abstract

The plasma membrane-localized plant resistance (R) protein RPM1 is degraded upon the induction of the hypersensitive response (HR) triggered in response to its own activation or that of other unrelated R proteins. We tested a role for RPM1 turnover in RPM1-mediated resistance and show that degradation of RPM1 is not associated with HR or resistance mediated by this R protein. Likewise, the runaway cell death phenotype in *lsd1* mutant was not associated with RPM1 degradation and did not alter RPM1-derived resistance. RPM1 stability and RPM1-mediated resistance were dependent on the double-stranded RNA binding (DRB) proteins 1 and 4. Interestingly, the function of DRB1 in RPM1-mediated resistance was not associated with its role in pre-miRNA processing. The DRB3 and DRB5 proteins negatively regulated RPM1 mediated resistance and a mutation in these completely or partially restored resistance in the *drb1*, *drb2* and *drb4* mutant backgrounds. Conversely, plants overexpressing *DRB5* showed attenuated *RPM1*-mediated resistance. A similar role for DRBs in basal and R-mediated resistance suggest that these proteins play a general role in bacterial resistance.

Introduction

Plants utilize active defense mechanisms such as specific induction of elaborate defense signaling pathways to counter microbes. The various modes of defenses induced upon the recognition of pathogen-derived molecules provide local resistance to race-specific pathogens, and basal resistance to virulent pathogens. Resistance (*R*) gene-mediated or species-specific immunity (also termed Effector-Triggered Immunity: ETI) is induced when a strain-specific effector or avirulence protein (AVR) from the pathogen associates directly/indirectly with the cognate plant R protein [reviewed in (Kachroo 2006; Jones and Dangl 2006; Chisholm et al. 2006)]. Some R proteins perceive the presence of the pathogen via direct physical interactions with the cognate Avr proteins (Scofield et al. 1996; Jia et al. 2000; Todd and Fumiaki 2000; Deslandes et al. 2003), but the majority of R proteins associate indirectly with cognate Avr proteins. A well-studied example of an indirect mode of effector recognition is that of the Arabidopsis (*Arabidopsis thaliana*) R protein, RPM1 (for resistance to *Pseudomonas syringae* pv *maculicola 1*). RPM1 mediates resistance against bacteria expressing two different Avr proteins, AvrRpm1 and AvrB. Although RPM1 does not directly interact with either AvrRpm1 or AvrB, it does associate with RIN4 (for RPM1-interacting 4), which interacts with AvrRpm1 and AvrB. RIN4 is required for RPM1-induced resistance to AvrRpm1/AvrB-expressing *P. syringae* pv. *tomato* (*Pst*) (Mackey et al. 2002). Both AvrRpm1 and AvrB induce the phosphorylation of RIN4, which is associated with RPM1 activation and the resulting resistance signaling.

R proteins are thought to transition from an inactive to active state upon direct or indirect binding to the AVR protein. The dormant state of RPM1 is stabilized by RAR1 (required for *Mla12*-mediated resistance) and DRB4 (double-stranded RNA binding protein 4) (Schulze-Lefert 2004; Zhu et al. 2013). RAR1 and DRB4 likely regulate RPM1 stability via independent processes because *drb4* mutant plants contain wild-type-like levels of RAR1. The Arabidopsis genome encodes four other DRB isoforms, which have been primarily studied for their roles in RNA silencing. Of these, DRB1 and DRB4 facilitate DCL1 (Dicer like 1)- and DCL4-mediated synthesis of miRNA and *trans*-acting siRNAs (tasiRNAs), respectively (Vazquez et al. 2004; Adenot et al. 2006). DRB2 is also involved in the biogenesis of specific miRNA subsets (Eamens et al. 2012a) and DRB3 and DRB5 are thought to function in the same non-canonical miRNA pathway as DRB2 (Eamens et al. 2012a). Interestingly, all DRB isoforms contribute to the stability of the R protein HRT (HR to Turnip Crinkle Virus), which confers resistance against turnip crinkle virus (TCV). In addition to stabilizing HRT, DRB1 also plays a role in HRT activation because a mutation in DRB1 compromises HR to TCV. The DRB1 and DRB4 proteins are positively regulated by COP1 (Cho et al. 2014; Lim et al. 2018), an E3 ubiquitin ligase that functions as a negative regulator of photomorphogenesis (Lau and Deng

2012). Thus, a mutation in COP1 affects the stability of both HRT and RPM1 (Lim et al. 2018). Consequently, the *cop1* mutant shows pronounced susceptibility to *Pst avrRpm1*.

Induction of R-mediated responses is often accompanied by the formation of a hypersensitive reaction (HR), a form of programmed cell death resulting in necrotic lesions, at the site of pathogen entry (Dangl et al. 1996). HR is one of the first visible manifestations of pathogen-induced host defenses. Interestingly, RPM1 is degraded in response to HR induced by *Pst avrRpm1* or the RPS2 (resistance to *Pst* 2)-mediated recognition of *Pst avrRpt2* (Boyes et al. 1998). This led to the suggestion that RPM1 turnover regulates the extent of cell death and the amplitude of resistance response at the site of infection (Boyes et al. 1998).

Here, we examined the roles of HR and DRB proteins in RPM1-mediated resistance against *Pst avrRpm1*. We show that degradation of RPM1 post pathogen infection is not associated with RPM1-mediated HR or the resistance response. Unlike their roles in the HRT-TCV pathosystem, DRB3 and DRB5 proteins negatively regulate defense against bacteria, and of these DRB5 negatively regulates the function of DRB2 in basal- and R-mediated resistance. We also show that DRB1 and DRB4 are required for RPM1 stability. Together, our results show that although some DRB isoforms have identical functions, others have more distinct roles in defense against bacterial and viral pathogens.

Results

DRB1, DRB2 and DRB4 proteins are required for RPM1-mediated resistance

The Arabidopsis genome encodes five DRB proteins and all contribute to HRT-mediated resistance against TCV (Lim et al., 2018). Of these, DRB4 is required for RPM1 stability and thereby RPM1-mediated resistance (Zhu et al., 2013). Based on the fact that HRT and RPM1 are both peripheral plasma membrane-localized proteins (Jeong et al. 2010; Boyes et al. 1998), we considered the possibility that DRB proteins function similarly in defense derived from these two R proteins. To test this, we analyzed DRB protein levels in response to *avrRpm1* infection in wild-type and transgenic plants expressing epitope-tagged DRB proteins in the respective *drb* mutant background (Table S1). The *drb* lines used here have been characterized in earlier studies (Curtin et al., 2008; Lim et al., 2018; Table S1). A time-course analysis of DRB levels was conducted after pathogen inoculation. Infection with *Pst avrRpm1* resulted in increased accumulation of DRB1, DRB2 and DRB4 within 24 h of infection (Figure 1A). The DRB3 and DRB5 proteins were undetectable in both mock- and *Pst avrRpm1*-infected plants. This suggested that DRB1 and DRB2 may contribute to RPM1-mediated resistance, as already shown for DRB4 (Zhu et al.,

4

81 2013). To test this, we evaluated resistance to *Pst avrRpm1* in *DRB* knock-out (KO)
82 lines. Notably, *drb1-2*, *drb2-1* and *drb4-1* showed prominent chlorotic symptoms (S.
83 Figure 1A) and supported ~7-10-fold higher levels of *Pst avrRpm1* (Figure 1B),
84 suggesting that these KO lines were compromised in RPM1-mediated resistance.
85 This was not the case for *drb3-1* or *drb5-1* (Figure 1B, S. Figure 1A), suggesting that
86 these DRB proteins either did not participate in resistance to *Pst avrRpm1* or had
87 other undiscernible roles. A similar trend was also seen in *drb* mutants infected with
88 *Pst avrRpt2* or virulent *Pst* (DC3000); *drb1*, *drb2*, and *drb4* showed enhanced
89 susceptibility while *drb3* and *drb5* showed wild-type-like resistance (S. Figures 1B,
90 1C). This suggested that DRB1, DRB2 and DRB4 proteins positively regulate
91 resistance to *Pst*. Noticeably, *drb1* plants showed higher susceptibility as compared
92 to *drb2* and *drb4* (Figure 1B), suggesting that DRB1 had a more significant role in
93 resistance against *Pst*. Consistent with their enhanced susceptibility phenotype, the
94 *drb1*, *drb2* and *drb4* plants showed significantly less ion-leakage compared to wild-
95 type or *drb3* and *drb5* plants (Figure 1C). This correlated with the significantly
96 reduced SA accumulation and *PR-1* expression in pathogen-infected *drb1*, *drb2* and
97 *drb4* plants (Figures 1D, 1E) (Zhu et al. 2013). These results indicated a role for
98 DRB1, DRB2 and DRB4 in RPM1-mediated resistance.
99   The canonical functions of DRB1 and DRB2 proteins are in miRNA
100 accumulation (Vaucheret et al. 2004; Vazquez et al. 2004; Han et al. 2004). Notably,
101 while the full-length DRB2 is essential for miRNA biosynthesis, the N-terminal
102 domain of DRB1 is sufficient for miRNA biosynthesis (Wu et al. 2007). We reasoned
103 that if the role of DRB1 in RPM1-mediated resistance was associated with its
104 function in miRNA processing, expression of the N-terminal DRB1 domain in *drb1*
105 plants (Δ^CDRB1 in Figure 2, Wu et al. 2007) should be sufficient to rescue their
106 defense-related defects. However, the *drb1::Δ^CDRB1* plants were as susceptible to
107 *Pst avrRpm1* or virulent *Pst* as *drb1* plants, whereas *drb1::DRB1* showed wild-type
108 like resistance (Figures 2A, 2B). This was consistent with the reduced *PR-1*
109 expression in *Pst avrRpm1* infected *drb1::Δ^CDRB1* plants (Figure 2C). In comparison,
110 full length transgenic expression of *DRB1* complemented the defense phenotypes
111 associated with *drb1* mutation (Figures 2A, 2B, 2C). Thus, the function of DRB1 in
112 RPM1-mediated resistance did not appear to be associated with its role in pre-
113 miRNA processing. We confirmed this further by evaluating RPM1-mediated
114 resistance in *dcl1* and *ago1* plants, since both DCL1 and AGO1 play important roles
115 in miRNA biosynthesis (Achkar et al. 2016); the *dcl1* and *ago1* plants showed wild-
116 type-like resistance to *avrRpm1 Pst* (Figure 2D).
117
118 **DRB1 regulates stability of the RPM1 protein**
119   DRB4 was previously shown to regulate RPM1 stability (Zhu et al., 2013) and
120 both DRB1 and DRB2 were required for RPM1-derived resistance (Figure 1B).

121   Therefore, we considered the possibility that the inhibition of RPM1-derived
122   resistance in *drb1* and *drb2* plants to *Pst avrRpm1* might be associated with reduced
123   stability of the RPM1 protein. We crossed the *drb* mutants with transgenic plants
124   expressing *RPM1-MYC* under the RPM1 native promoter. The *RPM1-MYC* line used
125   here has been characterized in several earlier studies (Boyes et al. 1998; Kawasaki
126   et al. 2005). At least five independent F2 plants were tested and all *drb1 RPM1-MYC*
127   or *drb4 RPM1-MYC* plants were found to contain dramatically reduced RPM1-MYC as
128   compared to wild-type plants (Figure 3A). The reduced RPM1 protein in these
129   plants was likely a post-transcriptional response because *drb1* and *drb4* plants
130   expressed wild-type like levels of *RPM1-MYC* transgene (S. Figure 2A). Unlike *drb1*
131   and *drb4* mutants, increased susceptibility seen in *drb2* was not associated with
132   RPM1-MYC levels; the *drb2* plants contained wild-type-like levels of RPM1-MYC
133   (Figure 3A). Notably, despite their reduced RPM1 levels the *drb1* and *drb4* plants
134   showed better resistance compared to *rpm1* plants suggesting that the RPM1
135   protein was not completely absent in these backgrounds (Figure 3B). We reasoned
136   that if *drb1* and *drb4* plants contained at least some RPM1 protein it should be
137   possible to restore *Pst avrRpm1* resistance in these plants by compensating for a
138   downstream factor such as SA. Indeed, *avrRpm1* infection increased SA levels in
139   wild-type plants (Figure 1D) and impaired biosynthesis of SA in *sid2* plants (which
140   are unable to synthesize pathogen-responsive SA; Wildermuth et al., 2001)
141   conferred enhanced susceptibility to *Pst avrRpm1* (Figure 3C). Furthermore, similar
142   expression of *PR-1* was detected in plants treated with SA, *Pst avrRpm1* or SA+ *Pst*
143   *avrRpm1* (S. Figure 2B). Together, these results suggest that SA operates
144   downstream of RPM1. Consistent with this notion, treatment with SA induced wild-
145   type-like *PR-1* expression in *drb* plants (S. Figure 2C) and enhanced resistance of
146   *drb1, drb2* and *drb4* plants to *Pst avrRpm1* (Figure 3C). Likewise, SA pretreatment
147   enhanced resistance of *sid2* plants to *Pst avrRpm1* (Figure 3C).
148   Activation of ETI is often associated with the induction of the systemic
149   acquired resistance (SAR) response and this is dependent on the presence of the R
150   protein and the SA pathway (Gao et al. 2014). We tested the SAR response of *drb1*,
151   *drb2* and *drb4* plants, which contained reduced RPM1 protein or showed reduced
152   accumulation of SA (Figures 1D, 3A). Wild-type (Col-0) and *drb* plants were
153   inoculated with $MgCl_2$ or *Pst avrRpm1* and 48 h later the distal leaves of all plants
154   were challenged with virulent *Pst* (*Pst Vir*). The growth of *Pst Vir* was monitored at 0
155   and 3 dpi. As expected, wild-type plants induced SAR; pre-exposure to *Pst avrRpm1*
156   resulted in ~10-fold reduction in the growth of the secondary pathogen *Pst Vir*
157   (Figure 3D). This was also the case for the *drb3* and *drb5* mutants. In contrast, *drb1*,
158   *drb2* and *drb4* mutant lines did not induce SAR. Together, these results indicate that,
159   DRB1, DRB2 and DRB4 proteins contribute to RPM1-mediated downstream
160   signaling leading to the establishment of SAR.

161

RPM1 turnover does not contribute to RPM1-mediated signaling

RPM1 is rapidly degraded after the onset of HR in response to pathogen infection and this is considered to feed-back regulate HR-associated cell death and the overall resistance response (Boyes et al. 1998). However, turnover of proteins involved in a signaling response can also play a regulatory role in physiological processes (Spoel et al. 2009; Zuo et al. 2012). This prompted us to examine whether turnover of RPM1 was required for RPM1-mediated resistance by evaluating the role of HR-associated cell death in RPM1 turnover and RPM1-derived resistance.

First, we examined whether HR-associated RPM1 degradation was derived from protease activity or the 26S proteasome. The *rpm1* RPM1-MYC plants (RPM1-MYC expressed under the *RPM1* native promoter) were treated with a protease inhibitor, the 26S proteasome-specific inhibitor MG132, or the solvent DMSO (as control). Infection with *Pst avrRpm1* resulted in RPM1-MYC degradation within 1 h in plants pretreated with DMSO or the protease-specific inhibitor, but not in plants pretreated with MG132 (Figure 4A). However, all treated plants exhibited significant ion-leakage in response to pathogen infection (data shown for MG132, Figure 4B). This suggested that the 26S proteasome was responsible for RPM1 turnover in response to pathogen-induced HR and that turnover of RPM1 was not essential for proper onset of HR. RPM1 turnover was also not essential for subsequent resistance signaling because the protease inhibitor- and MG132-treated plants exhibited similar *PR-1* induction and *Pst avrRpm1* resistance as the control plants (Figures 4C, 4D).

Next, we assayed RPM1 levels in *lsd1 RPM1-MYC* plants. The *lsd1* mutant exhibits runaway cell death phenotype in response to biotic and abiotic stresses (Dietrich et al. 1997; Mateo et al. 2004). Consistent with previous reports for *lsd1* (Xiaozhen et al. 2010), *lsd1 RPM1-MYC* plants grown in long day conditions (14 h day light), showed photo-oxidative stress and accumulated increased levels of reactive oxygen species (ROS), which was highly elevated after pathogen inoculation (S. Figure 3A). These plants contained wild-type-like levels of RPM1-MYC (Figure 4E). We next assayed RPM1-MYC levels after *avrRpm1* inoculation. As shown before, RPM1-MYC was degraded within 3 h of *avrRpm1* inoculation in the wild-type background (Figure 4F). In contrast, pathogen infection did not noticeably reduce RPM1-MYC protein levels in the *lsd1* background, even though these plants showed pronounced cell death (S. Figure 3B). These results suggested that *avrRpm1*-induced degradation of RPM1 is not merely a factor of the cell death response. It is possible that a more controlled HR is required for RPM1 turnover. Alternatively, increased accumulation of one or more defense compounds in the *lsd1* background may result in increased stability of the RPM1 protein in these plants. Consistent with earlier observations (Xiaozhen et al. 2010), the *lsd1 RPM1-MYC* plants showed increased accumulation of

201 ROS (S. Figure 3A) and elevated *RPM1* transcript levels (Figure 4G). Moreover, the
202 *lsd1* plants showed normal resistance to *avrRpm1* (Rustérucci et al. 2001). Together,
203 these data indicate that HR-associated turnover of RPM1 is not essential for RPM1-
204 mediated pathogen resistance. Thus, increased susceptibility in *drb1* and *drb4*
205 plants was likely due to reduced levels of RPM1 rather than turnover of RPM1. To
206 determine if MG132 treatment can bypass a requirement for DRB1 and DRB4
207 proteins, we assayed RPM1-mediated resistance in MG132 treated *drb1 drb4* plants.
208 Both mock- and MG132-treated *drb1 drb4* plants showed similar bacterial counts (S.
209 Figure 3D), indicating that MG132 pretreatment was unable to bypass a
210 requirement for DRB1 and DRB4 proteins in RPM1-mediated resistance response.
211
212
213 **DRB3 and DRB5 act as negative regulators of RPM1-mediated resistance**
214 Unlike DRB1 or 4, the DRB3 and DRB5 isoforms did not appear to influence
215 bacterial resistance or RPM1 protein stability (Figures 1B, 3A). One possibility was
216 that DRB3 and DRB5 were redundant in their functions at least for bacterial
217 resistance, because these isoforms do contribute individually to viral resistance
218 (Lim et al. 2018). We tested their potential functional redundancy by generating
219 *drb3 drb5* double mutant plants and evaluating their response to *Pst* (S. Figures 4A,
220 4B; Figures 5A, 5B, 5C). Interestingly, the *drb3 drb5* plants showed slightly enhanced
221 resistance to virulent *Pst* (Figures 5B, S4A) and wild-type-like resistance to *Pst*
222 *avrRpm1* (Figures 5C, S4B). This suggested that the functional redundancy between
223 *DRB3* and *DRB5* proteins was limited to their additive effect on basal resistance.
224 Considering their opposite effects on bacterial resistance, we next generated
225 double mutant combinations to evaluate epistasis between *drb3* or *drb5* and *drb1*,
226 *drb2*, or *drb4* (Figure 5A). Interestingly, the *drb5* mutation restored basal resistance
227 in *drb1* and *drb2* plants (Figure 5B). In contrast, the *drb3* mutation restored basal
228 resistance in the *drb4* background but did not alter the response of *drb2* plants
229 (Figure 5B). The *drb5* mutation also completely or partially restored resistance to
230 *Pst avrRpm1* in *drb1* and *drb2* plants, respectively, but did not alter the resistance
231 response of *drb4* plants (Figure 5C). In contrast, the *drb3* mutation partially
232 restored *Pst avrRpm1* resistance in *drb1*, but not in *drb2* or *drb4* plants (Figure 5C).
233 Together, these data suggest that the DRB5 negatively regulates the functions of the
234 DRB1 and DRB2 proteins during both basal and *R*-mediated resistance to *Pst* (Figure
235 5D). In comparison, DRB3 negatively regulated the function of DRB1 during both
236 basal and *R*-mediated resistance to *Pst* and that of DRB4 in basal resistance (Figure
237 5D).
238 We next tested if *DRB3* or *DRB5* overexpression altered the RPM1-mediated
239 resistance response in the wild-type (Col-0) background. Transgenic plants
240 overexpressing *DRB3* or *DRB5* were screened based on their transcript levels

241 (Figures 6A, 6B) and at least two independent transgenic lines each were evaluated
242 for their response to *Pst avrRpm1*. The *DRB3-* and *DRB5*-overexpressing plants
243 showed wild-type-like morphology (Figure 6A), and of these *35S-DRB5* plants
244 consistently exhibited enhanced susceptibility to *Pst avrRpm1* (Figure 6C). The
245 *DRB5* overexpressing plants also showed enhanced susceptibility to virulent *Pst* as
246 well as *Pst avrRpt2* (S. Figure 5). Their increased susceptibility correlated with
247 reduced ion-leakage in response to pathogen infection (Figure 6D). The *DRB3*
248 overexpressing plants showed a nominal reduction in ion-leakage but in most
249 experiments these plants showed wild-type-like resistance to *avrRpm1*, *avrRpt2* or
250 virulent *Pst* (Figures 6C, S5). Together, these results showed that DRB5-mediated
251 negative regulation had an effect on bacterial resistance.
252
253
254 **Discussion**
255
256 In this study, we evaluated five DRB isoforms, which are known to
257 participate in miRNA and siRNA biogenesis, for their roles in basal and R-mediated
258 resistance to *P. syringae*. Our results show that the various DRB isoforms have
259 specific and unique functions in bacterial defense; DRB1, DRB2 and DRB4 isoforms
260 function as non-redundant positive regulators of resistance, while DRB3 and DRB5
261 operate as negative regulators. The DRB2, DRB3 and DRB5 proteins are thought to
262 function in the same non-canonical pathway during miRNA biogenesis (Eamens et
263 al. 2012b). However, their differing functions in bacterial resistance suggests that
264 the functions of DRB proteins in defense signaling may not strictly overlap with
265 their functions in the miRNA pathway. This is further corroborated by the fact that
266 the C-terminal truncated form of DRB1, which is functional in miRNA biogenesis is
267 unable to complement resistance signaling in the *drb1* mutant background (Wu et
268 al. 2007; Yang et al. 2010). The C-terminal truncated DRB1 lacks the nuclear
269 localization signal (NLS) and protein-protein interaction (PPI) domains (Wu et al.
270 2007). Although neither the NLS or PPI domains are required for miRNA processing,
271 these regions are clearly important for DRB1-mediated defense against bacteria.
272 The roles of at least some of the DRB isoforms in plant defense appears to be
273 associated with their ability to regulate R protein stability, which in turn could be
274 mediated through direct or indirect interactions with the R protein. For instance, the
275 DRB1 and DRB4 proteins regulate viral resistance because they regulate the
276 stability of the R protein HRT (Lim et al. 2018). However, the DRB2 isoform which
277 also contributes to HRT stability and HRT-mediated resistance does not directly
278 interact with HRT. Notably, unlike their role in HRT-mediated resistance, DRB2 is
279 not required for the stability of RPM1. This suggests that DRB2 likely functions at a
280 downstream step in RPM1-mediated resistance.

281

Our results show that the various DRB proteins have differing functions in defense against *Pst* versus TCV. For instance, DRB2 functions as a positive regulator of defense against both *Pst* and TCV, whereas DRB3 and DRB5 negatively regulate defense against *Pst*, and positively regulate HRT-mediated resistance to TCV. The similar functions of DRB3 and DRB5 in defense against *Pst* are consistent with their common involvement in the non-canonical miRNA pathway (Eamens et al. 2012a). However, DRB3 and DRB5 have also been suggested to function in the DRB2-dependent miRNA pathway (Eamens et al. 2012a), but DRB2 and DRB3/DRB5 have opposing functions in bacterial defense. DRB3 was recently shown to regulate basal resistance to geminiviruses via its effect on changes to chromatin methylation (Raja et al. 2014). This suggests that DRB3 could contribute to bacterial resistance by regulating chromatin methylation, which are well known to play a role in bacterial resistance (Alexandre et al. 2012).

Both DRB2 and DRB4 proteins are part of a higher molecular weight complex (Clavel et al. 2015) and DRB2 interacts with proteins involved in the regulation of chromatin function (Clavel et al. 2015). Notably, the high molecular weight DRB2 complex contains MSI4, which functions as a substrate adaptor for CULLIN4 (CUL4)-Damaged DNA Binding Protein1 (DDB1) ubiquitin E3 ligases. CULLIN4 E3 ubiquitin ligases in turn interact with COP1, an important negative regulator of photomorphogenesis and a positive regulator of RPM1-mediated resistance. COP1 regulates RPM1 resistance by stabilizing DRB1 and DRB4 which are required for RPM1 stability (Cho et al. 2014; Lim et al. 2018). Thus, like the *drb1* and *drb4* mutants, the *cop1* mutant also contains little to no RPM1 protein and exhibits enhanced susceptibility to *Pst* (Lim et al. 2018).

In addition to COP1, RPM1 also interacts with two membrane bound E3 ligases RIN2 and RIN3. Although RIN2 and RIN3 positively regulate RPM1-triggered HR, they do not contribute to RPM1-mediated resistance (Tsutomu et al. 2005). RPM1 is unique in that it is the only known R protein that is degraded upon induction of HR (Boyes et al. 1998). COP1 is unlikely to contribute to this HR associated degradation of RPM1 because it is a positive regulator of RPM1-mediated resistance. Furthermore, mutations in RIN2/RIN3 has no effect on RPM1 level, suggesting that RIN2/RIN3 E3 ligases are not involved in pathogen-triggered degradation of RPM1 (Tsutomu et al. 2005). This is consistent with our results that show HR associated degradation of RPM1 is not essential for RPM1-derived resistance. This is based on our finding that 26S proteasome inhibitor MG132 inhibited RPM1 degradation but did not alter RPM1-mediated HR or resistance. In addition, RPM1 was not degraded in *lsd1* plants, which showed pronounced pathogen-induced cell death. It is possible that *lsd1* plants show normal resistance despite their lack of RPM1 turnover because they contain increased levels of ROS

321 (Dietrich et al., 1997; Mateo et al., 2004) or other defense-associated molecules. This
322 was consistent with the observation that exogenous application of glycerol resulted
323 in induction of *RPM1-MYC* transcript and conferred enhanced resistance to virulent
324 *Pst* (Venugopal et al. 2009).
325   Notably, overexpression of the *DRB5* protein compromised RPM1-mediated
326 resistance to *avrRpm1 Pst* but did not affect HRT-mediated resistance to TCV. This
327 together with the shared dependence of HRT- and RPM1-mediated resistance on SA
328 suggests that DRB3 and DRB5 proteins might not influence defense by perturbing
329 SA levels. Indeed, mutation or overexpression of *DRB3* or *DRB5* does not alter levels
330 of basal or pathogen induced SA. However, overexpression of *DRB3* or *DRB5* did
331 lower pathogen-induced ion-leakage with *35S-DRB5* plants showing more
332 pronounced reduction. Thus, it is possible that DRB3 and DRB5 proteins negatively
333 regulate a subset of RPM1-derived events leading to HR, and this in turn regulates
334 resistance. A nominal reduction of ion-leakage in *35S-DRB3* plant versus a more
335 pronounced effect seen in *35S-DRB5* correlates well with their respective resistance
336 response to *Pst avrRpm1*. Thus, while degradation of RPM1 is not essential for the
337 onset of HR or resistance, loss of HR might contribute to RPM1-mediated resistance.
338
339
340 **Experimental Procedures**
341
342 **Plant growth conditions, genetic analysis and generation of transgenic plants**
343   Plants were grown in MTPS 144 Conviron (Winnipeg, MB, Canada) walk-in-
344 chambers at 22 ^{0}C, 65% relative humidity and 14 hour photoperiod. The photon flux
345 density of the day period was 106.9 µmoles m^{-2} s^{-1} and was measured using a digital
346 light meter (Phytotronic Inc, Earth city, MO). Plants were grown on autoclaved Pro-
347 Mix soil (Premier Horticulture Inc., PA, USA). Soil was fertilized once using Scotts
348 Peter's 20:10:20 peat lite special general fertilizer that contained 8.1% ammoniacal
349 nitrogen and 11.9% nitrate nitrogen (Scottspro.com). Plants were irrigated using
350 deionized or tap water.
351   The *drb1-2*, *drb2-1*, *drb3-1*, *drb4-1*, *drb5-1* genotypes used in this study are
352 described in Table S1. Crosses were performed by emasculating the flowers of the
353 recipient genotype and pollinating with the pollen from the donor. F2 plants
354 showing the wild-type genotype at the mutant locus were used as controls in all
355 experiments. The wild-type and mutant alleles were identified by PCR, CAPS, or
356 dCAPS analysis.
357   For transgenic overexpression of *DRBs*, the cDNA spanning the coding region
358 in pGWB2 vector (Nakagawa et al., 2007), which after confirmation of the DNA
359 sequence was transformed into Col-0 plants. The transgenic plants were selected on
360 plates containing kanamycin (50 µg/ml) and hygromycin (17 µg/ml). For native

11

361 expression of *DRBs*, the MYC tagged DRBs along with their respective promoters
362 were cloned into pCambia 1300 derived vector and transformed into respective *drb*
363 mutant backgrounds (Clavel et al. 2015; Lim et al. 2018). Genetic complementation
364 was assayed by analyzing the levels of siRNA, as described before (Clavel et al.,
365 2015).
366
367 **RNA extraction, RNA gel-blot analyses and qRT-PCR**
368   Small-scale extraction of RNA from two or three leaves (per sample) was
369 performed with the TRIzol reagent (Invitrogen, CA), following the manufacturer's
370 instructions. RNA gel blot analysis and synthesis of random-primed probes for *PR-1*
371 were carried out as described previously (Kachroo et al., 2000).
372   RNA quality and concentration were determined by gel electrophoresis and
373 determination of A_{260}. Reverse transcription (RT) and first strand cDNA synthesis
374 were carried out using Superscript II (Invitrogen, CA). Quantitative RT-PCR was
375 carried out as described before (Zhang et al., 2009). Each sample was run in
376 triplicates and *ACTIN* expression levels were used as internal control for
377 normalization. Cycle threshold values were calculated by SDS 2.3 software. Gene-
378 specific primers used for real-time quantitative RT-PCR analyses are described in
379 Table S2.
380
381 **Trypan-blue staining**
382   The leaves were vacuum-infiltrated with trypan-blue stain prepared in 10
383 mL acidic phenol, 10 mL glycerol, and 20 mL sterile water with 10 mg of trypan
384 blue. The samples were placed in a heated water bath (90°C) for 2 min and
385 incubated at room temperature for 2-12 h. The samples were destained using
386 chloral hydrate (25 g/10 mL sterile water; Sigma), mounted on slides and observed
387 for cell death with a compound microscope. The samples were photographed using
388 an AxioCam camera (Zeiss, Germany) and images were analyzed using Openlab 3.5.2
389 (Improvision) software.
390
391 **Conductivity assays**
392   Electrolyte leakage was measured in four-week-old plants as described
393 earlier (Yu et al., 2013). Briefly, leaves were infiltrated with $MgCl_2$ or *avrRpt2 Pst*
394 (10^6 CFU/ml) and 5 leaf discs (7 mm) per plant were removed with a cork borer,
395 floated in distilled water for 50 min, and subsequently transferred to tubes
396 containing 5 ml of distilled water. Conductivity of the solution was determined with
397 an NIST traceable digital Conductivity Meter (Fisher Scientific). Standard deviation
398 was calculated from four replicate measurements per genotype per experiment.
399
400 **Pathogen infections**

401   The bacterial strain *avrRpm1* was grown overnight in King's B medium
402   containing rifampicin and kanamycin (Sigma, MO). The bacterial cells were
403   harvested, washed and suspended in 10 mM $MgCl_2$. The cells were diluted to a final
404   density of 10^5 CFU/mL (A_{600}) and used for infiltration. The bacterial suspension was
405   injected into the abaxial surface of the leaf using a needle-less syringe. Three leaf
406   discs from the inoculated leaves were collected at 0 and 3 dpi. The leaf discs were
407   homogenized in 10 mM $MgCl_2$, diluted 10^3 or 10^4 fold and plated on King's B
408   medium. For analysis of SAR, the primary leaves were inoculated with $MgCl_2$ or the
409   avr bacteria (10^7 cfu ml^{-1}) and, 48 h later, the systemic leaves were inoculated with
410   vir bacteria (10^5 cfu ml^{-1}). The samples from the systemic leaves were harvested at 3
411   dpi.
412
413   **Protein extraction and Immunoblot analysis**
414   Proteins were extracted in buffer containing 50mM Tris-HCl, pH7.5, 10%
415   glycerol, 150mM NaCl, 10mM $MgCl_2$, 5mM EDTA, 5 mM DTT, and 1 X protease
416   inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was
417   measured by the Bio-RAD protein assay (Bio-Rad, CA). For small scale extractions 2-
418   3 leaves were homogenized per sample. For Ponceau-S staining, PVDF membranes
419   were incubated in Ponceau-S solution (40% methanol (v/v), 15% acetic acid (v/v),
420   0.25% Ponceau-S). The membranes were destained using deionized water. Proteins
421   (30-50 μg) were fractionated on a 7-10% SDS-PAGE gel and subjected to
422   immunoblot analysis using α-MYC (Sigma-Aldrich, St. Louis, MO) antibody.
423   Immunoblots were developed using ECL detection kit (Roche) or alkaline-
424   phosphatase-based color detection.
425
426

Author contributions

Genetic analysis, pathogen infections, generation of *DRB* overexpressing plants, qRT, RNA gel-blot- and immunoblot-analysis was carried out by GHL with help from SZ, KZ and TH. PK supervised the project, PK and AK analyzed data, and wrote the manuscript with contribution from all the authors.

Competing financial interests

The authors declare no competing financial interests.

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Figure Legends

Figure 1. DRB proteins are required for local resistance to *Pseudomonas avrRpm1*. (**A**) Western blots showing relative levels of DRB1-MYC, DRB2-MYC and DRB4-MYC in *avrRPM1 P. syringae* (*Pst*) inoculated *drb* plants expressing *DRB* transgenes under their native promoters. Leaves were sampled at 0, 24 and 48 h post inoculation. Ponceau-S staining of the Western blot was used as the loading control. Numbers below each panel indicate relative levels of Rubisco quantified using Image Quant software. This experiment was repeated two times with similar results. (**B**) Growth of avirulent *avrRpm1 Pst* strain on Col-0 and *drb* mutants. Error bars indicate SD. Asterisks indicate data statistically significant from that of control (Col-0) (P<0.001, n=4). NS indicates that the data was not significantly different from Col-0. These experiments were repeated five times with similar results. (**C**) Electrolyte leakage in Col-0 plants infiltrated with $MgCl_2$ or *avrRpm1 Pst*. Error bars represent SD. This experiment was repeated two times with similar results. Asterisks indicate data significantly different from those of Col-0 (P<0.001, n=6). (**D**) Levels of free SA in mock ($MgCl_2$) and pathogen (*avrRpm1 Pst*) inoculated Col-0 and *drb* plants 48 h post treatments. Error bars indicate SD. Asterisks indicate statistically higher levels of SA compared to respective mock-inoculated plants (P<0.0001, n=4). "a" indicates significantly lower levels of SA in *drb1* and *drb2* plants compared to pathogen-inoculated Col-0 plants. (**E**) Quantitative RT-PCR analysis showing relative levels of *PR-1* transcript in mock and *avrRpm1 Pst* inoculated Col-0 and *drb* plants. This experiment was repeated twice using two or more independent cDNA preparations as templates. Asterisks indicate statistically higher expression compared to respective mock-inoculated plants (P<0.0001, n=4). "a" indicates significantly lower expression compared to pathogen-inoculated Col-0 plants.

Figure 2. C-terminal truncated DRB1 protein does not complement defense phenotypes of *drb1* plants. (**A** and **B**) Growth of *avrRpm1 Pst* (**A**) and virulent (**B**) DC3000 strains on Col-0, *drb1*, and *drb1* expressing wild-type (*drb1::DRB1*) or C-terminal truncated DRB1 (*drb1::ΔcDRB1*). Error bars indicate SD. Asterisks indicate data significantly different from those of Col-0 (P<0.0001, n=4). These experiments were repeated two times with similar results. (**C**) Quantitative RT-PCR analysis showing relative levels of *PR-1* transcript in mock and *avrRpm1 Pst* inoculated Col-0, *drb1*, and *drb1* expressing wild-type (*drb1::DRB1*) or C-terminal truncated DRB1 (*drb1::ΔcDRB1*). This experiment was repeated twice using two or more independent cDNA preparations as templates. Asterisks indicate data significantly higher compared to respective mock-inoculated plants (P<0.0001, n=4). "a"

indicates significantly lower expression compared to pathogen-inoculated Col-0 plants. (**D**) Growth of avirulent *avrRpm1 Pst* strain on Col-0, *ago1-27*, and *dcl1-7* plants. These experiments were repeated two times with similar results.

Figure 3. DRB1 and DRB4 proteins are required for the stability of RPM1. (**A**) Western blots showing relative levels of RPM1-MYC in *drb* mutants. Ponceau-S staining of the Western blot was used as the loading control. Arrow indicates RPM1-MYC protein. This experiment was repeated three times with similar results. (**B**) Growth of *avrRpm1 Pst* on Col-0, *drb1*, *drb4* and *rpm1* plants. Error bars indicate SD. Asterisks indicates significantly higher growth compared to respective mock-inoculated plants (P<0.0007, n=4). "b" indicates significant difference between *rpm1* and *drb* plants (P<0.003, n=4). (**C**) Growth of virulent *Pst* (DC3000) on Col-0, *drb* and *sid2* plants that were pretreated with water or SA (500 μm) prior to inoculation. Error bars indicate SD. Asterisks indicate data statistically significant from that of water treated plants (P<0.002, n=4). (**D**) SAR response in Col-0 and *drb* plants. Primary leaves were inoculated with MgCl$_2$ or *P. syringae* expressing *avrRpm1*. The distal leaves were inoculated with the virulent *P. syringae* and growth of the virulent bacteria was monitored at 3 dpi. Asterisks indicates statistically lower growth compared to respective MgCl$_2$ inoculated plants (P<0.001, n=4). This experiment was repeated three times with similar results.

Figure 4. Pathogen-triggered degradation of RPM1 is not required for its downstream signaling. (**A**) Western blot showing RPM1-MYC levels in Col-0::RPM1-MYC plants at 0-3 hours after *avrRpm1* inoculation. The plants were treated with DMSO solvent, protease inhibitor cocktail (PI), or MG132 and inoculated with 10^6 bacteria 24 h after treatment. Ponceau-S staining of the Western blot was used as the loading control. This experiment was repeated two times with similar results. (**B**) Electrolyte leakage in Col-0 plants infiltrated with MgCl$_2$ or *avrRpm1 Pst*. Error bars represent SD (n=6). This experiment was repeated two times with similar results. (**C**) RNA gel blot showing time-course expression analysis of *PR-1* in plants treated with DMSO, PI or MG132 24 h prior to inoculation with *avrRpm1 Pst*. Total RNA was extracted from inoculated leaves at 24 hours post inoculation (hpi). Ethidium bromide staining of rRNA was used as the loading control. The experiment was repeated twice with similar results. (**D**) Growth of *avrRpm1 Pst* on Col-0 plants treated with DMSO, PI or MG132 24 h prior to inoculation with *avrRpm1 Pst*. Error bars indicate SD. (**E**) Western blot showing RPM1-MYC levels in *LSD1* and *lsd1* plants. Both genotypes were derived from a cross between Col-0::RPM1-MYC x *lsd1* (Ws-0) plants and were homozygous for the *rpm1* locus. Ponceau-S staining of the Western blot was used as the loading control. Arrow

indicates RPM1-MYC protein. This experiment was repeated two times with similar results. (**F**) Western blot showing RPM1-MYC levels in *LSD1::RPM1-MYC* or *lsd1::RPM1-MYC* plants at 0, 3 or 6 hours after *avrRpm1* inoculation. The *RPM-MYC* genotypes were homozygous for the *rpm1* locus. Arrow indicates RPM1-MYC protein. This experiment was repeated two times with similar results. (**G**) Quantitative RT-PCR analysis showing relative levels of *RPM1* transcript in *LSD1* and *lsd1* plants. Error bars indicate SD. This experiment was repeated twice using two or more independent cDNA preparations as templates. Asterisks indicate statistically higher expression compared to *LSD1* plants (P<0.0001, n=4).

Figure 5. DRB3 and DRB5 negatively regulate resistance to bacterial pathogens. (**A**) Typical morphological phenotypes of soil grown four-week-old *drb* single and double mutants. Scale bar, 0.5 cm. (**B and C**) Growth of virulent DC3000 (**B**) and avirulent *avrRpm1 Pst* (**C**) *P. syringae* (*Pst*) strains on Col-0 and *drb* mutants. Error bars indicate SD. Asterisks indicate significantly higher growth compared to Col-0 (P<0.006, n=4). NS indicates that the data were not significantly different from Col-0. "b" indicates significant difference among indicated genotypes (P<0.003, n=4). (**D**) Simplified model showing genetic interaction between DRB1, DRB2, DRB4 and DRB3 and DRB5. A mutation in DRB1, DRB2 and DRB4 compromises both basal and R-mediated resistance against *Pseudomonas syringae* (*Pst*). In contrast, a mutation in DRB3 and DRB5 has no effect on host resistance to *Pst* but does suppress susceptibility caused by mutation in DRB1, DRB2 or DRB4 in a partial (dashed line) or complete (solid line) manner.

Figure 6. Overexpression of *DRB5* compromises RPM1-mediated resistance. (**A**) Typical morphological phenotypes of soil grown four-week-old Col-0 and transgenic plants overexpressing *DRB3* or *DRB5* transgenes in Col-0 background. Scale bar, 0.5 cm. (**B**) Quantitative RT-PCR analysis showing relative levels of *DRB* transcripts in transgenic Col-0 plants overexpressing *DRB3* or *DRB5* transgenes. This experiment was repeated twice using two independent cDNA preparations as templates. Error bars indicate SD. Asterisks indicate data significantly different from those of Col-0 (P<0.0001, n=4). (**C**) Growth of *avrRpm1 Pst* strain on Col-0 and *DRB* overexpressing plants. Error bars indicate SD. Asterisks indicate data significantly different from those of control (Col-0) (P<0.008, n=4). This experiment was repeated four times and while *35S-DRB5* plants showed enhanced susceptibility in all experiments, the *35S-DRB3* plants showed increased susceptibility in two of four experiments. (**D**) Electrolyte leakage in Col-0 or *35S-DRB* plants infiltrated with MgCl$_2$ or *avrRpm1 Pst*. Error bars represent SD (n=6). This experiment was repeated two times with similar results.

Supplementary Figure Legends

S. Figure 1. DRB proteins are required for local resistance to *Pseudomonas.* (A) Typical morphological phenotypes of *avrRpm1* inoculated leaves. The inoculated leaves were marked with silver ink and photographed 24 h post inoculation. (**B and C**) Growth of avirulent *avrRpt2* (**B**) or virulent (**C**) *Pst* (DC3000) on Col-0 and *drb* mutants. Error bars indicate SD. Asterisks indicate data significantly different from those of Col-0 (P<0.001, n=4). (**D**) RNA gel blot analysis showing expression of *PR-1* in mock- and *avrRpm1*-inoculated Col-0 and *drb* plants. Total RNA was extracted from inoculated leaves at 24 hpi. Ethidium bromide staining of rRNA was used as the loading control. The experiment was repeated twice with similar results.

S. Figure 2. *drb* mutants show normal SA responsiveness. (A) Quantitative RT-PCR analysis showing relative levels of *RPM1-MYC* transcript in Col-0 and *drb* mutants. This experiment was repeated twice using two independent cDNA preparations as templates. Error bars indicate SD. (**B)** Quantitative RT-PCR analysis showing relative levels of *PR-1* transcript in Col-0 plants treated with SA, *avrRpm1* or SA+*avrRpm1*. The leaves were sampled 24 h post treatment. This experiment was repeated twice using two or more independent cDNA preparations as templates. Asterisks indicate significantly higher expression compared to $MgCl_2$ (mock) infiltrated plants (P<0.0001, n=4). (**C**) RNA gel blot analysis showing expression of *PR-1* in water- and SA-treated Col-0 and *drb* plants. Total RNA was extracted from treated leaves at 24 hpi. Ethidium bromide staining of rRNA was used as the loading control. The experiment was repeated twice with similar results.
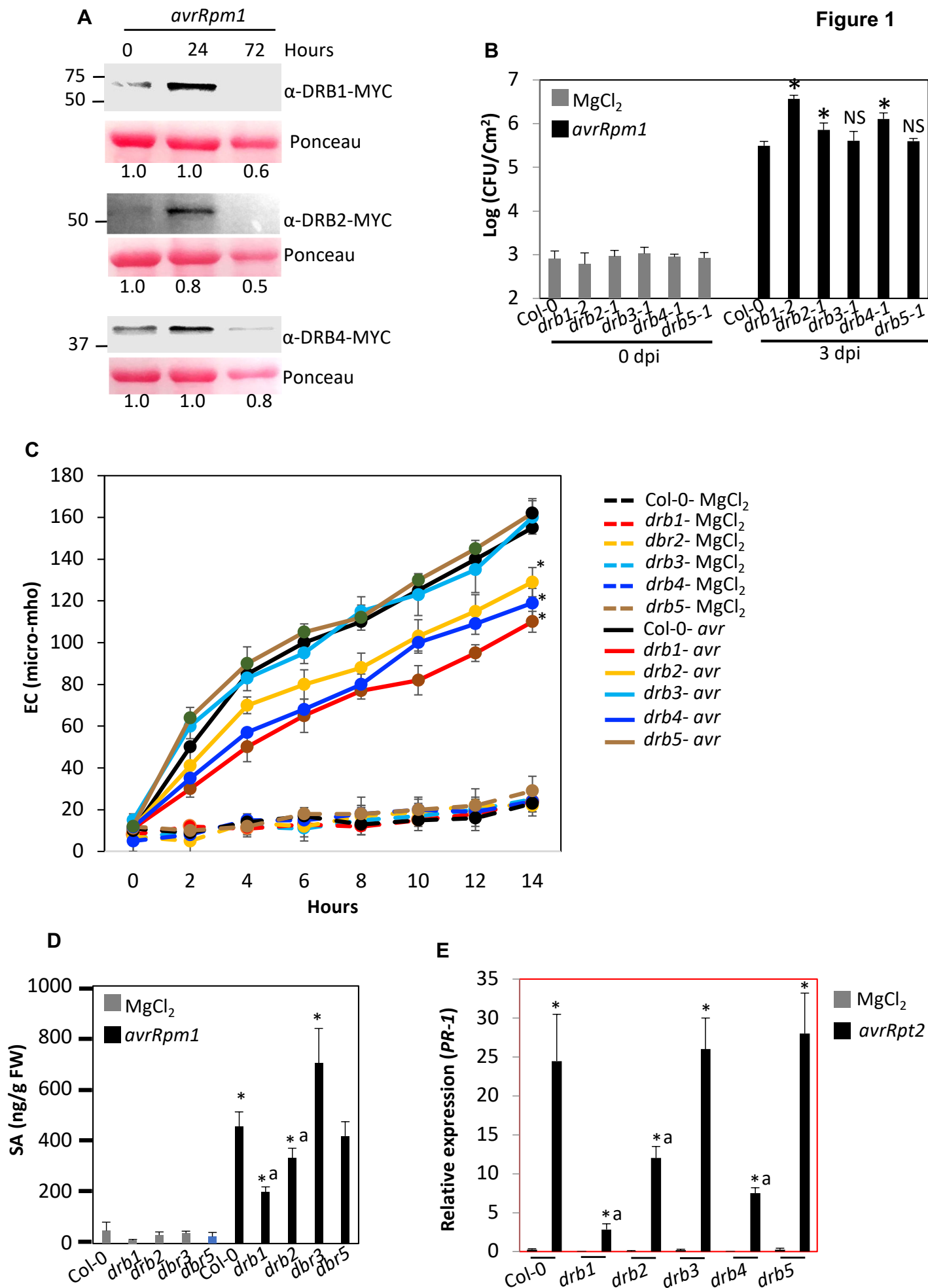
S. Figure 3. The *lsd1* plants accumulate higher levels of basal and pathogen-induced reactive oxygen species. (A) H_2O_2 levels in *LSD1::RPM1-MYC* and *lsd1::RPM1-MYC* plants after mock ($MgCl_2$) or pathogen (*avrRpm1*) inoculation. The leaves were sampled 24 h post treatments and stained with DAB (3, 3-diaminobenzidine). The experiment was repeated two times with similar results. (**B**) Typical morphological and microscopic cell death phenotypes shown by pathogen inoculated *LSD1::RPM1-MYC* and *lsd1::RPM1-MYC* plants. Leaves were photographed or stained 24 h post inoculation and at least six independent leaves were analyzed with similar results. (**C**) Growth of avirulent *avrRpm1 Pst* on *LSD1::RPM1-MYC* and *lsd1::RPM1-MYC* plants. The experiment was repeated twice with similar results. (**D**) Growth of *avrRpm1 Pst* on Col-0 and *drb1 drb4* plants treated with DMSO or MG132 24 h prior to inoculation. Error bars indicate SD.

Asterisks indicate significantly higher growth compared to Col-0 plants (P<0.0001, n=4).

S. Figure 4. The *drb3 drb5* double mutants show enhanced resistance to virulent bacteria (**A** and **B**) Growth of virulent (**A**) or avirulent *avrRpm1 Pst* (**B**) on Col-0, *drb3, drb5* and *drb3 drb5* double mutants. Error bars indicate SD. Asterisks indicate data significantly different from those of Col-0 (P<0.01, n=4). The experiment was repeated twice with similar results.
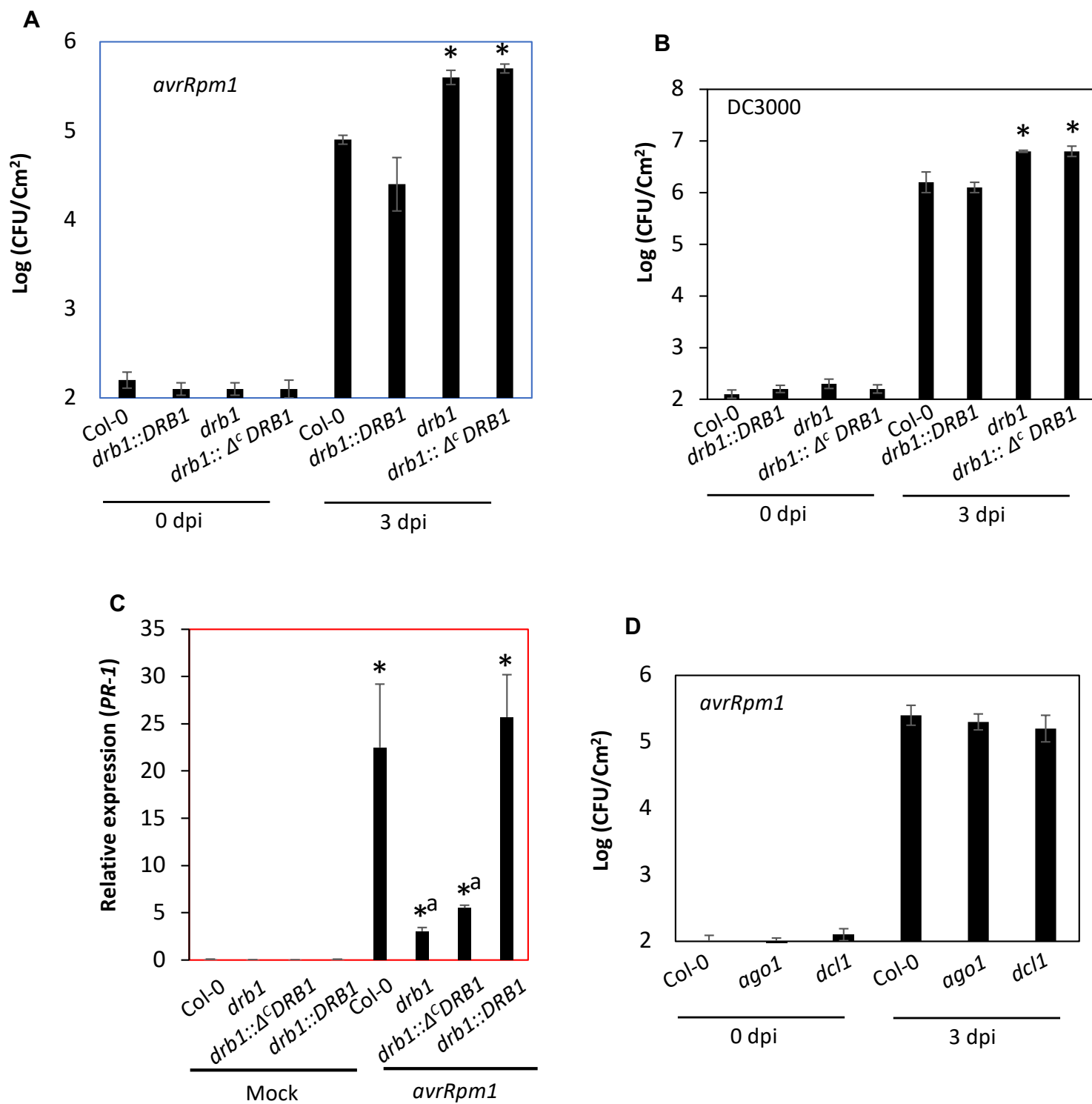
S. Figure 5. Overexpression of *DRB5* compromises basal and R-mediated resistance against bacterial pathogens. (**A** and **B**) Growth of avirulent *avrRpt2* (**A**) or virulent DC3000 (**B**) *Pst* on Col-0 and plants overexpressing *DRB3* or *DRB5*. Error bars indicate SD. Asterisks indicate data significantly different from those of Col-0 (P<0.01, n=4). These experiments were repeated four times and while *35S-DRB5* plants showed enhanced susceptibility in all experiments, the *35S-DRB3* plants showed increased susceptibility in two of four experiments.
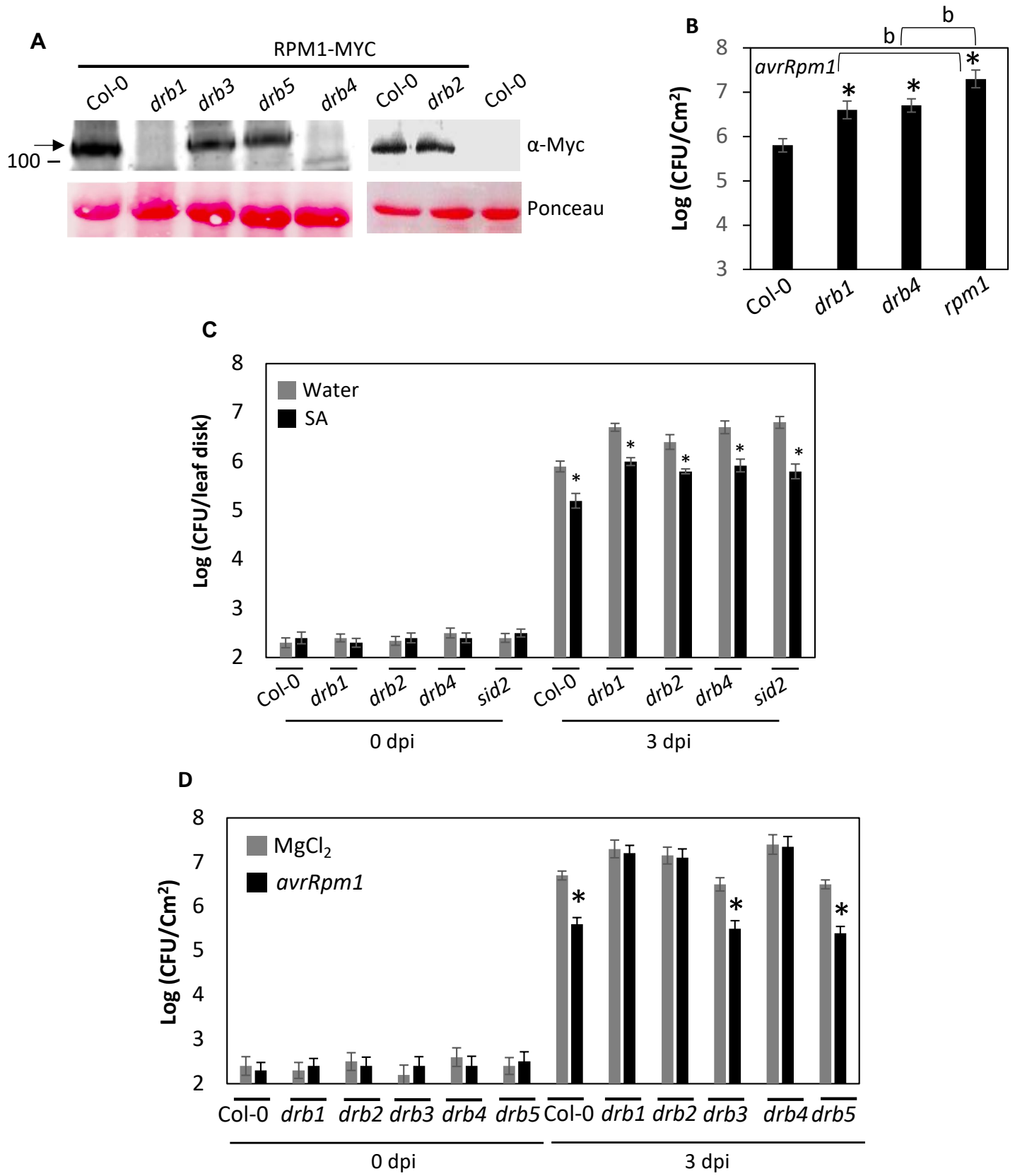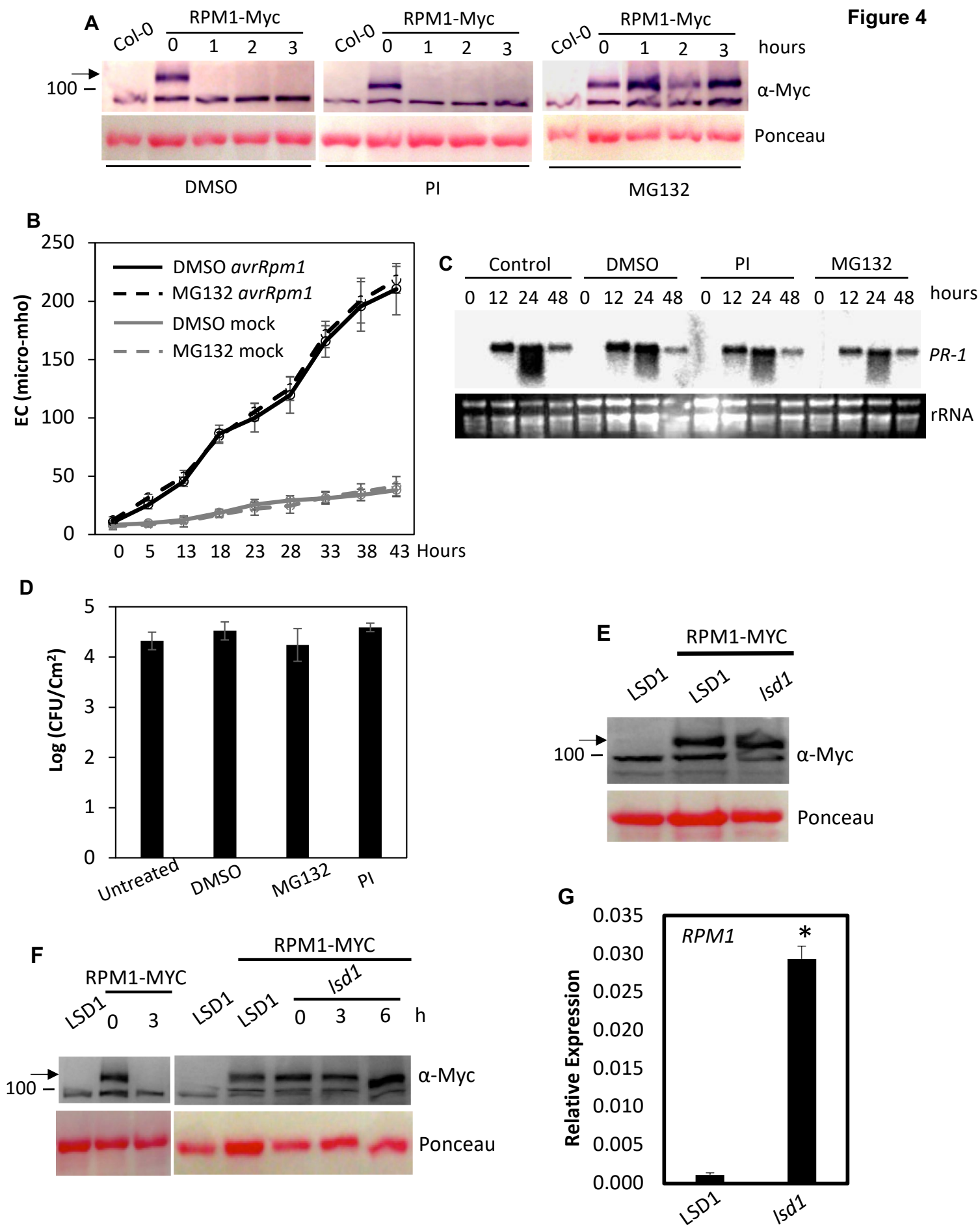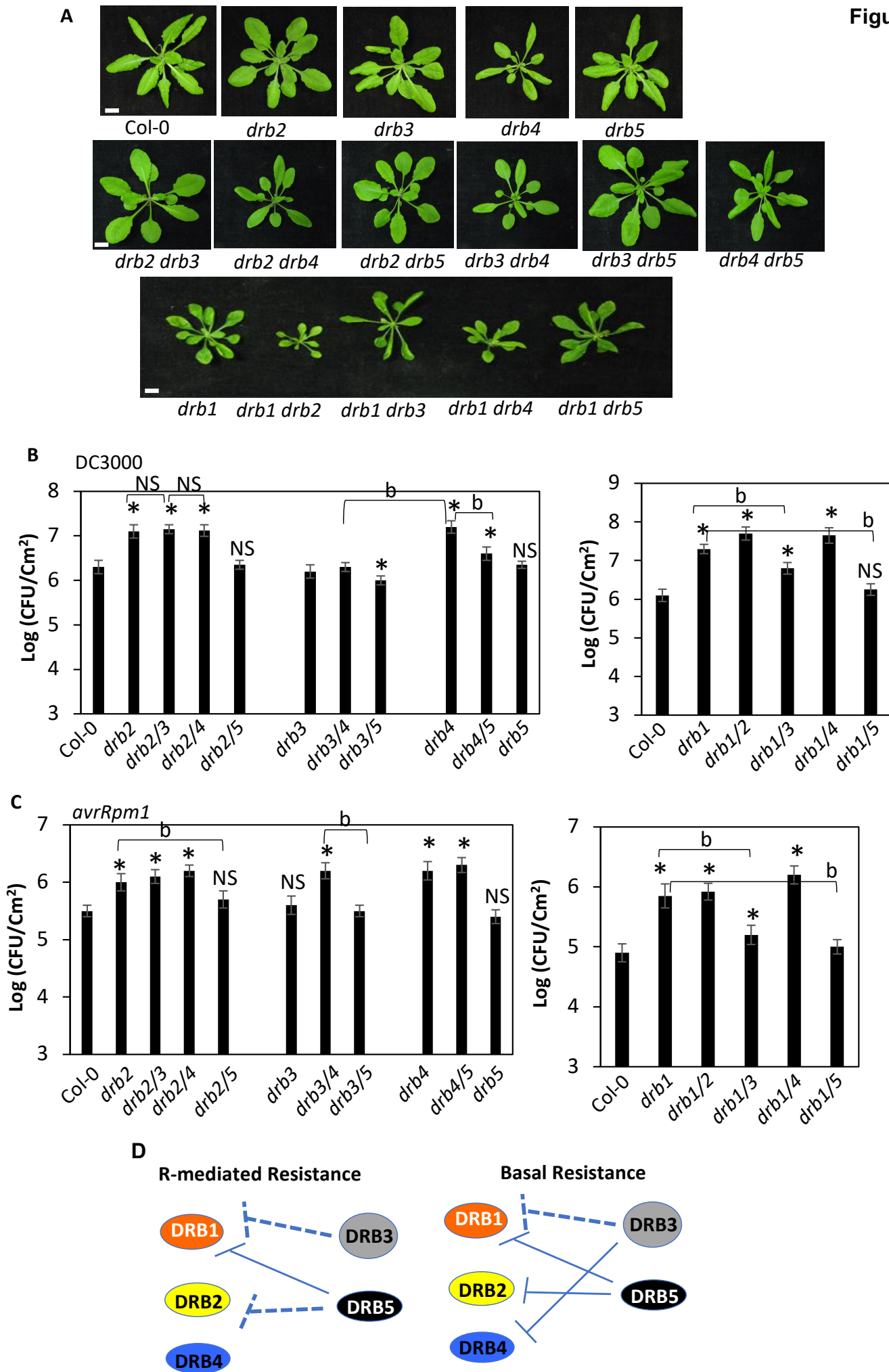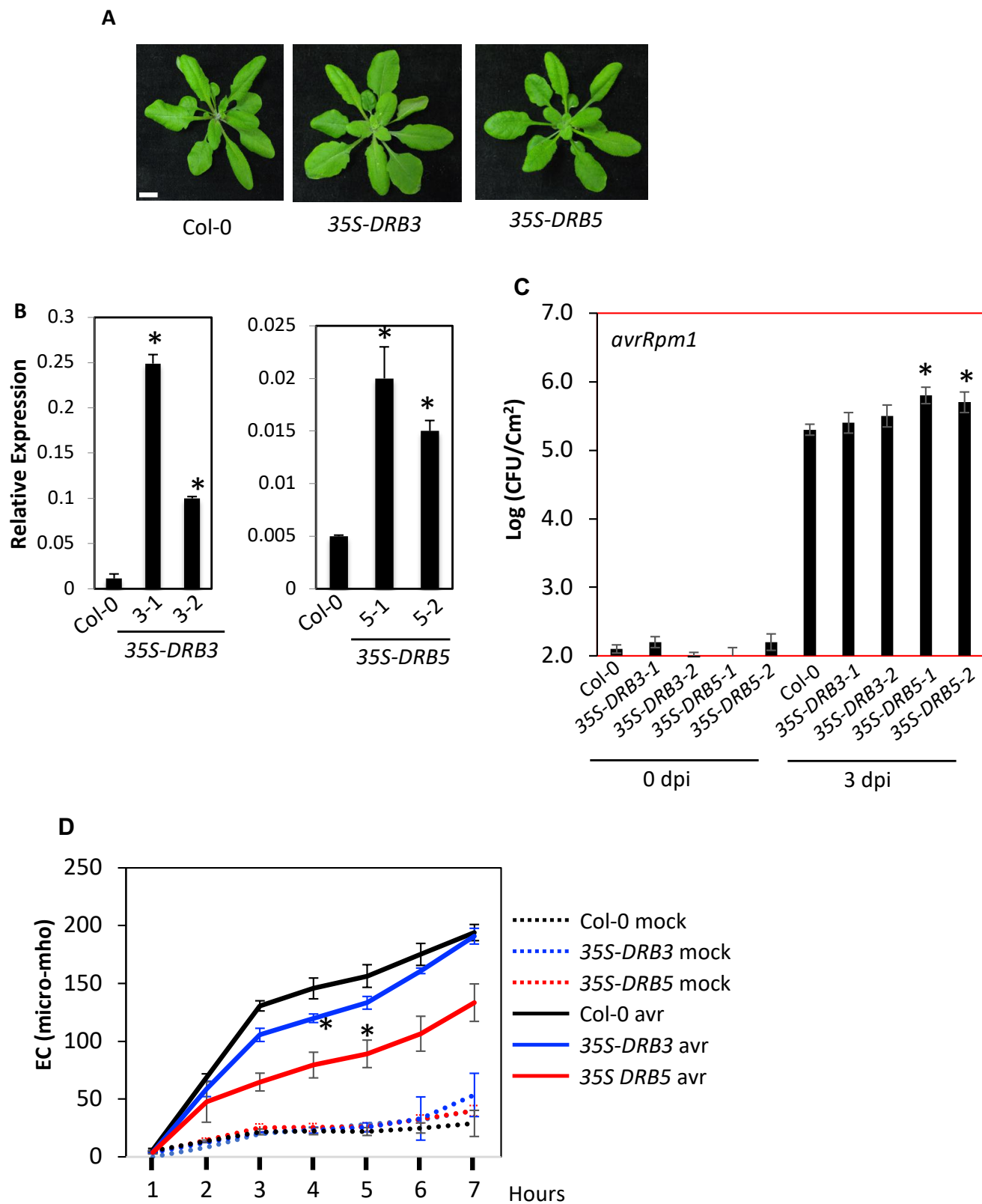
Figure 1

Figure 2

Figure 3

A

RPM1-MYC

	Col-0	drb1	drb3	drb5	drb4	Col-0	drb2	Col-0	

→
100 —   α-Myc

Ponceau

B

C

D

Figure 4

Figure 5

A

Col-0 drb2 drb3 drb4 drb5

drb2 drb3 drb2 drb4 drb2 drb5 drb3 drb4 drb3 drb5 drb4 drb5

drb1 drb1 drb2 drb1 drb3 drb1 drb4 drb1 drb5

B DC3000

C avrRpm1

D

R-mediated Resistance

DRB1   DRB3
DRB2   DRB5
DRB4

Basal Resistance

DRB1   DRB3
DRB2   DRB5
DRB4

Figure 6

A

Col-0   *35S-DRB3*   *35S-DRB5*

B

C

D

A

Col-0   drb1   drb2   drb3   drb4   drb5

B

C

D

A

LSD1::RPM1-MYC   lsd1::RPM1-MYC

Mock

Avr

B

LSD1::RPM1-MYC

RPM1-MYC

lsd1::RPM1-MYC

RPM1-MYC *lsd1*

C

D

A

B

A

B