

# Rhamnolipids Elicit Defense Responses and Induce Disease Resistance against Biotrophic, Hemibiotrophic, and Necrotrophic Pathogens That Require Different Signaling Pathways in Arabidopsis and Highlight a Central Role for Salicylic Acid<sup>1[C][W][OA]</sup>

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Plant resistance to phytopathogenic microorganisms mainly relies on the activation of an innate immune response usually launched after recognition by the plant cells of microbe-associated molecular patterns. The plant hormones, salicylic acid (SA), jasmonic acid, and ethylene have emerged as key players in the signaling networks involved in plant immunity. Rhamnolipids (RLs) are glycolipids produced by bacteria and are involved in surface motility and biofilm development. Here we report that RLs trigger an immune response in *Arabidopsis* (*Arabidopsis thaliana*) characterized by signaling molecules accumulation and defense gene activation. This immune response participates to resistance against the hemibiotrophic bacterium *Pseudomonas syringae* pv *tomato*, the biotrophic oomycete *Hyaloperonospora arabidopsidis*, and the necrotrophic fungus *Botrytis cinerea*. We show that RL-mediated resistance involves different signaling pathways that depend on the type of pathogen. Ethylene is involved in RL-induced resistance to *H. arabidopsidis* and to *P. syringae* pv *tomato* whereas jasmonic acid is essential for the resistance to *B. cinerea*. SA participates to the restriction of all pathogens. We also show evidence that SA-dependent plant defenses are potentiated by RLs following challenge by *B. cinerea* or *P. syringae* pv *tomato*. These results highlight a central role for SA in RL-mediated resistance. In addition to the activation of plant defense responses, antimicrobial properties of RLs are thought to participate in the protection against the fungus and the oomycete. Our data highlight the intricate mechanisms involved in plant protection triggered by a new type of molecule that can be perceived by plant cells and that can also act directly onto pathogens.

In their environment, plants are challenged by potentially pathogenic microorganisms. In response,

they express a set of defense mechanisms including preformed structural and chemical barriers, as well as an innate immune response quickly activated after microorganism perception (Boller and Felix, 2009). Plant innate immunity is triggered after recognition by pattern recognition receptors of conserved pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively) or by plant endogenous molecules released by pathogen invasion and called danger-associated molecular patterns (Boller and Felix, 2009; Dodds and Rathjen, 2010). This first step of recognition leads to the activation of MAMP-triggered immunity (MTI). Successful pathogens can secrete effectors that interfere or suppress MTI, resulting in effector-triggered susceptibility. A second level of perception involves the direct or indirect recognition by specific receptors of pathogen effectors leading to effector-triggered immunity (ETI; Boller and Felix, 2009; Dodds and Rathjen, 2010). Whereas MTI and ETI are thought to involve common signaling network, ETI is usually quantitatively stronger

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than MTI and associated with more sustained and robust immune responses (Katagiri and Tsuda, 2010; Tsuda and Katagiri, 2010).

The plant hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have emerged as key players in the signaling networks involved in MTI and ETI (Robert-Seilaniantz et al., 2007; Tsuda et al., 2009; Katagiri and Tsuda, 2010; Mersmann et al., 2010; Tsuda and Katagiri, 2010; Robert-Seilaniantz et al., 2011). Interactions between these signal molecules allow the plant to activate and/or modulate an appropriate spectrum of responses, depending on the pathogen lifestyle, necrotroph or biotroph (Glazebrook, 2005; Koornneef and Pieterse, 2008). It is assumed that JA and ET signaling pathways are important for resistance to necrotrophic fungi including *Botrytis cinerea* and *Alternaria brassicicola* (Thomma et al., 2001; Ferrari et al., 2003; Glazebrook, 2005). Infection of Arabidopsis (*Arabidopsis thaliana*) with *B. cinerea* causes the induction of the JA/ET responsive gene *PLANT DEFENSIN1.2* (*PDF1.2*; Penninckx et al., 1996; Zimmerli et al., 2001). Induction of *PDF1.2* by *B. cinerea* is blocked in *ethylene-insensitive2* (*ein2*) and *coronatine-insensitive1* (*coi1*) mutants that are respectively defective in ET and JA signal transduction pathways. Moreover, *ein2* and *coi1* plants are highly susceptible to *B. cinerea* infection (Thomma et al., 1998; Thomma et al., 1999). JA/ET-dependent responses do not seem to be usually induced during resistance to biotrophs, but they can be effective if they are stimulated prior to pathogen challenge (Glazebrook, 2005). Plants impaired in SA signaling are highly susceptible to biotrophic and hemibiotrophic pathogens. Following pathogen infection, SA hydroxylase (*nahG*), *enhanced disease susceptibility5* (*eds5*), or *SA induction-deficient2* (*sid2*) plants are unable to accumulate high SA levels and they display heightened susceptibility to *Pseudomonas syringae* pv *tomato* (*Pst*), *Hyaloperonospora arabidopsidis*, or *Erysiphe orontii* (Delaney et al., 1994; Lawton et al., 1995; Wildermuth et al., 2001; Nawrath et al., 2002; Vlot et al., 2009). Mutants that are insensitive to SA, such as *nonexpressor of PATHOGENESIS-RELATED (PR) genes1* (*npr1*), have enhanced susceptibility to these pathogens (Cao et al., 1994; Glazebrook et al., 1996; Shah et al., 1997; Dong, 2004). According to some reports, plant defense against necrotrophs also involves SA. Arabidopsis plants expressing the *nahG* gene and infected with *B. cinerea* show larger lesions compared with wild-type plants (Govrin and Levine, 2002). In tobacco (*Nicotiana tabacum*), acidic isoforms of *PR3* and *PR5* gene that are specifically induced by SA (Ménard et al., 2004) are up-regulated after challenge by *B. cinerea* (El Oirdi et al., 2010). Resistance to some necrotrophs like *Fusarium graminearum* involves both SA and JA signaling pathways (Makandar et al., 2010). It is assumed that SA and JA signaling can be antagonistic (Bostock, 2005; Koornneef and Pieterse, 2008; Pieterse et al., 2009; Thaler et al., 2012). In Arabidopsis, SA inhibits JA-dependent resistance against *A. brassicicola* or *B. cinerea* (Spoel et al., 2007; Koornneef

et al., 2008). Recent studies demonstrated that ET modulates the *NPR1*-mediated antagonism between SA and JA (Leon-Reyes et al., 2009; Leon-Reyes et al., 2010a) and suppression by SA of JA-responsive gene expression is targeted at a position downstream of the JA biosynthesis pathway (Leon-Reyes et al., 2010b). Synergistic effects of SA- and JA-dependent signaling are also well documented (Schenk et al., 2000; van Wees et al., 2000; Mur et al., 2006) and induction of some defense responses after pathogen challenge requires intact JA, ET, and SA signaling pathways (Campbell et al., 2003).

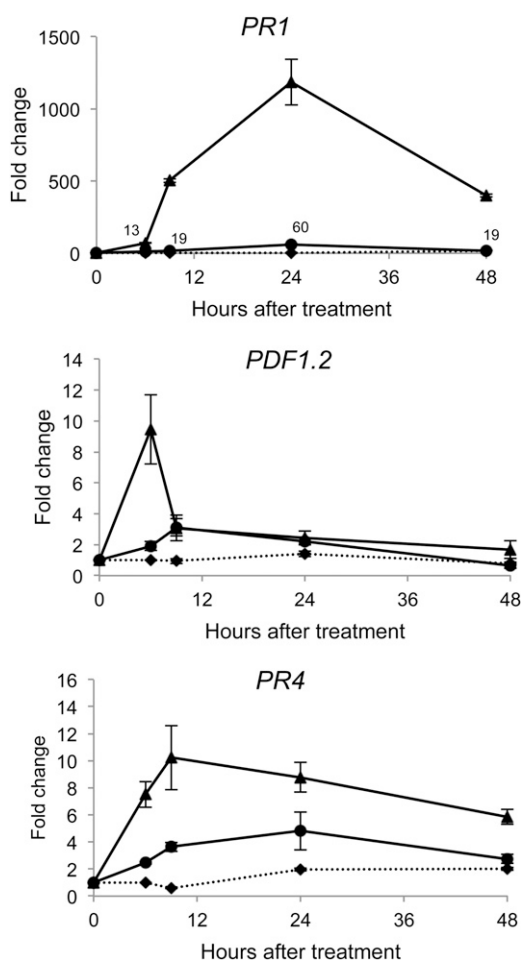
Isolated MAMPs trigger defense responses that also require the activation of SA, JA, and ET signaling pathways (Tsuda et al., 2009; Katagiri and Tsuda, 2010). For instance, treatment with the flagellin peptide *flg22* induces many SA-related genes including *SID2*, *EDS5*, *NPR1*, and *PR1* (Ferrari et al., 2007; Denoux et al., 2008), causes SA accumulation (Tsuda et al., 2008; Wang et al., 2009), and activates ET signaling (Bethke et al., 2009; Mersmann et al., 2010). Local application of lipopolysaccharides elevates the level of SA (Mishina and Zeier, 2007). The oomycete *Pep13* peptide induces defense responses in potato (*Solanum tuberosum*) that require both SA and JA (Halim et al., 2009). Although signaling networks induced by isolated MAMPs are well documented, the contribution of SA, JA, and ET in MAMP- or PAMP-induced resistance to biotrophs and necrotrophs is poorly understood.

Rhamnolipids (RLs) are glycolipids produced by various bacteria species including some *Pseudomonas* and *Burkholderia* species. They are essential for bacterial surface motility and biofilm development (Vatsa et al., 2010; Chrzanowski et al., 2012). RLs are potent stimulators of animal immunity (Vatsa et al., 2010). They have recently been shown to elicit plant defense responses and to induce resistance against *B. cinerea* in grapevine (*Vitis vinifera*; Varnier et al., 2009). They also participate to biocontrol activity of the plant beneficial bacteria *Pseudomonas aeruginosa* PNA1 against oomycetes (Perneel et al., 2008). However, the signaling pathways used by RLs to stimulate plant innate immunity are not known. To gain more insights into RL-induced MTI, we investigated RL-triggered defense responses and resistance to the necrotrophic fungus *B. cinerea*, the biotroph oomycete *H. arabidopsidis*, and the hemibiotroph bacterium *Pst* in Arabidopsis. Our results show that RLs trigger an innate immune response in Arabidopsis that protects the plant against these different lifestyle pathogens. We demonstrate that RL-mediated resistance involves separated signaling sectors that depend on the type of pathogen. In plants challenged by RLs, SA has a central role and participates to the restriction of the three pathogens. ET is fully involved in RL-induced resistance to the biotrophic oomycete and to the hemibiotrophic bacterium whereas JA is essential for the resistance to the necrotrophic fungus.

## RESULTS

## RLs Elicit Defense Responses in Arabidopsis

To assess the ability of RLs to induce defense responses in Arabidopsis and the potential links with SA, JA, and ET signaling, we monitored the expression pattern of *PR-1*, *PDF1.2*, and *PR-4* in plants challenged with RLs. In these experiments and the following, leaves were sprayed with the molecules to monitor local defense responses. *PR-1* is well known as a SA-dependent defense gene marker (Lebel et al., 1998; Vlot et al., 2009) whereas *PDF1.2* expression is regulated by JA/ET (Penninckx et al., 1996; Penninckx et al., 1998), and *PR-4* expression is dependent on ET (Lawton et al., 1994). We used two concentrations of RLs (0.2 and 1 mg mL<sup>-1</sup>) to compare the responses induced by



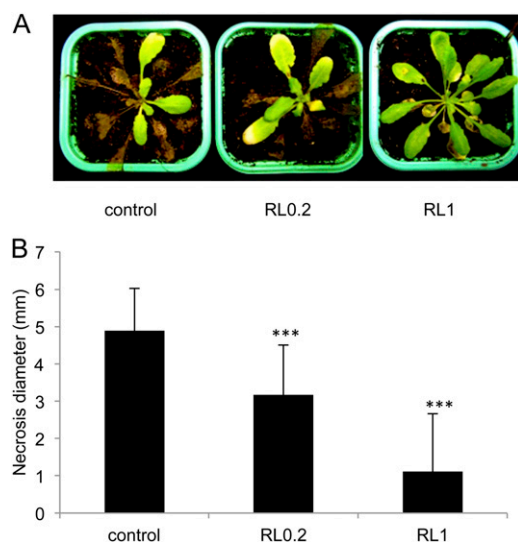
**Figure 1.** RLs elicit defense gene expression in Arabidopsis. Defense-related gene expression was monitored in control wild-type leaves (diamonds) and after treatment with RLs (0.2 [circles] and 1 mg mL<sup>-1</sup> [triangles]). Transcript accumulation of *PR1*, *PDF1.2*, and *PR4* genes was determined by qRT-PCR. Results are expressed as the fold increase in transcript level compared with time 0 h and are means  $\pm$  SD of duplicate data from one representative experiment among three independent repetitions.

low and strong RLs stimuli (Varnier et al., 2009). *PR-1* expression was induced at 6 h in response to both concentrations of RLs and peaked at 24 h post treatment (hpt; Fig. 1). *PDF1.2* expression was strongly and transiently induced in response to the highest dose of RLs, peaking at 6 hpt. A slight increase in *PDF1.2* expression was also observed with the lowest concentration of RLs. *PR4* expression was stimulated with both concentrations of RLs. Induction of gene expression was stronger with 1 mg mL<sup>-1</sup> of RLs. Concomitantly with gene expression, we measured a 2-fold increase in SA level at 24 hpt and a 200-fold increase in JA level at 6 h and 24 hpt with the lowest concentration of RLs (Supplemental Fig. S1). Using the Evans blue test, we did not detect any cell death in Arabidopsis leaves treated with 0.2 mg mL<sup>-1</sup> of RLs (Supplemental Fig. S2). At 1 mg mL<sup>-1</sup>, we observed few microlesions and clear necroses were present when we increased the concentration of RLs to 5 mg mL<sup>-1</sup>. These results suggest that high concentrations of RLs may trigger a hypersensitive response (HR)-like response as previously described for grapevine (Varnier et al., 2009).

RLs that we used are produced by *P. aeruginosa* and consist of a mix of mono- and di-RLs (Varnier et al., 2009). We previously described the ability of purified mono- and di-RLs to induce plant defense with the same intensity in grapevine cell suspensions (Varnier et al., 2009). To verify that both type of RLs can induce defense in Arabidopsis, we assayed induction of *PR1* in a *PR1::GUS* reporter line using flg22 (1  $\mu$ M) as positive control (Denoux et al., 2008; Supplemental Fig. S3). GUS expression was observed with similar intensity in Arabidopsis leaves after treatment with mono-RLs, di-RLs, and the mix of mono- and di-RLs at 0.2 mg mL<sup>-1</sup>. No induction of *PR1::GUS* was observed after elicitation with a concentration of 0.05 mg mL<sup>-1</sup>. We also quantified by quantitative reverse transcription-PCR (qRT-PCR) *PR1* gene expression at 24 h after flagellin and RLs (mix at 0.2 mg mL<sup>-1</sup>) treatment. *PR1* was induced 217-fold ( $\pm$ 3) over the control by flg22 and 160-fold ( $\pm$ 6) by RLs (data not shown).

#### RLs Induce Local Resistance against *B. cinerea*, *Pst*, and *H. arabidopsidis*

To assess the ability of RLs to enhance Arabidopsis resistance to different lifestyle pathogens, we performed infection experiments with *B. cinerea*, *Pst*, and *H. arabidopsidis*. These three pathosystems have been widely used to decipher disease resistance in Arabidopsis (Glazebrook, 2005; Coates and Beynon, 2010). Arabidopsis plants were sprayed with RLs at low or high concentrations and inoculated with *B. cinerea* 4 d after pretreatment. Twelve days after infection, most of the leaves from control plants were fully necrotized (Fig. 2A). Some protection effect was observed with 0.2 mg mL<sup>-1</sup> of RLs, and most of the leaves treated with 1 mg mL<sup>-1</sup> was symptomless or displayed only few necrotic lesions (Fig. 2A). Diameters of lesions on infected leaves



**Figure 2.** RLs induce resistance against *B. cinerea*. Plants were sprayed with RLs at 0.2 or 1 mg mL<sup>-1</sup> or water (control) and, 4 d later, leaves were inoculated with the fungus. A, Symptoms observed 12 d after infection by the fungus. B, Necroses diameter was measured 72 h after infection by the fungus in the control or RL-treated plants. Values shown are means  $\pm$  SD ( $n = 24$ ) from one representative experiment among three independent repetitions. Stars indicate significant differences between the RL-treated sample and the control according to Student's *t* test (\*\*\* $P < 0.005$ ). [See online article for color version of this figure.]

were also measured 72 h after *B. cinerea* challenge (Fig. 2B). Control leaves challenged with *B. cinerea* displayed very large necrotic lesions (mean of 5 mm). A significant reduction in lesion size was measured in plants treated with RLs at 0.2 mg mL<sup>-1</sup>, and a strong protective effect of RLs was found at the highest concentration with very small lesions (mean of 1 mm).

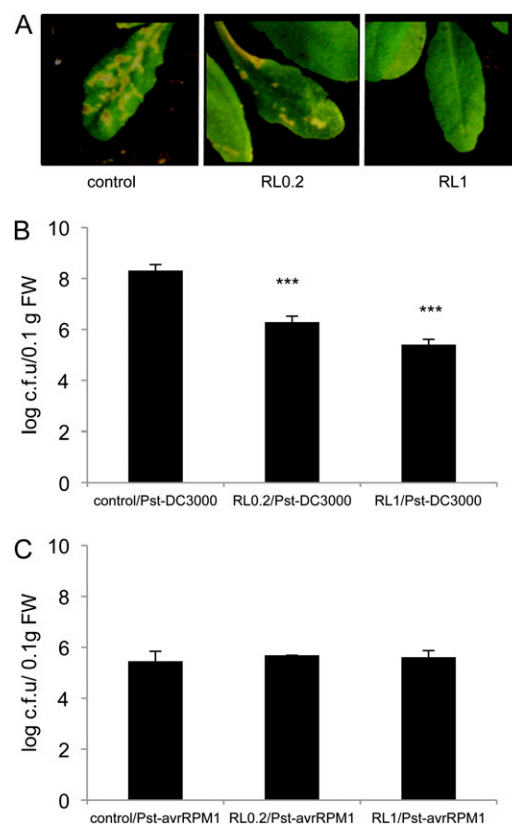
A *Pst* DC3000/*Arabidopsis* Columbia-0 (Col-0) pathosystem was used to assess the protective effect of RLs in the context of a compatible interaction. In these experiments, we also monitored the impact of RLs on a typical ETI triggered by the avirulent *Pst* carrying *avrRPM1* (*Pst-avrRPM1*). *Arabidopsis* plants dipped with the virulent strain of *Pst* exhibited typical disease symptoms 7 d post inoculation (Fig. 3A). Symptom development was strongly reduced by pretreatment with 0.2 mg mL<sup>-1</sup> of RLs and totally abolished with the highest concentration. We monitored the growth of *Pst* and *Pst-avrRPM1* over a time course in RL-treated and nontreated plants. *Pst* growth was stopped at 24 h post inoculation (hpi; data not shown) and was still strongly reduced at 72 hpi in RL-treated plants at the highest concentration (Fig. 3B). Bacterial growth was also stopped 24 hpi with 0.2 mg mL<sup>-1</sup> of RLs (data not shown) and significantly reduced at 72 hpi (Fig. 3B). No difference was observed between plants treated or not treated with RLs following inoculation with *Pst-avrRPM1*, so RLs did not interfere with the ETI triggered by the bacteria (Fig. 3C). It is interesting that resistance induced

by RLs against the virulent strain of *Pst* was very similar in intensity with the resistance observed during the typical *Pst-avrRPM1*-triggered ETI (Fig. 3, B and C).

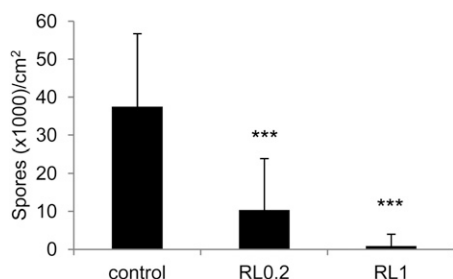
*Arabidopsis* Col-0 plants pretreated with RLs were also infected by the compatible strain of the biotrophic oomycete *H. arabidopsidis* Noco2. Conidiospores were harvested 7 d after *Arabidopsis* infection and counted. A strong and significant protection against *H. arabidopsidis* was observed after treatment with RLs at 0.2 mg mL<sup>-1</sup> (Fig. 4). Moreover, at 1 mg mL<sup>-1</sup>, RLs restricted almost completely pathogen sporulation.

#### RL-Driven Potentiation of Gene Expression in Plants Challenged by *B. cinerea*, *Pst*, and *H. arabidopsidis*

The expression of defense-related genes *PR1*, *PDF1.2*, and *PR4* was compared at 6 and 24 hpi between pathogen-inoculated or mock-inoculated plants



**Figure 3.** RLs protect *Arabidopsis* against *Pst* DC3000 infection. Plants were sprayed with RLs at 0.2 or 1 mg mL<sup>-1</sup> or water (control) and 4 d later leaves were dipped with *Pst*  $\pm$  *avrRPM1*. A, Symptoms observed 7 d after infection with the virulent strain *Pst* DC3000. Bacterial growth in *Arabidopsis* at 72 hpi with *Pst* DC3000 (B) and *Pst-avrRPM1* (C). Asterisks indicate significant differences between the RL-treated sample and the control according to Student's *t* test (\*\*\* $P < 0.005$ ). Values shown are means  $\pm$  SD ( $n = 24$ ) from one representative experiment among three independent repetitions. [See online article for color version of this figure.]



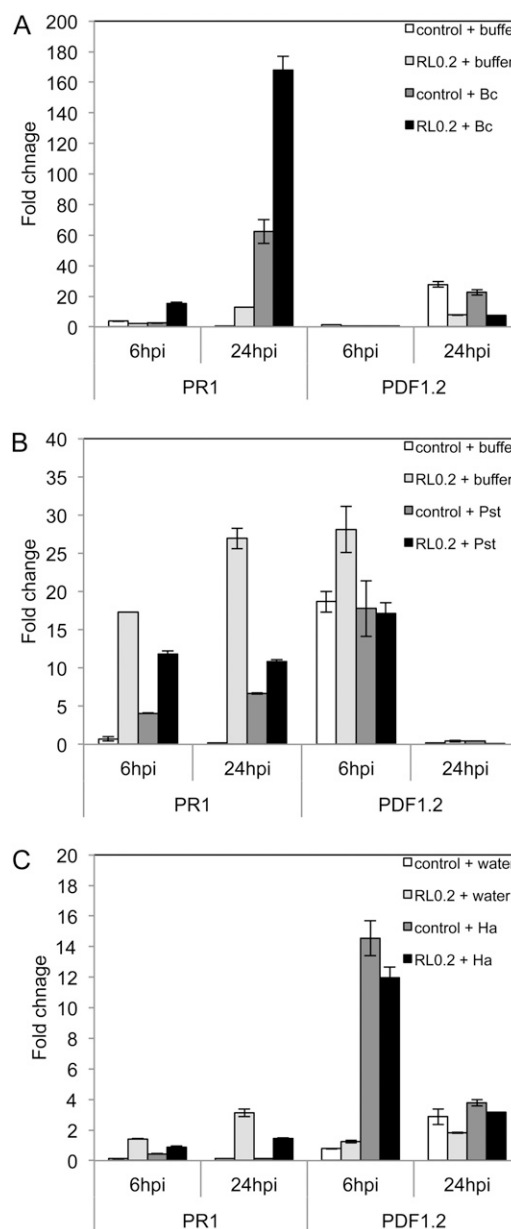
**Figure 4.** RLs induce resistance against *H. arabidopsidis* in *Arabidopsis*. Plants were treated with RLs (0.2 and 1 mg mL<sup>-1</sup>) or with water (control) and, 4 d later, leaves were inoculated with the oomycete. Spores were washed from infected leaves (7 d post inoculation) with water and an aliquot of spore suspension was counted under a microscope. Values shown are means  $\pm$  SD ( $n = 15$ ) from one representative experiment among three independent repetitions. Asterisks indicate significant differences between the RL-treated sample and the control according to Student's *t* test (\*\*\* $P < 0.005$ ).

pretreated 4 d before with water control or RLs at 0.2 mg mL<sup>-1</sup> (Fig. 5) or at 1 mg mL<sup>-1</sup> (Supplemental Fig. S4). Our results indicate that only *B. cinerea* infection caused a significant induction of *PR1* at 24 hpi and that pretreatment with 0.2 mg mL<sup>-1</sup> of RLs potentiates *PR1* gene expression in response to the fungus as soon as 6 hpi (Fig. 5A). No significant potentiation in *PDF1.2* expression (Fig. 5) and *PR4* expression (data not shown) was observed for all the conditions tested. Using a higher concentration of RLs (1 mg mL<sup>-1</sup>), we measured a strong potentiation of *PR1* response 24 h after *B. cinerea* infection or 6 h after *Pst* challenge and a very small potentiation effect at 24 h after *H. arabidopsidis* challenge (Supplemental Fig. S4). Again, no significant potentiation of *PDF1.2* expression (Supplemental Fig. S4) or *PR4* expression (data not shown) was observed in these conditions.

#### Effect of RLs on Growth and Swarming Motility of *Pst* and *B. cinerea* Spore Germination

RLs are known to inhibit oomycete mycelial growth and to decrease zoospore germination and/or motility in vitro (Stanghellini and Miller, 1997; Yoo et al., 2005). Recently, Varnier et al. (2009) showed that RLs also have a direct effect against the strain T4 of *B. cinerea*. We performed protection experiments with the strain B05.10 of the fungus, which is widely used for infection tests in *Arabidopsis* (Williamson et al., 2007). Using in vitro tests, we observed that after incubation with RLs at 0.2 mg mL<sup>-1</sup>, there was no significant difference in spore germination and hyphae growth of strain B0510 compared with the control (Supplemental Fig. S5). However, we estimated that 1 mg mL<sup>-1</sup> of RLs led to around 95% of spore germination inhibition at 24 h. To monitor the potential effect of RLs on *Pst* and *Pst-avrRPM1* growth, bacterial strains were cultivated in King's B medium supplemented or not with RLs (0.2 and 1 mg mL<sup>-1</sup>). No effect on bacterial growth

was observed in the presence of RLs compared with the control (data not shown). RLs are known to be involved in *P. aeruginosa* swarming motility (Köhler et al., 2000; Déziel et al., 2003; Caiazza et al., 2005). Swarming motility was assessed by examining and measuring the circular turbid zone formed by the bacterial cells migrating on swarm agar plates supplemented

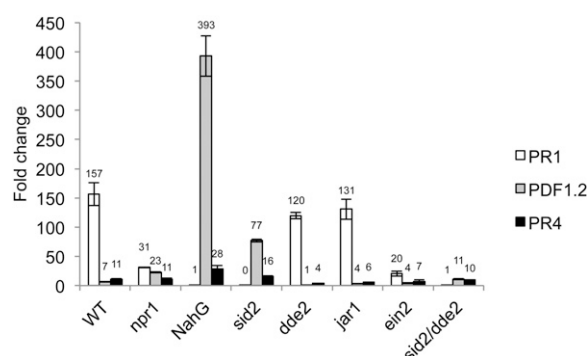


**Figure 5.** RL-mediated potentiation of gene expression after pathogen challenge. Plants were sprayed with 0.2 mg mL<sup>-1</sup> of RLs or not treated (control) 4 d before inoculation with pathogen or mock inoculation. Transcript accumulation of *PR1* and *PDF1.2* genes was determined by qRT-PCR 6 and 24 hpi with *B. cinerea* (Bc; A), *Pst* DC3000 (Pst; B), or *H. arabidopsidis* (Ha; C). Results are expressed as the fold increase in transcript level compared with nontreated leaves just before pathogen inoculation (control 0 hpi). Values shown are means  $\pm$  SD of duplicate data from one representative experiment among three independent repetitions.

or not with 0.2 and 1 mg mL<sup>-1</sup> of RLs. In these conditions, we did not observe any swarming motility effect of RLs on *Pst* and *Pst-avrRPM1* (data not shown).

### Changes in Gene Expression after Perception of RLs in Arabidopsis Mutants Affected in SA, JA, and ET Signaling Pathways

To assess the role of SA, JA, and ET in RL-mediated resistance to pathogens, we used Arabidopsis mutants impaired in their ability to accumulate or perceive these signal molecules. In these experiments, we chose *NahG* plants that totally degrade SA to be sure that no remaining traces of SA would be detected after RLs treatment and before protection assays (Heck et al., 2003; Ménard et al., 2004; Ferrari et al., 2007). We also used *sid2* plants that are impaired in SA synthesis (Nawrath and Métraux, 1999; Wildermuth et al., 2001), *npr1* that is insensitive to SA (Cao et al., 1994), and *ein2* mutants that are insensitive to ET (Guzmán and Ecker, 1990). *jasmonate-resistant1* (*jar1*) plants insensitive to JA (Staswick et al., 2002), *delayed-dehiscence2* (*dde2*) mutants affected in JA biosynthesis (von Malek et al., 2002), and the double mutant *sid2/dde2* affected in both SA and JA pathways (Tsuda et al., 2009) were also used in the following experiments. We first monitored signaling-specific marker gene expression in these mutants and in wild-type plants after RL treatments. In these experiments and the following, we used the lowest concentration of RLs to be in conditions where there is no direct effect of the molecules on the pathogens. *PR1* expression was totally abolished in *NahG* plants, *sid2*, and *sid2/dde2* mutants and strongly reduced in *npr1* and *ein2* mutants (Fig. 6). This unexpected result for *ein2* mutant can be explained by a high basal level of the mock-treated control (9×) compared with the basal level



**Figure 6.** Defense-related gene expression in leaves of wild-type, *npr1*, *NahG*, *sid2*, *dde2*, *jar1*, *ein2*, and *sid2/dde2* plants 24 h after treatment with RLs at 0.2 mg mL<sup>-1</sup>. Transcript accumulation of *PR1*, *PDF1.2*, and *PR4* genes was determined by qRT-PCR. Results are expressed as the fold increase in transcript level compared with mock-treated leaves and are means  $\pm$  SD of duplicate data from one representative experiment among three independent repetitions.

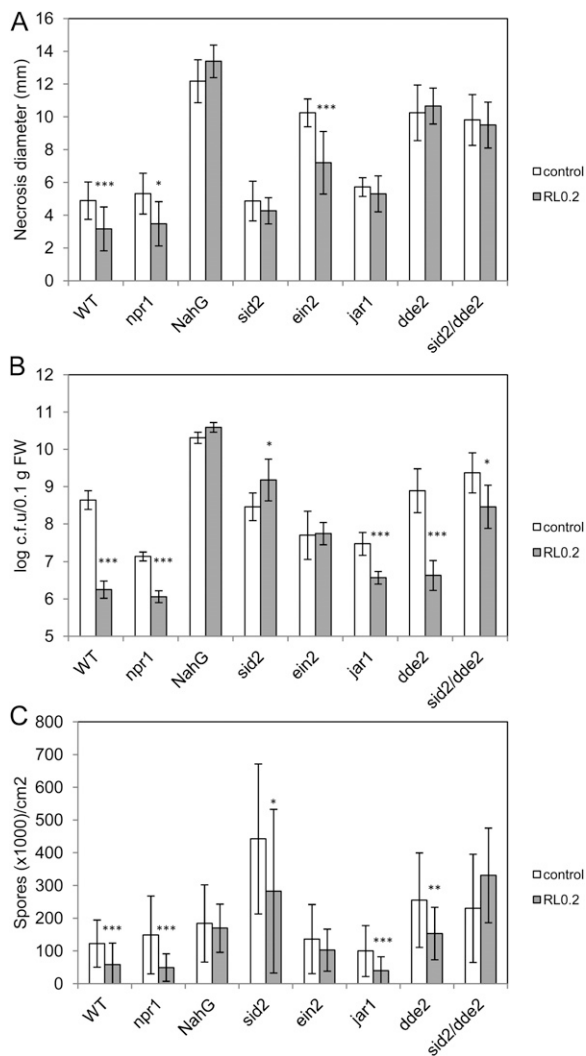
in the wild type. Otherwise, the *PR1* expression in the treated plants is similar in *ein2* mutant and wild-type plants (data not illustrated). The expression of *PR1* was not significantly affected in *jar1* and *dde2* mutants. *PDF1.2* was slightly overexpressed in *npr1* mutants but was strongly overexpressed in *sid2* and *NahG* plants. A 2-fold reduction in *PDF1.2* expression was observed in *jar1* or *ein2* mutants compared with wild-type plants, and its expression was totally abolished in the *dde2* plants. *PR4* expression was similar in *npr1*, *sid2/dde2*, and wild-type plants, whereas slightly overexpressed in *NahG* and *sid2* mutants. As for *PDF1.2*, *PR4* expression was weaker in *jar1*, *dde2*, and *ein2* mutants compared with wild-type plants.

### Role of SA, JA, and ET in RL-Mediated Resistance to *B. cinerea*, *Pst*, and *H. arabidopsidis*

To further elucidate the mechanisms responsible for the resistance triggered by RLs, wild-type plants, and plants affected in the different signaling pathways were analyzed in protection experiments after pretreatment with RLs and challenge with pathogens. As expected, control *ein2*, *dde2*, *sid2/dde2*, and *NahG* plants were more susceptible to *B. cinerea* compared with wild-type plants (Fig. 7A; Thomma et al., 1999; Ferrari et al., 2003; Raacke et al., 2006; Ferrari et al., 2007). Surprisingly, we did not observe any differences in susceptibility to the fungus between *jar1* and wild-type controls. Resistance induced by RLs was not compromised in *ein2* mutants, suggesting that the ET pathway is not involved in the process. No protection was observed in *NahG*, *sid2*, *jar1*, *dde2*, and *sid2/dde2* RL-treated plants (and to a lesser extent in *npr1* mutants), suggesting that SA and JA participate in the induced resistance (Fig. 7A).

Consistent with previous reports, we observed that the profile of *Pst* growth was similar in *npr1*, *jar1*, and wild-type plants (Laurie-Berry et al., 2006; Niu et al., 2011) and that *NahG* plants were more susceptible to *Pst* (Delaney et al., 1994; Fig. 7B). Surprisingly, we did not observe any differences in susceptibility to the bacterium between *sid2*, *dde2*, and wild-type plants as it was previously described (Nawrath and Métraux, 1999; Raacke et al., 2006). These differences may be explained by the method of bacterial inoculation (infiltration versus dipping in our study). *Npr1*, *jar1*, and *dde2* mutations did not compromise RL-mediated reduction of bacterial growth. However, susceptibility of *NahG*, *sid2*, *ein2* (and to a lesser extent, *sid2/dde2*) plants to *Pst* were unchanged after treatment with RLs. These results suggest that SA and ET play a role in RL-induced resistance to the hemibiotrophic bacterium, but that this resistance is *NPR1* independent and does not involve JA.

*NahG* plants were more susceptible to *H. arabidopsidis* in absence of treatment, which is in accordance with previous work (Donofrio and Delaney, 2001; Fig. 7C). Moreover, we observed a strong susceptibility of *sid2* mutant to *H. arabidopsidis* as described previously with *Peronospora parasitica* (Nawrath and Métraux,



**Figure 7.** RLs induce disease resistance that requires different signaling sectors depending on the pathogen. Wild-type, *npr1*, *NahG*, *sid2*, *dde2*, *jar1*, *ein2*, and *sid2/dde2* plants were pretreated with 0.2 mg mL<sup>-1</sup> of RLs or water (control) and infected with *B. cinerea* (A), *Pst* (B), and *H. arabidopsidis* (C). Protection assays were performed as described in Figures 2–4. Asterisks indicate significant differences between the RL-treated samples and controls according to Student's *t* test (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.005). The figures represent means ± SD (*n* = 24) from one representative experiment. Each experiment was repeated three times.

1999). We did not observe any change in the protective effect of RLs toward *H. arabidopsidis* in *npr1*, *jar1*, and *dde2* mutants compared with wild-type plants (Fig. 7C), suggesting that resistance against the oomycete does not go through JA alone and is NPR1 independent. Resistance induced by RLs was compromised in *NahG* and *ein2*, suggesting that both SA and ET may be involved in RL-mediated resistance to *H. arabidopsidis*. The mixed results obtained with *sid2* plants could be due to the high level of susceptibility of the mutant to *H. arabidopsidis*. It is interesting that resistance induced by RLs to the oomycete was completely compromised

in *sid2/dde2* plants, suggesting that SA and JA may act in synergy to account for the protection.

## DISCUSSION

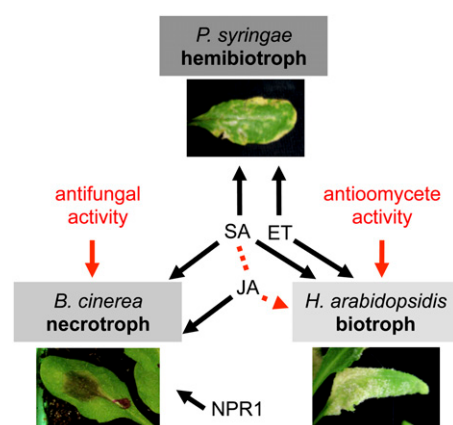
RLs are glycolipids produced by various bacterial species including some *Pseudomonas* spp. and *Burkholderia* spp. RLs have several potential functions in bacteria. They are involved in the uptake and biodegradation of poorly soluble substrates and are essential for surface motility and biofilm development (Abdel-Mawgoud et al., 2010). Recently, they have been highlighted as potential molecules recognized by animal cells that stimulate innate immunity (Andr  et al., 2006; Bauer et al., 2006; Howe et al., 2006; Vatsa et al., 2010). RLs have also been shown to induce defense responses in grapevine, wheat (*Triticum durum*), and tobacco cells (Varnier et al., 2009; Vatsa et al., 2010). We demonstrated here that RLs induce the typical Arabidopsis defense marker genes *PR1*, *PDF1.2*, and *PR4*, suggesting that Arabidopsis cells perceive these glycolipids as elicitors. Therefore, RLs display a nonspecific perception profile affecting a broad range of plant genera. RLs behaviors are very similar to those of cyclic lipopeptides that are involved in bacterial motility and biofilm development and that have also been recently described as inducers of plant innate immunity (Ongena et al., 2007; Raaijmakers et al., 2010). Surfactin, the most studied cyclic lipopeptide from *Bacillus subtilis*, has been shown to trigger early signaling events and late defense responses in tobacco cell suspensions (Jourdan et al., 2009). Other cyclic lipopeptides including massetolide A and fengycin were also identified as elicitors inducing a systemic resistance in tomato (*Solanum lycopersicum*) and bean (*Phaseolus vulgaris*; Ongena et al., 2007; Tran et al., 2007). Owing to their physical and chemical properties (i.e. amphiphilic molecules) and their potential mode of perception, RLs and lipopeptides can be considered as a new class of MAMPs produced by either pathogenic or nonpathogenic bacteria (Raaijmakers et al., 2010; Vatsa et al., 2010). Recently, some data indicated that surfactin perception relies on a lipid-driven process at the plasma membrane level (Henry et al., 2011). Such a sensor role of the lipid bilayer is quite uncommon considering that plant basal immunity is usually triggered upon recognition of microbial molecular patterns by high-affinity proteic receptors. It is yet unclear whether the induction of the defense response by RLs requires a specific pattern recognition receptor in the plant plasma membrane, as it is the case for flg22 and oligogalacturonides (G mez-G mez and Boller, 2000; Brutus et al., 2010) or whether they interfere directly with the plant plasma membrane as it has been postulated for surfactin or Nep1-like proteins (Qutob et al., 2006; Ottmann et al., 2009; Raaijmakers et al., 2010; Henry et al., 2011). However, the similarities in physical and chemical properties of lipopeptides and RLs suggest that RLs could be perceived in the same manner. Further experiments will be needed to clarify this point.



We demonstrated that RLs induced a resistance to *B. cinerea*, *Pst*, and *H. arabidopsidis*, three pathogens that are members of different lifestyle categories. This is to our knowledge the first report that describes a MAMP-induced resistance, at the local level, to a necrotrophic fungus, a hemibiotrophic Gram-negative bacterium, and a biotrophic oomycete. RLs are known to have antimicrobial properties (Varnier et al., 2009; Vatsa et al., 2010), but the contribution of both the direct effect of RLs, and the induced defense responses in the resistance process is not known. Our results confirm that at high concentrations, RLs have strong inhibitory effects on *B. cinerea*, and this inhibition is not restricted to a specific strain of the fungus. This direct effect of RLs is thought to participate in the protection that we observed at high concentration. However, resistance to *B. cinerea* is impaired in *NahG*, *sid2*, *jar1*, and *dde2* plants pretreated with a low concentration of RLs, demonstrating that activation of defense responses participate in RL-mediated resistance to the fungus. Similarly, although RLs are known to induce the direct lysis of oomycete zoospores (Stanghellini and Miller, 1997; Vatsa et al., 2010), our results show that RL-mediated resistance to *H. arabidopsidis* requires functional signaling pathways in the plant. RLs do not directly affect *Pst* growth or swarming motility, so bacterial resistance induced by RLs is essentially due to activation of plant defense responses.

In our study, levels of resistance to the virulent strain *Pst* observed in plants treated with RLs at 1 mg mL<sup>-1</sup> were strong and very similar to those observed in control plants infected with the avirulent strain *Pst-avrRPM1*. At this concentration, RLs can induce micronecroses reminiscent to micro-HR in Arabidopsis (Supplemental Fig. S2) and in grapevine leaves (Varnier et al., 2009). HR induction and robust defense responses are characteristic of the ETI (Tsuda and Katagiri, 2010), and our results strengthen the similarities between RLs and some general elicitors/MAMPs or toxins including Nep-1-like proteins (Qutob et al., 2006), fungal toxin fumonisin B1 (Asai et al., 2000), lipopolysaccharides (Desaki et al., 2006), or elicitors (Baillieul et al., 2003) that display ETI-like defense responses associated with a HR. Our data further reinforce the new concept that there is a continuum between MTI and ETI (Thomma et al., 2011) and that distinction between MAMPs and effectors is not completely relevant, at least in terms of physiological responses.

Our results with defense signaling in deficient mutants showed that RL-induced resistance to *B. cinerea*, *H. arabidopsidis*, and *Pst* requires some common signaling pathways but also differs for others. SA is essential for the resistance to the three pathogens, whereas ET is involved in the resistance to the hemibiotrophic bacterium and the biotrophic oomycete, and JA participates in the protection against the necrotrophic fungus (Fig. 8). It is interesting that RL-induced resistance to *B. cinerea* differs from resistance induced by typical MAMPs and danger-associated molecular patterns in terms of signaling pathways. Indeed, it has been shown that protection against *B. cinerea* in flg22- and oligogalacturonide-treated



**Figure 8.** Proposed model showing how RLs protect Arabidopsis against biotrophic hemibiotrophic and necrotrophic pathogens. RL-mediated resistance to *Pst* (hemibiotroph) and *H. arabidopsidis* (biotroph) involves SA and ET signaling (JA synergy with SA is illustrated by the dotted arrow). RL-mediated resistance to *B. cinerea* (necrotroph) involves SA and JA signaling and is affected by *NPR1* mutation. Direct antimicrobial activities of RLs participate to protection against fungus and oomycete pathogens (far left and far right arrows). [See online article for color version of this figure.]

Arabidopsis plants is independent of SA, ET, and JA signaling (Ferrari et al., 2003; Ferrari et al., 2007). We found that RL-mediated *PDF1.2* expression is over-induced in *NahG* and *sid2* plants. This result is in agreement with a compensation of JA/ET signaling in SA-depleted plants, but this compensation effect does not allow for better protection in RL-treated *NahG* and *sid2* plants. Moreover, RLs potentiate the expression of the SA marker *PR-1* after *B. cinerea* challenge, reinforcing the potential role of SA-dependent responses for the resistance to the fungus. Signaling pathways involved in MAMP-mediated resistance against biotrophic bacteria seem to be more conserved because flg22 (like RLs) triggers resistance against *Pst* that is compromised in SA-deficient *sid2* mutants (Kunze et al., 2004; Zipfel et al., 2004; Mishina and Zeier, 2007). Before our study, few data were available concerning elicitor-induced resistance against *H. arabidopsidis*. Only recently, Massoud et al. (2012) presented evidence that phosphite could prime Arabidopsis defenses against this oomycete. SA-dependent defenses are thought to play a role in limiting oomycete growth as demonstrated by experiments carried out in *eds5* and *sid2* plants (Nawrath and Métraux, 1999). Moreover, no enhanced susceptibility to oomycete was observed in *npr1-1* (Col-0 background; Bowling et al., 1997). SA-dependent and NPR1-independent resistance responses that limit growth of oomycetes seem to be conserved in RL-induced resistance but not in phosphite-induced protection, which is SA and NPR1 dependent (Massoud et al., 2012). Until now, there was no evidence that ET-dependent responses were normally active in limiting *H. arabidopsidis* (Glazebrook, 2005), but our data suggest that ET is involved in RL-mediated resistance to the oomycete.



The similarity between RLs and lipopeptides could suggest that common signaling pathways may be involved in induced resistance to different pathogens by these molecules. However, the sole available study suggests that this seems not to be the case for resistance to oomycetes because tomato protection mediated by massetolide A against *Phytophthora infestans* is independent of SA signaling (Tran et al., 2007). Unfortunately, there is no data available on the most studied lipopeptide, surfactin, regarding the signaling sectors involved in induced resistance to pathogens.

In conclusion, we propose a model in which RLs mediate a MTI that efficiently restricts *Arabidopsis* colonization of biotrophic, hemibiotrophic, and necrotrophic pathogens (Fig. 8). SA is a central signaling sector in overall RL-induced resistance, whereas ET and JA are differentially required depending on the pathogen life-style. In addition to activation of plant defense responses, RLs possess antimicrobial properties that reinforce their efficiency in restricting fungi and oomycete spread.

## MATERIALS AND METHODS

### Plant Material and Elicitation Treatments

*Arabidopsis* (*Arabidopsis thaliana*) Col-0 plants were used in this work. The mutants *npr1-1* (Cao et al., 1994), *jar1.1* (Staswick et al., 2002), *dde2-2* (von Malek et al., 2002), *ein2-1* (Guzmán and Ecker, 1990), *sid2-2* (Wildermuth et al., 2001), *sid2-2/dde2-2* (Tsuda et al., 2009) or transgenic *nahG* plants (Delaney et al., 1994) were all in the Col-0 background. Plants were grown in soil (Gramoflor) at 21°C with 60% relative humidity and a 12-h-light/12-h-dark cycle (light intensity 150  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) for 5 weeks. RLs from *Pseudomonas aeruginosa* (mix of  $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate; RL-1,2<sub>10</sub>: 40%) and 2-O- $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate (RL-2,2<sub>10</sub>: 60%) used in this study were previously characterized in Varnier et al. (2009). Fully expanded leaves were sprayed with different concentrations of RLs or water (control). For gene expression, leaves (from at least five plants) were collected and mixed 6, 9, 24, and 48 h after elicitation and conserved in liquid nitrogen for RNA extraction.

### Pathogen Assays in Planta

All of the protection experiments were repeated three times, unless otherwise indicated in the figure legends. *Botrytis cinerea* B05.10 cultures were initiated by transferring pieces of solid tomato (*Solanum lycopersicum*)/agar medium containing mycelium to fresh solid tomato/agar medium and incubated at 28°C. Conidia were collected from 3-week-old cultures in 2 mL of growth culture medium ( $\text{KH}_2\text{PO}_4$  1.75 g L<sup>-1</sup>,  $\text{MgSO}_4$  0.75 g L<sup>-1</sup>, Glc 4 g L<sup>-1</sup>, peptone 4 g L<sup>-1</sup>, Tween 20 0.02% [v/v]). The suspension was adjusted at 10<sup>5</sup> conidia mL<sup>-1</sup> of culture medium and agitated (130 rpm) during 9 h at 22°C to initiate spore germination. For each protection experiment, at least six plants and five leaves per plant were inoculated 4 d after elicitation with one droplet containing 1  $\times$  10<sup>5</sup> germinative conidia mL<sup>-1</sup>. The diameter of each lesion was measured 48, 72, and 96 hpi.

Inoculation with the bacterial leaf pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 or *Pst AvrRPM1* was realized by dipping. Briefly, bacteria were cultured overnight at 28°C in liquid King's B medium, supplemented with rifampicin (50  $\mu\text{g mL}^{-1}$ ) and kanamycin (50  $\mu\text{g mL}^{-1}$ ). Subsequently, bacterial cells were collected by centrifugation and resuspended in 10 mM  $\text{MgCl}_2$  Silwet L77 0.02% to a final density of 10<sup>8</sup> colony forming units (cfu) mL<sup>-1</sup> (optical density = 0.1). Plants were dipped in a suspension of *Pst* at 10<sup>8</sup> cfu mL<sup>-1</sup> or in 10 mM  $\text{MgCl}_2$  Silwet L77 0.02% as control. At 3, 24, and 72 hpi, 10 foliar discs from five leaves were excised using a cork borer, weighed, and ground in 1 mL  $\text{MgCl}_2$  (10 mM) with a plastic pestle. Appropriate dilutions were plated on King's B medium containing rifampicin (50  $\mu\text{g mL}^{-1}$ ) and kanamycin (50  $\mu\text{g mL}^{-1}$ ), and bacterial colonies were counted. Data are

reported as means and SD of the log (cfu 0.1 g<sup>-1</sup> fresh weight) of three replicates. Growth assays were performed three times with similar results.

*Hyaloperonospora arabidopsidis* isolate Noco2 was propagated at 7-d intervals in the *Arabidopsis* Col-0 wild type. Inoculum was prepared by placing heavily sporulating leaves into water, gently vortexing, and centrifuging the liquid to collect the conidiospores, which were resuspended in water ( $5 \times 10^4$  conidiospores mL<sup>-1</sup>). Infections were performed by spray-inoculation with asexual inoculum suspension ( $5 \times 10^4$  mL<sup>-1</sup>) on 5-week-old plants. The inoculated plants were maintained in a box for 7 d at 16°C with 8 h of light/day (100  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) and high humidity (80%–100%), which is optimal for *H. arabidopsidis* germination and growth. For the resistance test, each infected leaf was collected 7 d after infection and photographed to determine foliar surface (using ImageJ software) and placed in 400  $\mu\text{L}$  of distilled water. Spores were then washed from infected leaves by vortexing, and an aliquot of spore suspension was counted under a microscope.

### Spore Germination Assay

*B. cinerea* spore germination assay was realized as described by Prost et al. (2005). Briefly, *B. cinerea* strain B05.10 was grown in sterile, flat-bottom, 96-well microplates in a final volume of 100  $\mu\text{L}$  growth culture medium ( $\text{KH}_2\text{PO}_4$  1.75 g L<sup>-1</sup>,  $\text{MgSO}_4$  0.75 g L<sup>-1</sup>, Glc 4 g L<sup>-1</sup>, peptone 4 g L<sup>-1</sup>, Tween 20 0.02% [v/v]). Cultures were started with 5,000 spores and RLs were added after 16 h of growth. Growth was monitored by measuring the absorbance of the microcultures at 595 nm with a microplate reader (Bio-Rad or DYNEX technologies) at 0 h and after 5, 8, and 24 h of incubation in the presence or absence of RLs. Germ tube growth was observed using inverted light microscopy (Leica) 5, 8, and 24 h after RLs addition.

### Swarming Motility Assays

Swarm motility plates (0.5% agar) consisted of TSB/10 medium supplemented or not with RLs (0.2 and 1 mg mL<sup>-1</sup>). Once poured, swarm plates were allowed to dry at room temperature for 16 to 18 h prior to inoculation. Five microliters of overnight culture of bacteria (*Pst* or *Pst-avrRPM1*) grown in King's B medium with appropriate antibiotics, was spot inoculated into the middle of the plate, allowed to dry, and incubated at 37°C for 16 to 18 h. The assay was performed at least three times for each condition.

### RNA Extraction and Real-Time qRT-PCR

For each sample, 100 mg of leaves were ground in liquid nitrogen. Total RNA was isolated using Extract'All (Eurobio), and 1  $\mu\text{g}$  was used for reverse transcription using the Absolute MAX 2-Step qRT-PCR SYBR Green Kit (ThermoElectron) according to the manufacturer's instructions. The transcript levels were determined by real-time qRT-PCR using the Chromo4 system (BIO-RAD) and the SYBR Green Master Mix PCR kit as recommended by the manufacturer (Applied Biosystems).

PCR reactions were carried out in duplicates in 96-well plates (15  $\mu\text{L}$  per well) in a buffer containing 1 $\times$  SYBR Green I mix (including Taq polymerase, deoxyribonucleotide triphosphates, SYBR green dye), 280 nm forward and reverse primers, and 1:10 dilution of reverse transcript RNA. After denaturation at 95°C for 15 min, amplification occurred in a two-step procedure: 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C, with a total of 40 cycles. Identical thermal cycling conditions were used for all targets. Specific primers were designed using the Primer Express software (Applied Biosystems) and are presented in Supplemental Table S1. PCR efficiency of the primer sets was calculated by performing real-time PCR on serial dilutions. For each experiment, PCR reactions were performed in duplicate, and three independent experiments were analyzed. Results correspond to means  $\pm$  SD of duplicate reactions of one representative experiment out of three. Relative gene expression was determined with the formula fold induction:  $2^{-\Delta\Delta\text{Ct}}$ , where  $\Delta\Delta\text{Ct} = (\text{Ct GI [unknown sample]} - \text{Ct GI [reference sample]}) - (\text{Ct actin [unknown sample]} - \text{Ct actin [reference sample]})$ . GI is the gene of interest. Actin is used as internal control. The reference sample is the nontreated sample chosen to represent 1 $\times$  expression of the gene of interest.

### Seedling Assay and Histochemical GUS Detection

Seedling assay and GUS detection was performed according to Denoux et al. (2008) with minor modifications. For aseptic growth of seedlings, seeds were sterilized by treating them for 1 min in a mix of 95% ethanol/2%

commercial bleach (9:1), supplemented with Tween 20 (final concentration 0.01% [v/v]), followed by three quick washes with 99% ethanol and placement to dry under the hood. Ten to 15 seeds were dispensed into each well of a 12-well tissue culture plate with 1 mL of Murashige and Skoog Basal medium with vitamins (Duchefa) supplemented with 0.5% Suc and 0.5 g L<sup>-1</sup> 2-(N-morpholino)ethanesulfonic acid, pH 5.7. Plates were sealed with Parafilm to prevent evaporation of the medium. Seedlings were grown at 22°C with a 16-h photoperiod at a light intensity of 100  $\mu\text{m}^{-2}\text{s}^{-1}$  for 10 d before treatment. On the eighth day, the media were replaced with 1 mL of fresh media. Seedlings were treated with elicitors by adding directly to the medium either mono-RLs, di-RLs, or a mix of 40% mono- and 60% di-RLs. RLs were purified according to Varnier et al. (2009). flg22, a synthetic peptide of 22 amino acids (Boller and Felix, 2009), was used as positive control to a final concentration of 1  $\mu\text{M}$ . GUS enzyme activity of *PR-1::GUS* Arabidopsis seedlings was determined histochemically. Seedling medium in each well was removed and after a quick wash with sodium phosphate buffer, was replaced by 2 mL of 50 mM sodium phosphate, pH 7, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (Duchefa). Seedlings were incubated for 8 h at 37°C. The samples were then fixed with acetic acid/ethanol 1:3 (v/v); the chlorophyll was entirely removed by several washes in 70% ethanol, and the seedlings were mounted in 100% lactic acid.

## Cell Death Assay

Cell death was measured in the leaves by staining with Evans blue according to the method described by Kato et al. (2007), with some modifications. Excised leaflets from Arabidopsis plants were vacuum infiltrated with 0.2% (w/v) Evans blue (Sigma-Aldrich, France) for 10 min in eppendorfs to maintain plant tissues in the dying solution. After staining, the leaves were washed three times with distilled water until they were fully decolorized. All experiments were repeated at least three times, and at least 10 leaves collected from multiple seedlings (5 weeks old) were inspected in each experiment. Pictures of representative leaves were taken with a Canon Powershot G12 digital camera.

## Supplemental Data

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

**Supplemental Figure S1.** RLs induce SA and JA accumulation in Arabidopsis.

**Supplemental Figure S2.** RL-induced cell death in Arabidopsis.

**Supplemental Figure S3.** Purified mono- and di-RLs induce Arabidopsis defense response.

**Supplemental Figure S4.** RL-mediated potentiation of gene expression after pathogen challenge.

**Supplemental Figure S5.** Effect of RLs on *B. cinerea* spore germination.

**Supplemental Table S1.** Primers sequences used in qRT-PCR

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