GDSL lipase-like 1 regulates systemic resistance associated with ethylene signaling in Arabidopsis

Sun Jae Kwon[1], Hak Chul Jin[1], Soohyun Lee[2], Myung Hee Nam[3], Joo Hee Chung[3], Soon Il Kwon[1], Choong-Min Ryu[2] and Ohkmae K. Park[1,*]

[1]*School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea,*
[2]*Systems Microbiology Research Center, KRIBB, Daejeon 305-600, Korea, and*
[3]*Metabolome Analysis Team, Korea Basic Science Institute, Seoul 136-701, Korea*

Summary

Systemic resistance is induced by necrotizing pathogenic microbes and non-pathogenic rhizobacteria and confers protection against a broad range of pathogens. Here we show that Arabidopsis GDSL LIPASE-LIKE 1 (GLIP1) plays an important role in plant immunity, eliciting both local and systemic resistance in plants. GLIP1 functions independently of salicylic acid but requires ethylene signaling. Enhancement of *GLIP1* expression in plants increases resistance to pathogens including *Alternaria brassicicola*, *Erwinia carotovora* and *Pseudomonas syringae*, and limits their growth at the infection site. Furthermore, local treatment with GLIP1 proteins is sufficient for the activation of systemic resistance, inducing both resistance gene expression and pathogen resistance in systemic leaves. The *PDF1.2*-inducing activity accumulates in petiole exudates in a GLIP1-dependent manner and is fractionated in the size range of less than 10 kDa as determined by size exclusion chromatography. Our results demonstrate that GLIP1-elicited systemic resistance is dependent on ethylene signaling and provide evidence that GLIP1 may mediate the production of a systemic signaling molecule(s).

Keywords: Arabidopsis, GDSL lipase, systemic resistance, ethylene, salicylic acid, jasmonic acid.

Introduction

Plants possess multiple layers of immunity against pathogen attacks. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two types of systemic defense responses induced by necrotizing pathogens and plant growth-promoting rhizobacteria, respectively (Dong, 2004; Durrant and Dong, 2004; Pieterse and Van Loon, 2004; Ryu *et al.*, 2004b; Van Loon *et al.*, 1998). Systemic acquired resistance is the best-studied systemic immune response and is characterized by micro-oxidative bursts, accumulation of salicylic acid (SA), and expression of pathogenesis-related (*PR*) genes such as *PR1*, *PR2* and *PR5* in systemic tissues (Malamy *et al.*, 1990; Ryals *et al.*, 1996; Alvarez *et al.*, 1998). In contrast, ISR is dependent on jasmonic acid (JA) and ethylene (ET) pathways, and is not associated with *PR* expression (Pieterse *et al.*, 1996, 1998). Whereas SAR and ISR show distinct effectiveness against some pathogens (e.g. Turnip crinkle virus and *Alternaria brassicicola*), they confer resistance to the common spectrum of pathogens (Ton *et al.*, 2002). There seems to be substantial cross-talk

between SAR and ISR signaling pathways, as evidenced by the dependency of two pathways on NPR1, known as a key positive regulator of SAR (Pieterse *et al.*, 1998; Pieterse and Van Loon, 2004; Ryu *et al.*, 2004b).

Signaling is a central part of systemic resistance that requires a systemic mobile signal (Dong, 2004; Durrant and Dong, 2004; Fobert and Despres, 2005; Wang *et al.*, 2005). It is believed that a signal is generated at the primary infection site and translocated to the rest of the plant tissues to activate defense mechanisms (Uknes *et al.*, 1992; Ryals *et al.*, 1996; Grant and Lamb, 2006). A key step for understanding the signaling mechanism of systemic resistance is the identification of the mobile signals. Several candidates have been suggested as the signaling molecules that induce SAR. Although SA has long been suspected to be a systemic signal, accumulating data demonstrate that SA is not the signal itself, but its accumulation is important for establishing and maintaining SAR (Yalpani *et al.*, 1991; Vernooij *et al.*, 1994). Salicylic acid-binding protein 2 (SABP2) in

tobacco, belonging to the α/β hydrolase superfamily, has emerged as an essential component in SAR (Kumar and Klessig, 2003). Structural and biochemical analyses indicate that SABP2 has strong esterase activity toward the substrate methyl SA (MeSA) and converts MeSA to SA, and that SA is a potent feedback inhibitor of SABP2 (Forouhar *et al.*, 2005). In line with these findings, a series of grafting experiments using wild-type (WT) and *SABP2* mutant transgenic plants led to the conclusion that MeSA is a SAR mobile signal and SABP2 is required for the conversion of MeSA to SA in systemic tissue (Park *et al.*, 2007). Many lines of recent evidence suggest that lipids play critical roles in SAR signaling. Mutations in *Suppressor of Fatty Acid Desaturase 1* (*SFD1*) encoding dihydroxyacetone phosphate reductase compromise the activation of SAR, suggesting the involvement of plastidic glycerolipids in SAR (Nandi *et al.*, 2004; Chaturvedi *et al.*, 2008). The importance of plant lipid transfer proteins (LTPs) as components in systemic signaling has also been emphasized (Maldonado *et al.*, 2002; Buhot *et al.*, 2004). *Defective in Induced Resistance 1* (*DIR1*) encoding a putative LTP appears to function in long-distance signaling. The *dir1-1* mutant fails to develop SAR, suggesting that a lipid-derived molecule may be the systemic mobile signal and interact with DIR1 (Maldonado *et al.*, 2002). In an *in vitro* binding analysis, tobacco LTP1 preferentially loads JA over fatty acids and the LTP1–JA complex induces protection against *Phytophthora parasitica* in tobacco plants (Buhot *et al.*, 2004). These reports together indicate that a lipid signaling process is essential for systemic resistance.

Our previous studies demonstrated that GDSL LIPASE-LIKE 1 (GLIP1) plays important roles in resistance against the necrotrophic fungus *A. brassicicola* (Oh *et al.*, 2005). In this work, we provide evidence that Arabidopsis GLIP1 functions in both local and systemic resistance, and in cooperation with ET signaling. *GLIP1*-overexpressing transgenic Arabidopsis (*35S:GLIP1*) exhibited enhanced resistance against necrotrophic pathogens including *A. brassicicola* and *Erwinia carotovora*, and the hemibiotrophic pathogen *Pseudomonas syringae*. Application of GLIP1 proteins or petiole exudates from *35S:GLIP1* plants, but not from *glip1*, induced *PDF1.2* and *GLIP1* expression in distal leaves, which was accompanied by the activation of systemic resistance against multiple pathogens. We propose that GLIP1 has the activity to generate and propagate a systemic signal that is required for ET-mediated systemic resistance.

Results

Pathogen resistance phenotypes of glip1 and 35S:GLIP1 plants

In our previous studies, *glip1* mutant plants were found to be highly susceptible to *A. brassicicola* infection, unlike the incompatible parental Arabidopsis ecotype Columbia-0

(Col-0) (Oh *et al.*, 2005). To extend functional analysis of GLIP1, we generated *35S:GLIP1* plants that constitutively overexpressed *GLIP1* from the cauliflower mosaic virus 35S promoter. Five T_3 homozygous lines were recovered, two of which (3–2 and 8–6) displayed strong *GLIP1* expression and were used for analysis (Figure S1a). *35S:GLIP1* plants showed enhanced growth with an apparent increase in the size of leaves and primary root length, in contrast to *glip1* plants (Figure S1b). Inoculation with *A. brassicicola* induced the formation of hypersensitive response-like lesions in *35S:GLIP1(3-2)* plants as in WT plants (Figure 1a). Fungal growth at the infection sites of plants was examined under a scanning electron microscope. In WT and *35S:GLIP1(3-2)* plants, spore germination and initial hyphal growth occurred, but further growth was largely hindered. We observed that the hyphal surface was damaged in WT plants and this appeared more severe in *35S:GLIP1(3-2)* plants, where the hyphae underwent even fragmentation at multiple places. In contrast, the lesions in *glip1-1* plant leaves were heavily colonized by fungal hyphae as compatible interaction developed. Plants were also subjected to infection with the necrotrophic bacterial pathogen *E. carotovora* subsp. *carotovora* SCC1 (Kariola *et al.*, 2005) expressing green fluorescent protein (GFP) (Figure 1b). The disease symptoms that developed in *glip1-1* were abolished in *35S:GLIP1(3-2)* plants, which correlates well with bacterial growth as monitored under a fluorescence microscope. In contrast, when challenged with the hemibiotrophic bacterial pathogen *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), *glip1-1* displayed no differences in bacterial growth and disease symptoms compared with WT (Figure 1c), as shown in our previous report (Oh *et al.*, 2005). In *35S:GLIP1(3-2)*, however, the growth of both virulent and avirulent strains of *Pst* DC3000 was significantly suppressed. These results suggest that secreted GLIP1 proteins are involved in resistance against fungal and bacterial pathogens, but differentially to necrotrophic and hemibiotrophic pathogens.

GLIP1 induces systemic resistance

We have previously shown that GLIP1 may play a role in systemic resistance signaling when plants are challenged with *A. brassicicola* (Oh *et al.*, 2005). The role of GLIP1 in systemic resistance was further investigated by assaying recombinant GLIP1 proteins for the systemic activity against the virulent pathogens *Pst* DC3000 and *E. carotovora*. Along with active GLIP1, we prepared an inactive form of GLIP1 (GLIP1[TM]) in which residues of the catalytic triad (Ser, Asp and His) were replaced with Ala. The growth of *Pst* DC3000 and *E. carotovora* was determined in systemic leaves after GLIP1 treatment of primary leaves (Figure 2a,b). In WT, bacterial growth following primary GLIP1 treatment was significantly suppressed to levels comparable with those
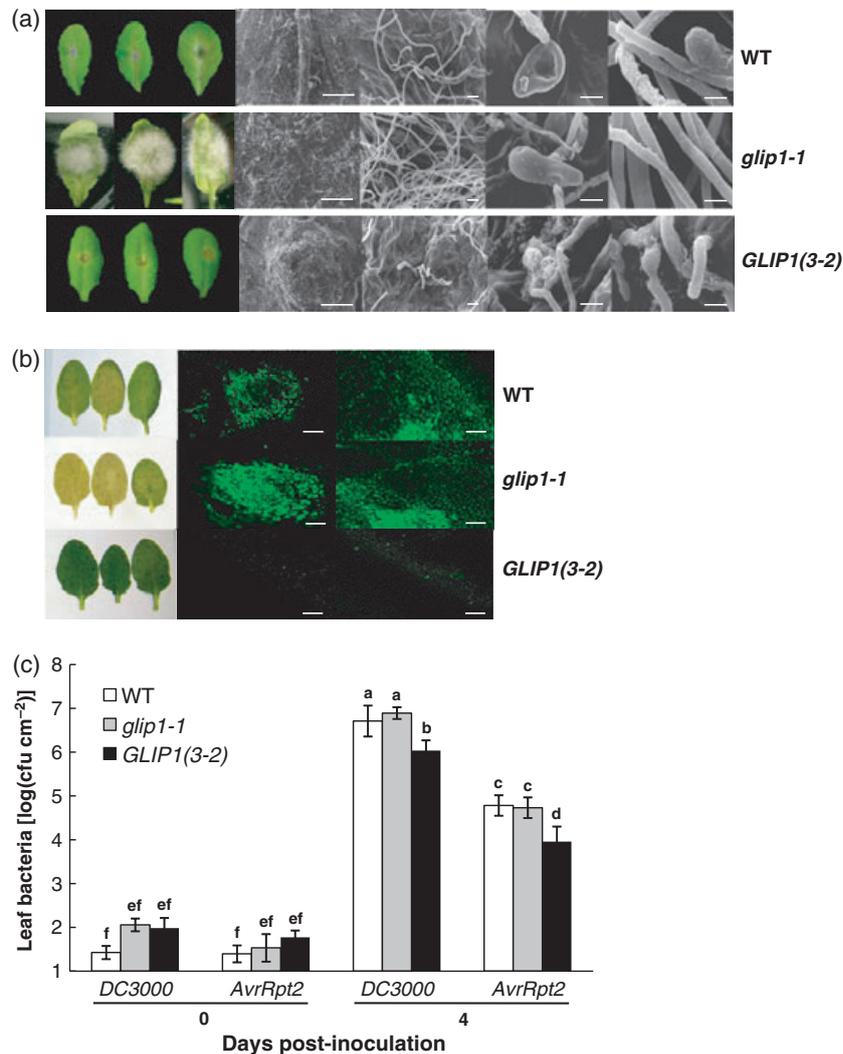
Figure 1. Pathogenic response of WT, *glip1* and *35S:GLIP1* plants.
(a) Growth of *Alternaria brassicicola* visualized by scanning electron microscopy (right panel); scale bar, 500 μm (left) and 5 μm (the rest).
(b) Growth of *Erwinia carotovora* visualized by fluorescence microscopy (right panel); scale bar, 100 μm.
(c) Growth of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and *Pst* DC3000 (*avrRpt2*) measured as colony-forming units (cfu). Experiments were repeated three times with similar results, using five plants for each respective treatment. Different letters indicate significant differences between treatments (least significant difference test; $P = 0.05$, $n = 5$).
Four-week-old plants were challenged with 10 μl of spore suspension of *A. brassicicola* (5×10^5 spores ml^{-1}) (a) or *E. carotovora* (10^7 cfu ml^{-1}) (b), or 20 μl of *P. syringae* bacterial suspension (10^6 cfu ml^{-1}) (c). Pathogen growth was determined 4 days after inoculation.

observed after primary inoculation with avirulent *Pst* DC3000 (*avrRpt2*) and benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) (Lawton *et al.*, 1996) for *Pst* DC3000 and *E. carotovora*, respectively. GLIP1® had little effect on bacterial growth, indicating that the acyl hydrolase activity of GLIP1 is critical for systemic resistance. In the *glip1-1* mutant background, however, primary GLIP1 treatment failed to systemically suppress bacterial growth, but *Pst* DC3000 (*avrRpt2*) and BTH treatments continued to be effective in systemic resistance to *Pst* DC3000 and *E. carotovora*, respectively. Constitutive expression of *GLIP1* in *35S:GLIP1(3-2)* appeared to confer resistance to pathogens regardless of the pathogen and primary treatment applied (Figures 1 and 2).

GLIP1-mediated pathogen resistance is dependent on ET signaling

How plant immunity induced by GLIP1 proteins interacts with hormones was examined in the ET-insensitive mutant *etr1-1*, the JA-resistant mutant *jar1-1*, and *salicylate hydroxylase*-overexpressing transgenic *NahG* plants (Figure 3a). All three mutant plants were more susceptible to *Pst* DC3000 than WT plants in control (PBS), indicating that SA, JA, and ET signaling pathways contribute to pathogen resistance. GLIP1-elicited systemic resistance to *Pst* DC3000 was effective in WT and to a lesser extent in *jar1-1* and *NahG* plants, but was impaired in *etr1-1*. This indicates that the ET
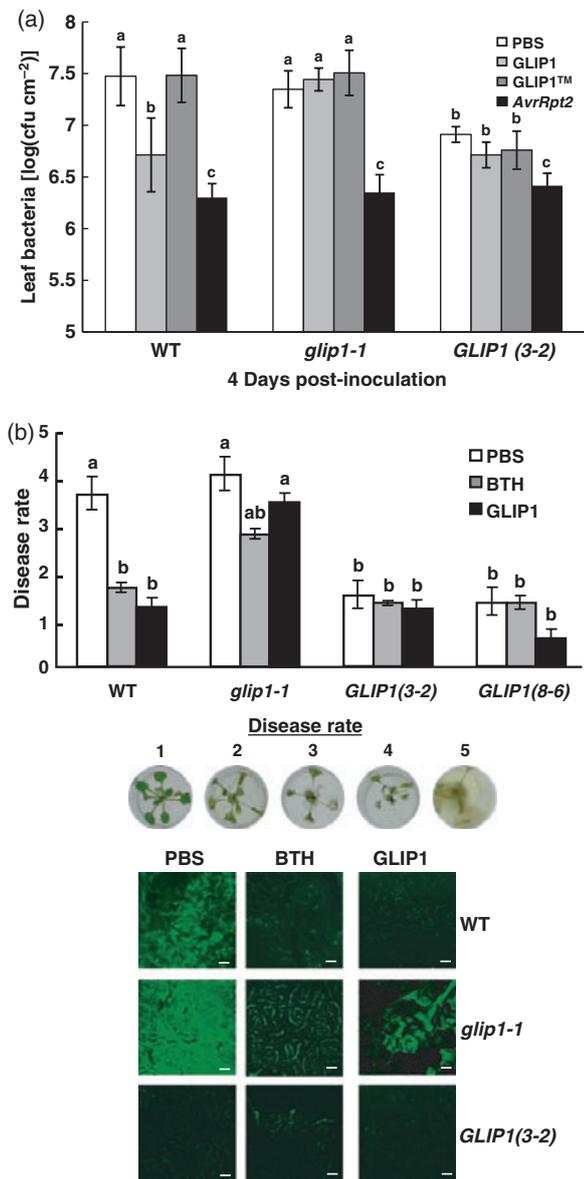
Figure 2. GLIP1-dependent systemic suppression of bacterial growth.
(a) Systemic resistance against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) in wild-type (WT), *glip1* and *35S:GLIP1* plants. Four-week-old plants were infiltrated at one site on the primary leaf with PBS (control), GLIP1 or GLIP^{TM} proteins (0.1 μg), or 20 μl of *Pst* DC3000 (*avrRpt2*) suspension [10^6 colony-forming units (cfu) ml^{-1}], incubated for 3 days, and then inoculated with 20 μl of *Pst* DC3000 suspension (10^6 cfu ml^{-1}) on the secondary leaves. This experiment was designed as a randomized complete block with five replications and one plant per replication. The experiment was repeated three times with similar results. Different letters indicate significant differences using Fisher's least significant difference (LSD) test at *P* = 0.05.
(b) Systemic resistance against *Erwinia carotovora*. Disease severity estimations (0–5) were made 2 days after pathogen inoculation, which occurred 3 days after infiltration of 3-week-old seedlings with PBS (control), benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH; 1 mM), or GLIP proteins (0.1 μg). This experiment was designed as a randomized complete block with 10 replications and one plant per replication. The experiment was repeated three times with similar results. Different letters indicate significant differences using Fisher's LSD test at *P* = 0.05. The growth of *E. carotovora* was visualized by fluorescence microscopy (bottom panel); scale bar, 50 μm.
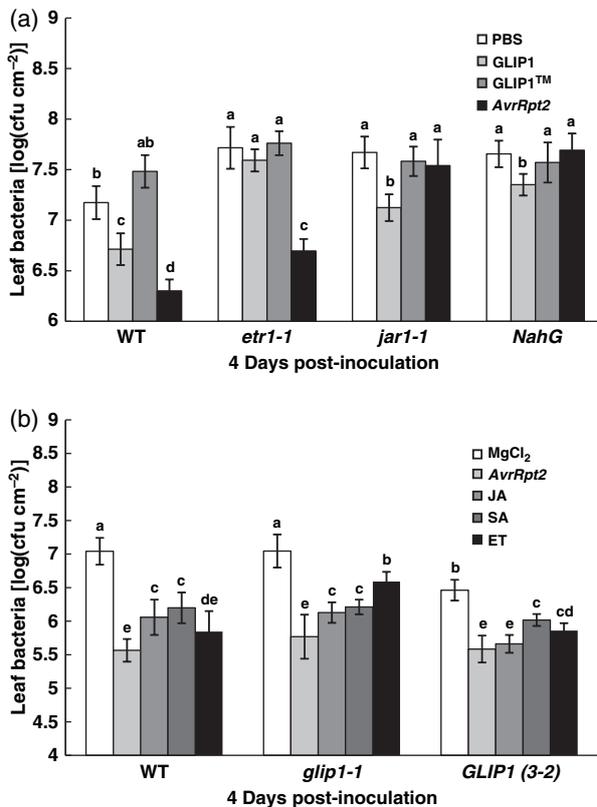
Figure 3. GLIP1-induced systemic resistance in hormone response mutant plants.
(a) Systemic resistance against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) in wild-type (WT), *etr1-1*, *jar1-1* and *NahG* plants. Primary leaves of 4-week-old plants were infiltrated with PBS (control), GLIP1 or GLIP^{TM} proteins (0.1 μg), or 20 μl of *Pst* DC3000 (*avrRpt2*) suspension [10^6 colony-forming units (cfu) ml^{-1}], incubated for 3 days, and then inoculated with 20 μl of *Pst* DC3000 suspension (10^6 cfu ml^{-1}) on the secondary leaves. Different letters indicate significant differences using Fisher's least significant difference (LSD) test at *P* = 0.05.
(b) Effect of hormones on bacterial growth in WT, *glip1* and *35S:GLIP1* plants. Four-week-old plants were infiltrated with *Pst* DC3000 (*avrRpt2*) or sprayed with salicylic acid (SA; 1 mM), methyl jasmonic acid (MeJA) (50 μM), or ethephon (1.5 mM) 3 days before *Pst* DC3000 inoculation. Different letters indicate significant differences using Fisher's LSD test at *P* = 0.05.
Pathogen growth was determined 4 days after inoculation. This experiment was designed as a randomized complete block with five replications and one plant per replication. The experiment was repeated three times with similar results.

response is necessary for GLIP1 function, consistent with our previous observation (Oh *et al.*, 2005). In contrast, *Pst* DC3000 (*avrRpt2*)-mediated resistance was significantly compromised in *jar1-1* and *NahG*, but not in the *etr1-1* background. Moreover, bacterial growth in WT, *glip1-1* and *35S:GLIP1(3-2)* plants pre-treated with hormones was compared with bacterial growth in plants pre-challenged with *Pst* DC3000 (*avrRpt2*) (Figure 3b). In WT plants, ethephon, methyl JA (MeJA) and SA were as effective in restricting virulent bacterial growth as *Pst* DC3000 (*avrRpt2*) pre-inoculation. However, resistance was induced much less in

glip1-1 compared with WT plants upon ethephon treatment, unlike MeJA and SA. These results suggest that the ET–GLIP1 signaling pathway may operate in plants independently of JA and SA which are associated with *avrRpt2*-induced immunity.

GLIP1 treatment is sufficient for the induction of systemic gene expression

We further examined whether the activation of systemic resistance is accompanied by systemic gene induction by GLIP1. To test this, we used *PDF1.2:GUS* transgenic plants where the promoter region of *PDF1.2* gene was fused to the GUS reporter (Ryu *et al.*, 2004a). GUS expression was monitored in *PDF1.2:GUS* transgenic plants in response to applications of *A. brassicicola* and recombinant GLIP1 proteins to determine systemic gene expression (Figure 4a). As expected, inoculation of *A. brassicicola* activated systemic GUS expression and led to strong GUS staining in plants. A significant GUS induction was observed upon treatment with GLIP1, but not with GLIP1®. The local and systemic gene expression by *A. brassicicola*
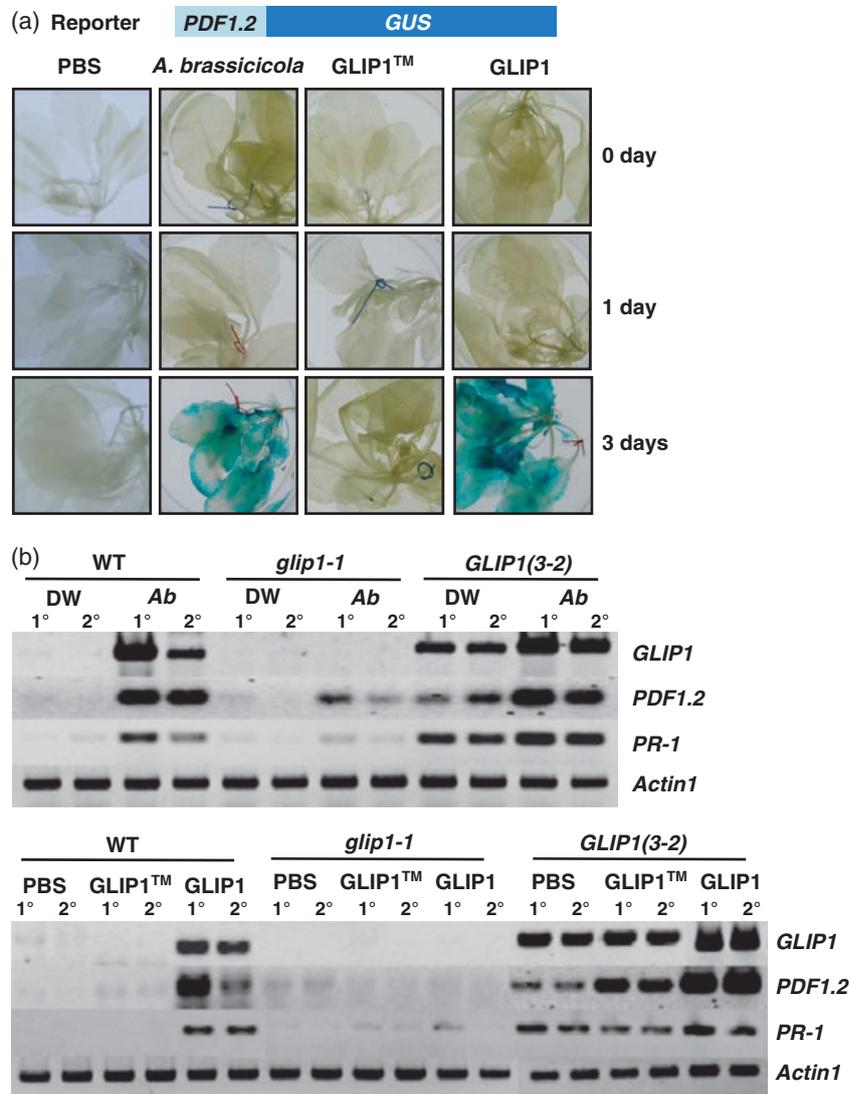

Figure 4. GLIP1-dependent systemic gene activation.

(a) Systemic GUS expression in *PDF1.2:GUS* transgenic plants in response to *Alternaria brassicicola* and GLIP1 proteins. Histochemical GUS staining of whole plants was performed at 0, 1 and 3 days after the respective treatments.

(b) Ribonucleic acid analysis of systemic gene induction of *GLIP1*, *PDF1.2* and *PR-1* in response to *A. brassicicola* and GLIP1 proteins. Total RNAs from both local (primary) and systemic (secondary) leaves were isolated 3 days after the indicated treatments and RNA levels were semiquantitatively determined by RT-PCR analysis. *Actin1* was used as a control.

A single leaf from 4-week-old plants was inoculated with PBS (control), distilled water (DW; control), 10 μl of spore suspension of *A. brassicicola* (Ab; 5×10^5 spores ml^{-1}), or GLIP1 or GLIP1^{TM} proteins (0.1 μg).

and GLIP1 proteins was then evaluated by RT-PCR (Figure 4b). In WT plants, the expression of *GLIP1*, *PDF1.2* and *PR-1* was induced in both local and systemic leaves by *A. brassicicola* and GLIP1 treatments. These WT results are consistent with those from *PDF1.2:GUS* plants and indicate that local treatment with GLIP1 allows the activation of systemic gene expression. In *glip1-1* plants, this gene induction was not significant although *PDF1.2* expression slightly increased in response to *A. brassicicola*, suggesting that systemic induction of *GLIP1* following local treatment is also necessary for the systemic induction of genes such as *PDF1.2* and *PR-1*. We previously showed that *PR-1* expression is induced in *glip1-1* plants by *A. brassicicola* treatment (Oh *et al.*, 2005). This discrepancy is explained because *PR-1* expression is time dependent in *A. brassicicola*-treated *glip1-1* plants, increasing for the first day of treatment but decreasing afterwards (Figure S2). In *35S:GLIP1(3-2)* plants, constitutive expression of *GLIP1*, *PDF1.2* and *PR-1* was observed.

PDF1.2-*inducing activity accumulates in the phloem in a GLIP1-dependent manner*

Since GLIP1 may activate systemic resistance through the production of a systemic signal, we examined whether the activity to systemically induce gene expression accumulated in the phloem in a GLIP1-dependent manner. Petiole exudates were prepared using a previously described method (Taylor *et al.*, 1990; Maldonado *et al.*, 2002). Exudates were collected from WT, *glip1-1* and *35S:GLIP1(3-2)* (Figure S3) and used to inoculate *PDF1.2:GUS* plants (Figure 5a). GUS induction was detected in plants inoculated with *35S:GLIP1(3-2)* exudates, and when prepared from *A. brassicicola*-treated plants both WT and *35S:GLIP1(3-2)* exudates were able to induce *PDF1.2* as indicated by GUS expression; however, *glip1-1* exudates failed. We checked for the possibility that overexpression of *GLIP1* may release GLIP1 proteins into the phloem. Total cell extracts and petiole exudates were prepared from *35S:GLIP1-His* transgenic plants in which His-tagged GLIP1 proteins were overexpressed and subjected to western blot analysis with the anti-His antibody (Figure S4). GLIP1 proteins were detected in cells but not in petiole exudates, confirming the lack of GLIP1 contamination of the phloem. In addition, microbial contamination was not detected in petiole exudates of plants (data not shown). These results suggest that a systemic signal can be produced constitutively in *35S:GLIP1(3-2)* plants, and induced in WT. For quantitative measurement of GUS activity, protoplasts prepared from *PDF1.2:GUS* plants were incubated with petiole exudates for 30 min (Figure 5b). GUS expression was highly induced by *35S:GLIP1(3-2)* exudates and slightly induced by WT exudates, but not by *glip1-1* exudates. A single treatment with recombinant GLIP1 proteins was not capable of enhancing GUS expres-
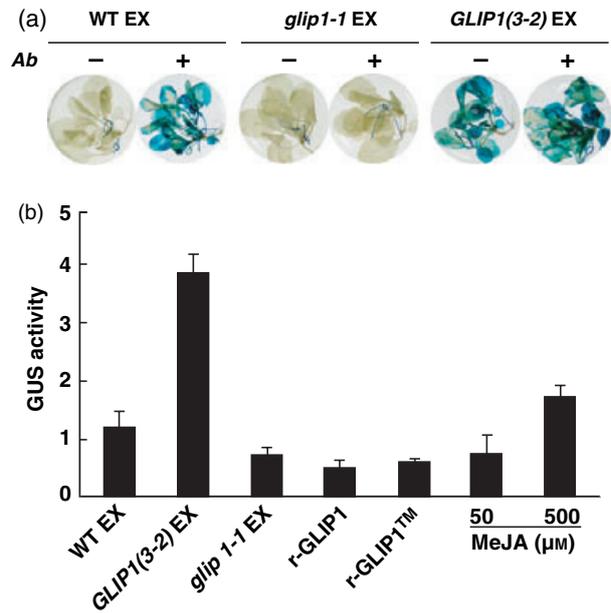

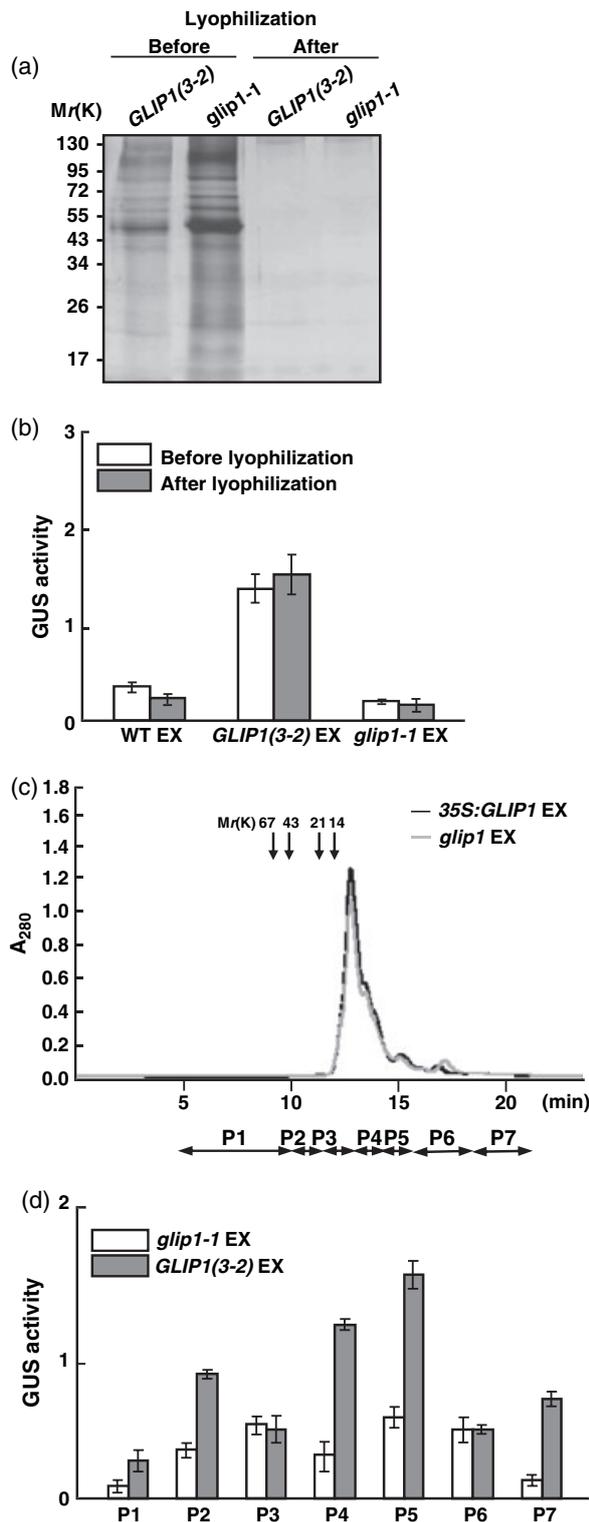**Figure 5.** Detection of *PDF1.2*-inducing activity in petiole exudates.
(a) Systemic GUS expression in *PDF1.2:GUS* transgenic plants in response to petiole exudates. Petiole exudates (EX) were isolated from wild-type (WT), *glip1-1* and *35S:GLIP1(3-2)* plants untreated or treated with *Alternaria brassicicola* (*Ab*) for 3 days. Histochemical GUS staining of whole plants was performed 3 days after infiltration with 20 μl of exudates (0.3 μg μl^{-1}).
(b) *PDF1.2* activation in *PDF1.2:GUS* protoplasts. Protoplasts (2×10^5 cells) were treated with 20 μl of exudates (0.3 μg μl^{-1}), recombinant proteins (1 μg), or 50 and 500 μM methyl jasmonic acid (MeJA), and incubated for 30 min. Results represent means of GUS activities obtained after subtraction of control (PBS-treated) activities. All data are representative of three independent replicates.

sion, implying the requirement for cell wall components, probably the GLIP1 substrate. The addition of MeJA also led to GUS induction to a certain level.

We then assessed whether the *PDF1.2*-inducing activity in petiole exudates could be chromatographically fractionated. To reduce the sample volume, the collected exudates were concentrated in a lyophilizer and then resuspended in the buffer. During this process, most high-molecular-weight proteins became insoluble (Figure 6a), but all the activity remained in the concentrated soluble fraction as determined by a GUS assay in *PDF1.2:GUS* protoplasts (Figure 6b). This procedure was found to be highly effective in excluding a range of contaminating proteins present in petiole exudates. Consequently, the partially purified *35S:GLIP1(3-2)* and *glip1-1* exudates could then be loaded for size exclusion chromatography (Figure 6c). Chromatographic profiles displayed proteins in the range of less than 20 kDa. The fractions were collected, combined into seven pools (P1 to P7), and used for a GUS assay in *PDF1.2:GUS* protoplasts (Figure 6d). In contrast to *glip1-1* exudates, *35S:GLIP1(3-2)* exudates showed a significant level of GUS-inducing activity

in P4 and P5. GUS-inducing activity in *35S:GLIP1(3-2)* exudates was fractionated in the size range of less than 10 kDa, although its identity is yet to be determined.

Figure 6. Lyophilization and chromatographic fractionation of petiole exudates.
(a) Protein profiles in soluble fractions of petiole exudates before and after lyophilization. Proteins were separated by SDS gel electrophoresis and visualized by silver staining.
(b) *PDF1.2* activation in *PDF1.2:GUS* protoplasts. Protoplasts (2×10^5 cells) were treated with soluble fractions of petiole exudates before and after lyophilization. The same portions of petiole exudates were taken out of the soluble fractions before and after lyophilization to estimate the recovery of systemic acquired resistance (SAR)-inducing activity after lyophilization.
(c) Size fractionation of *glip1* and *35S:GLIP1* exudates. Petiole exudates collected from *glip1-1* and *35S:GLIP1(3-2)* plants were fractionated by Zorbax GF-450 size exclusion chromatography. The distribution of standard protein markers is indicated at the top. The fractions were combined into seven pools (P1–P7) and used for the GUS assay.
(d) *PDF1.2* activation in *PDF1.2:GUS* protoplasts. Protoplasts (2×10^5 cells) were treated with pools (P1–P7) of fractions collected by Zorbax GF-450 size exclusion chromatography.
In (b) and (d) GUS activity is expressed as pmol 4-methylumbelliferone (MU) min^{-1} mg^{-1} protein. Results represent means of GUS activities obtained after subtraction of control (PBS-treated) activities. All data are representative of three independent replicates.

Discussion

GLIP1 functions as an elicitor of systemic resistance linked to ET signaling

Our data demonstrate that GLIP1 plays a crucial role in plant systemic immunity. In our previous work (Oh *et al.*, 2005), *glip1* mutant plants were markedly susceptible to *A. brassicicola* but not to both virulent and avirulent strains of *Pst* DC3000, suggesting that GLIP1 may specifically function in the resistance to necrotrophic pathogens. However, over-expression of GLIP1 in plants enhanced resistance to both *A. brassicicola* and *P. syringae* (Figure 1). These results suggest that growth inhibition of *P. syringae* in *35S:GLIP1(3-2)* plants may be due to the systemic resistance activated throughout the plant by constitutively expressed GLIP1, but not due to the direct bacteriostatic activity of GLIP1. This is further supported by the observations that direct treatment of recombinant GLIP1 proteins had little effect on *P. syringae* (data not shown), in contrast to the severe damage to both structural integrity and growth sustained by *A. brassicicola* (Oh *et al.*, 2005). Taken together, our current and previous (Oh *et al.*, 2005) work leads us to propose dual roles for GLIP1: direct interference with pathogen growth and activation of systemic resistance. The former may be a specific response to necrotrophic pathogens, and differs from the latter systemic resistance response which exhibits broad specificity. It was also found that systemic resistance is not induced in *glip1* plants (Figures 2 and 4), suggesting that secondary induction of GLIP1 is required for signal amplification and propagation in systemic tissues (Figure 7). However, we cannot rule out the possibility that systemic resistance induced by GLIP1 application or overexpression in plants may be due to mis-expression of GLIP1 where endogenous GLIP1 proteins do not normally accumulate in
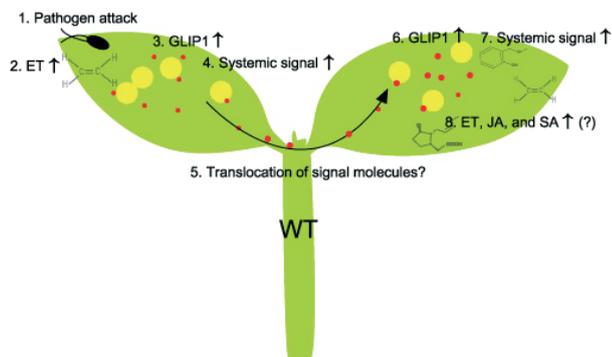
Figure 7. A model for GLIP1 in systemic resistance signaling.
We propose a role for GLIP1 in systemic immunity. Pathogen infection (1) induces *GLIP1* expression that may depend on ethylene (ET) signaling (2, 3). GLIP1 proteins mediate the generation of the systemic signal that translocates to other uninfected tissues (4, 5). The mobile signal leads to the production of GLIP1 which in turn induces the accumulation of signaling molecules in systemic tissues (6, 7). This chain-reaction of GLIP1 and the systemic signal may amplify and propagate systemic resistance throughout the plant. The level of salicylic acid (SA), jasmonic acid (JA) and ET may increase in systemic tissues, conferring broad spectrum resistance to plants (8). GLIP1 proteins and signaling molecules are indicated as yellow and red symbols.

plants. Further examination would be required to clarify this point.

Here, and in previous work (Oh *et al.*, 2005), we show that GLIP1 is associated with the ET pathway. Systemic resistance against *Pst* DC3000 could be induced by either *Pst* DC3000 (*avrRpt2*) or GLIP1 treatment that appeared to be linked to JA and SA, and to ET, respectively (Figure 3). Although SAR was repressed in *NahG* and *jar1-1* plants but was still activated in *etr1-1* upon treatment with *Pst* DC3000 (*avrRpt2*), the opposite results were obtained with GLIP1 treatment (Figure 3a). However, pre-treatments with SA, MeJA and ethephon were individually effective in inhibiting the growth of *Pst* DC3000 (Figure 3b). Whereas JA/ET and SA have been considered antagonistic in resistance to *P. syringae* (Kloek *et al.*, 2001; Kunkel and Brooks, 2002; Glazebrook *et al.*, 2003), our results together with other studies provide evidence about positive roles for JA, SA and ET in systemic resistance (Xu *et al.*, 1994; Broekaert *et al.*, 2006; Mur *et al.*, 2006). In *glip1* plants, only ET lost the positive effect on systemic resistance among these hormones, suggesting again that the GLIP1-ET pathway is independent of the SA and JA pathways. Based on our results, GLIP1 may form a type of systemic resistance that is distinct from SAR. The supporting evidence can be summarized as follows: (i) GLIP1-associated systemic resistance is not dependent on SA, but requires ET signaling; (ii) *Pst* DC3000 (*avrRpt2*)-induced local and systemic resistance is not affected in *glip1* plants; (iii) *A. brassicicola*-induced resistance gene expression is abolished in *glip1* plants; and (iv) *Pst* DC3000 (*avrRpt2*)-induced SAR does not confer resistance to *A. brassicicola* (Ton *et al.*, 2002).

GLIP1 shares characteristics with previously reported defense regulators enhanced disease susceptibility 1 (EDS1) (Falk *et al.*, 1999), phytoalexin deficient 4 (PAD4) (Jirage *et al.*, 1999), and senescence-associated gene 101 (SAG101) (Feys *et al.*, 2005) that belong to the α/β hydrolase superfamily and have been implicated in systemic resistance. In contrast to others, GLIP1 has unique properties in that it is localized in the cell wall and possesses systemic resistance-inducing activity associated with ET signaling. In contrast to the implication of these regulators in SA-dependent basal resistance to biotrophic and hemibiotrophic pathogens, defense against necrotrophic pathogens such as *A. brassicicola* is mediated by ET and JA pathways (Penninckx *et al.*, 1998; Pieterse and van Loon, 1999; Kunkel and Brooks, 2002). It has been shown that MAP kinase 4 (MPK4) represses SA-dependent defense but stimulates JA and ET signaling (Petersen *et al.*, 2000). Recent studies demonstrate that EDS1/PAD4 controls the antagonism between SA and ET/JA, and MPK4 negatively regulates the antagonistic effect of EDS1/PAD4, stimulating JA/ET-dependent resistance to *A. brassicicola* (Brodersen *et al.*, 2006). It remains to be determined how GLIP1 is linked to ET signaling, i.e. whether GLIP1, like MPK4, has a negative effect on the ET-repressing functions of EDS1/PAD4 or directly connects to the known ET signaling pathway involving ETR1, CTR1, EIN2, EIN3 and ERF1.

PDF1.2-*inducing activity accumulates in the phloem of 35S:GLIP1 plants*

Systemic immunity requires long-distance signaling that is mediated by the mobile signal(s) generated at the infection site and translocated to systemic tissues. Accumulation of systemic resistance-inducing activity in petiole exudates is thus an indispensable condition for a molecule to be a systemic signal. A recent study demonstrates that MeSA is a SAR signal in tobacco and accumulates in phloem exudates in a SABP2-dependent manner (Park *et al.*, 2007). Truman *et al.* (2007) show that JA is a critical molecule for RPM1-specified systemic immunity in Arabidopsis. Jasmonic acid, but not SA, accumulates in petiole exudates, accompanied by the induction of JA biosynthesis genes in systemic leaves when challenged with *Pst* DC3000 (*avrRpm1*). However, the increase in JA level and gene expression is a rapid and transient response, suggesting that JA is an early signal and possibly potentiates SA-dependent signaling processes. Many reports demonstrate that lipids or lipid derivatives function as important signaling molecules in SAR signaling. The Arabidopsis *sfd1* compromises SAR-associated SA accumulation, *PR1* expression and resistance to *P. syringae* pv. *maculicola* in systemic leaves (Nandi *et al.*, 2004). In addition to *SFD1*, *Fatty acid desaturase 7* (*FAD7*), *SFD2* and

Monogalactosyldiacylglycerol synthase 1 (*MGD1*) are required for the accumulation of SAR-inducing activity in phloem exudates from *Pst* DC3000 (*avrRpt2*)-inoculated Arabidopsis leaves (Chaturvedi *et al.*, 2008). Since these genes are involved in biosynthesis of plastid glycerolipids such as phosphatidylglycerol, galactolipid and sulfoglycerolipid, glycerolipid metabolism may be important for the production of lipid signaling molecules for SAR activation.

Here we show that the *PDF1.2*-inducing activity accumulates in petiole exudates of *35S:GLIP1* plants (Figure 5). The HPLC analysis of petiole exudates indicates that GLIP1-dependent *PDF1.2*-inducing activity could be fractionated on columns (Figure 6). The activity was detected in the range of less than 10 kDa by size exclusion chromatography. Although the putative mobile signal corresponding to the *PDF1.2*-inducing activity remains to be identified, it may be associated with proteins. It is intriguing to note that the less than 10 kDa size determined for the activity is similar to those of LTPs (Maldonado *et al.*, 2002; Beisson *et al.*, 2003; Buhot *et al.*, 2004). Further studies involving a series of chromatographic and mass spectrometric analyses should be carried out in order to identify the mobile signal and enhance our understanding of the systemic resistance signaling pathway in plants.

Experimental procedures

Plant materials, growth conditions and pathogen treatments

All of the *Arabidopsis thaliana* plants (WT, *glip1-1*, *35S:GLIP1*, *PDF1.2:GUS*, *etr1-1*, *jar1-1* and *NahG*) were grown at 23°C under long-day conditions in a 16-h light/8-h dark cycle. For infection with *P. syringae*, plants were grown under short-day conditions in an 8-h light/16-h dark cycle. To generate transgenic plants overexpressing *GLIP1*, PCR products amplified using *GLIP1* cDNA were cloned into the binary vector pBI121 (Clontech, http://www.clontech.com/) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. For *35S:GLIP1-His* plants, PCR products were inserted into the pET26b vector (Novagen, http://www.merckbiosciences.co.uk/g.asp?f=NVG/home.html) and amplified again to add a His-tag at the C-terminus of GLIP1 before cloning into the binary vector pBI121. To generate transgenic plants expressing *PDF1.2:GUS*, the *PDF1.2* promoter region was amplified by PCR using primers 5′-GAG AGA ATT CGG TGC TTG ATC GTG TGT G-3′ and 5′-GAG AAC TAG TGA TGA TTA TTA CTA TTT TGT TTT C-3′. The product was inserted into a pCAMBIA1303 binary vector. These constructs were transformed into *Agrobacterium tumefaciens* strain GV3101.

Treatment with *A. brassicicola* and *P. syringae* were performed as described previously (Oh *et al.*, 2005). *Erwinia carotovora* was inoculated on detached leaves or on 3-week-old seedlings by applying a 10 μl drop of bacterial cell suspension (1 × 10^7 cells ml^{-1}). For pre-treatment with GLIP1 proteins, plants were infiltrated with 10 μl of GLIP1 proteins (0.1 μg) in PBS and incubated for 3 days before pathogen inoculation. This experiment was designed as a randomized complete block with five replications and one plant per replication. The experiment was repeated at least three times.

Microscopic analysis

The leaves of WT, *glip1-1* and *35S:GLIP1* plants treated with *A. brassicicola* and *E. carotovora* were visualized with a scanning electron microscope (Hitachi, http://www.hitachi.com/) and a confocal laser scanning microscope (Zeiss, http://www.zeiss.com/), respectively. Leaf samples were taken 3–4 days after pathogen infection.

RNA analysis

Northern blot analysis and RT-PCR were performed as described previously (Oh *et al.*, 2005). The primers used in the RT-PCR were as follows: for *GLIP1*, 5′-CGA TTG TGC ACC AGC CTC ATT GGT T-3′ and 5′-CAG CGC TTT GAG ATT ATA GGG TCC-3′; for *PR-1*, 5′-TCG TCT TTG TAG CTC TTG TAG GTG-3′ and 5′-TAG ATT CTC GTA ATC TCA GCT CT-3′; and for *PDF1.2*, 5′-GCT AAG TTT GCT TCC ATC ATC ACC CTT-3′ and 5′-AAC ATG GGA CGT AAC AGA TAC ACT TGT G-3′.

Preparation of GLIP1 recombinant proteins

The inactive form of GLIP1 (GLIP1^{TM}) was generated by mutagenic PCR. The *GLIP1* mutant with a single mutation (S44A) was used as a template to generate GLIP1^{TM} in which catalytic Ser, Asp and His residues were replaced with Ala (S44A/D276A/H341A). Site-directed mutagenesis was done by PCR using the following primers with the sequences for base substitutions (lowercase): for D276A mutation, 5′-ATT GGA CTC AAC Gcg TAT CAA TAT CCA TTC ACT AC-3′ and 5′-GTG AAT GGA TAT TGA TAc gCG TTG AGT CCA ATA TGA AAC-3′; for H341A mutation, 5′-GTT CTT TGA TCC TTT Tgc TTT GAC GGA AAA GGC TAA TCG-3′ and 5′-CGA TTA GCC TTT TCC GTC AAA gcA AAA GGA TCA AGA AAC-3′. The recombinant GLIP1 proteins were purified as previously described (Oh *et al.*, 2005).

Isolation of protoplasts

Protoplasts were prepared using the method described previously (Yoo *et al.*, 2007) with some modifications. Arabidopsis leaves of 2–3-week-old seedlings were dissolved in enzyme solution [1% cellulase R10, 0.25% Macerozyme R10, 0.4 M mannitol, 8 mM CaCl$_2$, 0.1% BSA, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.6] and incubated in the dark at 23°C for 4 h with gentle agitation (50–75 rpm). After filtering the protoplasts through a 100-μm mesh, an equal volume of W5 solution (154 mM NaCl, 125 mM CaCl$_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES, pH 5.6) was added to the isolated protoplasts. The protoplasts were then centrifuged at 100 ***g*** for 5 min, resuspended in W5 solution (5 ml), and transferred into a round-bottomed tube containing 21% sucrose (5 ml). After centrifugation, the protoplasts were carefully transferred into a new tube, resuspended in W5 solution and spun down at 100 ***g*** for 5 min. The isolated protoplasts were resuspended in W5 solution and adjusted to a density of 2 × 10^5 cells ml^{-1} for use in the GUS assay.

Preparation of petiole exudates

Petiole exudates were prepared as described previously (Taylor *et al.*, 1990; Maldonado *et al.*, 2002). Petioles of WT, *glip1-1* and *35S:GLIP1* plants untreated or treated with *A. brassicicola* were cut above the stem. The cut surface was briefly sterilized in a solution containing 50% ethanol and 0.0006% bleach and rinsed in sterile distilled water. Exudates were collected in distilled water for 2 days at 28°C. Exudates collected from 50–60 leaves

contained 0.5–1 mg of total proteins (Bio-Rad Protein Assay Kit, http://www.bio-rad.com/).

Size exclusion chromatography

The prepared exudates were lyophilized and dissolved in 0.2 M sodium phosphate buffer (pH 7.5). After centrifugation, the supernatant was applied to size exclusion chromatography on a Zorbax GF-450 column (9.4 × 250 mm) (Agilent, http://www.chem.agilent.com). The column was run with 0.2 M sodium phosphate buffer (pH 7.5) at a flow rate of 1 ml min^{-1}. Fractions (0.3 ml) were combined, lyophilized and dialyzed against 1 × PBS (pH 7.3) for use in the GUS assay.

GUS assay

Protoplasts were incubated with the desired samples such as exudates, column fractions, recombinant proteins and MeJA in the dark at 23°C for 30 min with gentle agitation (50–75 rpm). The treated protoplasts were pelleted by centrifugation at 100 *g* for 10 min and incubated with 100 μl of lysis buffer (50 mM sodium phosphate, pH 7.4, 10 mM EDTA, 0.1% sarkosyl, 0.1% Triton X-100, 0.008% β-mercaptoethanol). An aliquot of lysed protoplasts (50 μl) was incubated with 100 μl of 1 mM 4-methylumbelliferyl β-D-glucuronide (Sigma, http://sigmaaldrich.com/) at 37°C for 30 min as described previously (Jefferson *et al.*, 1987). The reaction was stopped by adding 1 ml of 0.2 M Na_2CO_3. The fluorescence of 4-methylumbelliferone (MU) was measured in a fluorometer.

Data analysis

Data were subjected to analysis of variance using JMP IN version 4 software (SAS Institute, http://www.sas.com/). The significance of each treatment effect was determined by the magnitude of the *F* value at *P* = 0.05. When a significant *F* value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) at *P* = 0.05. Results of repeated trials of each experiment outlined above were similar. Hence, one representative trial of each experiment is reported in the results section.

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Figure S1. Overexpression of *GLIP1* in Arabidopsis.
Figure S2. Analysis of *PR-1* expression in Col-0 and *glip1-1* leaves inoculated with *Alternaria brassicicola*.
Figure S3. Proteins isolated from petiole exudates.
Figure S4. Western analysis of GLIP1-His expression in *35S:GLIP1-His* plants.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell*, **92**, 773–784.

Beisson, F., Koo, A.J., Ruuska, S. *et al.* (2003) Arabidopsis genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. *Plant Physiol.* **132**, 681–697.

Brodersen, P., Petersen, M., Bjørn Nielsen, H., Zhu, S., Newman, M.A., Shokat, K.M., Rietz, S., Parker, J. and Mundy, J. (2006) Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant J.* **47**, 532–546.

Broekaert, W.F., Delaure, S.L., De Bolle, M.F. and Cammue, B.P. (2006) The role of ethylene in host-pathogen interactions. *Annu. Rev. Phytopathol.* **44**, 393–416.

Buhot, N., Gomes, E., Milat, M.L., Ponchet, M., Marion, D., Lequeu, J., Delrot, S., Coutos-Thevenot, P. and Blein, J.P. (2004) Modulation of the biological activity of a tobacco LTP1 by lipid complexation. *Mol. Biol. Cell*, **15**, 5047–5052.

Chaturvedi, R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, A.A., Roth, M.R., Welti, R. and Shah, J. (2008) Plastid omega3-fatty acid desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. *Plant J.* **54**, 106–117.

Dong, X. (2004) NPR1, all things considered. *Curr. Opin. Plant Biol.* **7**, 547–552.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42**, 185–209.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J. and Parker, J.E. (1999) EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proc. Natl Acad. Sci. USA*, **96**, 3292–3297.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A. and Parker, J.E. (2005) Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell*, **17**, 2601–2613.

Fobert, P.R. and Despres, C. (2005) Redox control of systemic acquired resistance. *Curr. Opin. Plant Biol.* **8**, 378–382.

Forouhar, F., Yang, Y., Kumar, D. *et al.* (2005) Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc. Natl Acad. Sci. USA*, **102**, 1773–1778.

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T. and Katagiri, F. (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J.* **34**, 217–228.

Grant, M. and Lamb, C. (2006) Systemic immunity. *Curr. Opin. Plant Biol.* **9**, 414–420.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999) *Arabidopsis thaliana*

PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl Acad. Sci. USA*, **96**, 13583–13588.

Kariola, T., Brader, G., Li, J. and Palva, E.T. (2005) Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. *Plant Cell*, **17**, 282–294.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F. and Kunkel, B.N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. *Plant J.* **26**, 509–522.

Kumar, D. and Klessig, D.F. (2003) High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc. Natl Acad. Sci. USA*, **100**, 16101–16106.

Kunkel, B.N. and Brooks, D.M. (2002) Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* **5**, 325–331.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* **10**, 71–82.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science*, **250**, 1002–1004.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J. and Cameron, R.K. (2002) A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature*, **419**, 399–403.

Mur, L.A., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* **140**, 249–262.

Nandi, A., Welti, R. and Shah, J. (2004) The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene *SUPPRESSSOR OF FATTY ACID DESATURASE DEFICIENCY1* is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell*, **16**, 465–477.

Oh, I.S., Park, A.R., Bae, M.S., Kwon, S.J., Kim, Y.S., Lee, J.E., Kang, N.Y., Lee, S., Cheong, H. and Park, O.K. (2005) Secretome analysis reveals an Arabidopsis lipase involved in defense against *Alternaria brassicicola*. *Plant Cell*, **17**, 2832–2847.

Park, S.W., Kaimoyo, E., Kumar, D., Mosher, S. and Klessig, D.F. (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science*, **318**, 113–116.

Penninckx, I.A., Thomma, B.P., Buchala, A., Métraux, J.P. and Broekaert, W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell*, **10**, 2103–2113.

Petersen, M., Brodersen, P., Naested, H. *et al.* (2000) Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103**, 1111–1120.

Pieterse, C.M. and van Loon, L.C. (1999) Salicylic acid-independent plant defence pathways. *Trends Plant Sci.* **4**, 52–58.

Pieterse, C.M. and Van Loon, L.C. (2004) NPR1: the spider in the web of induced resistance signaling pathways. *Curr. Opin. Plant Biol.* **7**, 456–464.

Pieterse, C.M., Van Wees, S.C., Hoffland, E., Van Pelt, J.A. and Van Loon, L.C. (1996) Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell*, **8**, 1225–1237.

Pieterse, C.M., Van Wees, S.C., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and Van Loon, L.C. (1998) A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell*, **10**, 1571–1580.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell*, **8**, 1809–1819.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper, J.W. and Pare, P.W. (2004a) Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiol.* **134**, 1017–1026.

Ryu, C.M., Murphy, J.F., Mysore, K.S. and Kloepper, J.W. (2004b) Plant growth-promoting rhizobactia systemically protect *Arabidopsis thaliana* against *Cucumber mosaic virus* by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. *Plant J.* **39**, 381–392.

Taylor, J.S., Thompson, B., Pate, J.S., Atkins, C.A. and Pharis, R.P. (1990) Cytokinins in the phloem sap of white lupin (Lupinus albus L.). *Plant Physiol.* **94**, 1714–1720.

Ton, J., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M. (2002) Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. *Mol. Plant Microbe Interact.* **15**, 27–34.

Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C. and Grant, M. (2007) Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc. Natl Acad. Sci. USA*, **104**, 1075–1080.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. (1992) Acquired resistance in Arabidopsis. *Plant Cell*, **4**, 645–656.

Van Loon, L.C., Bakker, P.A. and Pieterse, C.M. (1998) Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* **36**, 453–483.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell*, **6**, 959–965.

Wang, D., Weaver, N.D., Kesarwani, M. and Dong, X. (2005) Induction of protein secretory pathway is required for systemic acquired resistance. *Science*, **308**, 1036–1040.

Xu, Y., Chang, P., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M. and Bressan, R.A. (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell*, **6**, 1077–1085.

Yalpani, N., Silverman, P., Wilson, T.M., Kleier, D.A. and Raskin, I. (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell*, **3**, 809–818.

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**, 1565–1572.