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

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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 \( \alpha / \beta \) 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 Suppresser of Fatty Acid Desaturase 1 (SFD1) encoding dihydroxyacetone phosphate reductase compromise the activation of SAR, suggesting the involvement of plasticid 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 T3 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<sup>TM</sup>) 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...
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

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**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 × 10⁵ spores ml⁻¹) (a) or *E. carotovora* (10⁷ cfu ml⁻¹) (b), or 20 μl of *P. syringae* bacterial suspension (10⁶ cfu ml⁻¹) (c). Pathogen growth was determined 4 days after inoculation.
3 days after infiltration of 3-week-old seedlings with PBS (control), inspections (0–5) were made 2 days after pathogen inoculation, which occurred (b) Systemic resistance against using Fisher’s least significant difference (LSD) test at times with similar results. Different letters indicate significant differences with similar results. 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.

Figure 2. GLIP1-dependent systemic suppression of bacterial growth. (a) Systemic resistance against Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) in wild-type (WT), glip1-1 and 3SS:GLIP1 plants. Four-week-old plants were infiltrated at one site on the primary leaf with PBS (control), GLIP1 or GLIP1TM proteins (0.1 µg), or 20 µl of Pst DC3000 (avrRpt2) suspension (10^5 colony-forming units (cfu ml^-1), incubated for 3 days, and then inoculated with 20 µl of Pst DC3000 suspension (10^5 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) Effect of hormones on bacterial growth in WT, glip1-1 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.

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 GLIP1TM proteins (0.1 µg), or 20 µl of Pst DC3000 (avrRpt2) suspension (10^5 colony-forming units (cfu ml^-1), incubated for 3 days, and then inoculated with 20 µl of Pst DC3000 suspension (10^5 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, glip1-1 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. The 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 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*

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**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 μl).

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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 expression, 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.

### 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, overexpression 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...
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) (Fey 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 and ET signaling (Petersen et al., 2000). 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

![Figure 7. A model for GLIP1 in systemic resistance signaling.](image-url)
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 activation accumulate 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, 3SS:GLIP1, PDF1.2:GUS, etr1-1, jar1-1* and NahG1) 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 DNA were cloned into the binary vector pBI121 (Clontech, www.clontech.com/) under the control of the cauliflower mosaic virus (CaMV) 3SS promoter. For 3SS:GLIP1-His plants, PCR products were inserted into the pET26b vector (Novagen, http://www.merckbiosciences.co.uk/g.asp?N=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 CGT TGT G-3′ and 5′-GAG AAC TAG TGA TGA TTA CTA TTT TGT 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 × 10^8 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 TGC TTG ACC AGC CCT ATT GTG T-3′ and 5′-CGG CTT TTT TGA AGT ATA GGG TCC -3′; for PR-1, 5′-TGG TCT TTG TAG CTC CCT TGG TGT-3′ and 5′-TGG ATT CTC GTA ATC TCA GCT CT-3′; and for PDF1.2, 5′-GCT AAG TTT GCT TCC ATC ATT GGT T-3′.

**Preparation of GLIP1 recombinant proteins**

The inactive form of GLIP1 (GLIP1TM) was generated by mutagenic PCR. The GLIP1 mutant with a single mutation (S44A) was used as a template to generate GLIP1TM 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 GTA ACA AGG ATT ATA GGG TCC-3′, 5′-CAG CGC TTT GAG ATT ATA GGG TCC-3′, and 5′-AAC ATG GGA CGT AAC AGA TAC ACT TGT G-3′. Northern blot analysis and RT-PCR were performed as described previously (Oh et al., 2005). The inactive form of GLIP1 was 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 mM mannosite, 8 mM CaCl₂, 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 CaCl₂, 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 10% bleach solution containing 50% ethanol 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% l-sarkosyl, 0.1% Triton X-100, 0.008% β-mercaptoethanol). An aliquot of lysed protoplasts (50 μl) was incubated with 100 μl of 1× 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 Na₂CO₃. 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.

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**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 glip-1-1 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.

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**References**


