GDSL LIPASE1 Modulates Plant Immunity through Feedback Regulation of Ethylene Signaling[1][W]

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Ethylene is a key signal in the regulation of plant defense responses. It is required for the expression and function of GDSL LIPASE1 (GLIP1) in Arabidopsis (*Arabidopsis thaliana*), which plays an important role in plant immunity. Here, we explore molecular mechanisms underlying the relationship between GLIP1 and ethylene signaling by an epistatic analysis of ethylene response mutants and *GLIP1*-overexpressing (*35S:GLIP1*) plants. We show that *GLIP1* expression is regulated by ethylene signaling components and, further, that *GLIP1* expression or application of petiole exudates from *35S:GLIP1* plants affects ethylene signaling both positively and negatively, leading to *ETHYLENE RESPONSE FACTOR1* activation and ETHYLENE INSENSITIVE3 (EIN3) down-regulation, respectively. Additionally, *35S:GLIP1* plants or their exudates increase the expression of the salicylic acid biosynthesis gene *SALICYLIC ACID INDUCTION-DEFICIENT2*, known to be inhibited by EIN3 and EIN3-LIKE1. These results suggest that GLIP1 regulates plant immunity through positive and negative feedback regulation of ethylene signaling, and this is mediated by its activity to accumulate a systemic signal(s) in the phloem. We propose a model explaining how GLIP1 regulates the fine-tuning of ethylene signaling and ethylene-salicylic acid cross talk.

The gaseous plant hormone ethylene plays important roles in growth and development, senescence, fruit ripening and abscission, and pathogen resistance (Abeles et al., 1992; van Loon et al., 2006; Cho and Yoo, 2009). The components of ethylene signaling have been identified through genetic screens for mutants in Arabidopsis (*Arabidopsis thaliana*) that exhibit defects in ethylene responses, the so-called triple response, exemplified by short, thick hypocotyl and root and exaggerated apical hook of ethylene-treated etiolated seedlings (Ecker, 1995; Chang, 1996; Woeste and Kieber, 1998). Ethylene response mutants include the constitutive triple response mutants *constitutive triple response1* (*ctr1*), *ethylene overproducer1* (*eto1*), *eto2*, and *eto3* as well as the ethylene-insensitive mutants *ethylene response1* (*etr1*), *etr2*, *ethylene insensitive2* (*ein2*), *ein3*, *ein4*, *ein5*, *ein6*, and *ein7* (Kieber et al., 1993; Guo and Ecker, 2004). In addition to these, mutants with an enhanced ethylene sensitivity have been isolated, including *EIN3-binding F-box protein1* (*ebf1*), *ebf2*, *enhanced ethylene response1* (*eer1*), *eer3*, *eer4*, *eer5*, and *reversion-to-ethylene sensitivity1* (*rte1*; Larsen and Chang, 2001; Guo and Ecker, 2003; Resnick et al., 2006; Christians and Larsen, 2007; Robles et al., 2007; Christians et al., 2008). Epistasis and molecular analyses of mutants have revealed a linear ethylene signaling pathway and the core components involved in ethylene perception, signal cascading, and transcriptional regulation (Solano and Ecker, 1998; Chen et al., 2005).

Ethylene is recognized by a family of five endoplasmic reticulum-located integral membrane receptors in Arabidopsis, ETR1, ETR2, EIN4, ETHYLENE RESPONSE SENSOR1 (ERS1), and ERS2 (Chang et al., 1993; Hua et al., 1998; Sakai et al., 1998). In the absence of ethylene, ethylene receptors associate with the Raf-like Ser/Thr kinase CTR1 and repress downstream ethylene signaling (Kieber et al., 1993). When bound to ethylene, receptor signaling and CTR1 become inactivated, leading to derepression of the downstream positive regulators EIN2 and EIN3 (Chao et al., 1997). EIN2 has sequence similarity to mammalian NRAMP metal transporters and localizes at the endoplasmic reticulum membrane (Alonso et al., 1999; Bisson et al., 2009). Recent findings reveal that EIN2 undergoes proteolytic cleavage in response to ethylene, and the resultant C-terminal fragment of EIN2 moves into the nucleus and stabilizes EIN3 to activate ethylene responses (Ju et al., 2012; Qiao et al., 2012; Wen et al.,

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2012). In the absence of ethylene, the F-box proteins EIN2 TARGETING PROTEIN1 (ETP1) and ETP2 lead to proteasomal degradation of EIN2 and thus negatively regulate ethylene signal transduction (Qiao et al., 2009). EIN3 and EIN3-LIKE1 (EIL1) are critical transcription factors downstream of EIN2 and also subjected to proteasomal degradation by EBF1 and EBF2 in the absence of ethylene (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Ethylene stabilizes EIN3 and EIL1 by inducing the proteasomal degradation of EBF1 and EBF2 (An et al., 2010). EIN3 and EIL1 bind to a specific sequence in the target gene promoter and induce transcription (Chao et al., 1997; Solano et al., 1998). *ETHYLENE RESPONSE FACTOR1* (*ERF1*), which contains the promoter sequence for EIN3 and EIL1, is thought to be the direct target gene of these proteins in Arabidopsis (Solano et al., 1998). ERF1 itself is a transcription factor belonging to the ethylene-responsive element-binding protein family, which can bind to the GCC box, and functions positively by activating ethylene responses (Fujimoto et al., 2000).

Ethylene, salicylic acid (SA), and jasmonic acid (JA) are key hormones regulating disease resistance, and they interact both synergistically and antagonistically in the signaling networks (Wang et al., 2002; Bostock, 2005; Beckers and Spoel, 2006; Broekaert et al., 2006). In Arabidopsis, each of these hormones is involved in different host-pathogen interactions. Whereas the SA-dependent pathway is generally implicated in resistance to biotrophic pathogens such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*, ethylene and JA pathways primarily confer resistance to necrotrophic pathogens such as *Alternaria brassicicola* and *Botrytis cinerea* (Penninckx et al., 1998; Pieterse and van Loon, 1999; Glazebrook, 2005; Spoel et al., 2007). There are many reports that SA antagonizes JA/ethylene signaling (Koornneef et al., 2008), but there is also evidence that positive interactions of these pathways lead to induced resistance (Penninckx et al., 1998; Thomma et al., 1998, 1999; Kwon et al., 2009). Systemic acquired resistance (SAR) is the best-studied SA-requiring induced immune response (Sticher et al., 1997), and other types of JA/ethylene-dependent induced resistance, including rhizobacteria-mediated induced systemic resistance (ISR), have also been demonstrated (Heil and Bostock, 2002; Kwon et al., 2009). Whereas SAR and ISR are differentially effective against some pathogens (e.g. *Turnip crinkle virus* and *A. brassicicola*), they also additively enhance resistance against others (e.g. *P. syringae*; van Wees et al., 2000; Ton et al., 2002). EIN3 and EIL1 transcription factors can both positively and negatively regulate pathogen-associated molecular pattern-triggered immunity at the transcriptional level, through up-regulation of *FLAGELLIN-SENSING2* (*FLS2*), which is required for pathogen-associated molecular pattern flagellin binding, or down-regulation of *SALICYLIC ACID INDUCTION-DEFICIENT2* (*SID2*), which is required for SA biosynthesis (Chen et al., 2009; Boutrot et al., 2010). These results suggest a mechanism of EIN3/EIL1-mediated cross talk between the ethylene and SA pathways.

Previously, we found that Arabidopsis GDSL LIPASE1 (GLIP1) is an ethylene-responsive secreted protein and regulates plant immunity (Oh et al., 2005; Kwon et al., 2009). Whereas GLIP1 is specifically involved in local resistance against necrotrophic pathogens, *GLIP1* overexpression in plants induces resistance to a range of pathogens, including the necrotrophic pathogens *A. brassicicola* and *Erwinia carotovora* and the hemibiotrophic pathogen *P. syringae* pv *tomato* (*Pst*) DC3000 (Kwon et al., 2009). Local inoculation of GLIP1 proteins elicits systemic resistance, which is abolished in the ethylene-insensitive mutant *etr1-1*, suggesting that ethylene signaling plays a vital role in GLIP1 action. However, the mechanism of this interaction remains unknown. Here, we present data indicating that *GLIP1* expression depends on ethylene signaling components. Furthermore, we show that activated *GLIP1* or petiole exudates from *GLIP1*-overexpressing plants (*35S*:*GLIP1*) both positively and negatively modulate ethylene responses, thereby enhancing JA/ethylene- and SA-mediated pathogen resistance, respectively.

RESULTS

GLIP1 Is Linked to the Ethylene Signaling Pathway

Our previous results demonstrated that *GLIP1* is strongly induced by ethylene-releasing ethephon treatment and that it requires the ethylene pathway for the induction of pathogen resistance (Oh et al., 2005; Kwon et al., 2009). *GLIP1* expression was additionally examined in wild-type plants treated with the ethylene precursor 1-aminocyclopropane-carboxylic acid (ACC) or ethylene (Supplemental Fig. S1A). All three treatments, ethephon, ACC, and ethylene, significantly induced *GLIP1* expression. To gain insights into the relationship between ethylene and GLIP1, we evaluated *GLIP1* expression in various ethylene mutants, including *etr1-1*, *ctr1-1*, *ein2-1*, and *ein3-1 eil1-1* (Fig. 1A). Whereas *GLIP1* expression was compromised in *etr1-1*, *ein2-1*, and *ein3-1 eil1-1* mutant plants, strong and constitutive expression of *GLIP1* was observed in the *ctr1-1* mutant. In addition, *GLIP1* expression was markedly higher in *ERF1*-overexpressing plants (Fig. 1A). These results demonstrate that *GLIP1* expression is regulated by ethylene signaling components.

We then evaluated whether the expression of ethylene-responsive genes is altered in *glip1-1* mutant and *35S*:*GLIP1* plants (Fig. 1C; Supplemental Fig. S1, B–F). We analyzed the expression levels of *ERF1*, *HOOKLESS1* (*HLS1*), required for apical hook curvature (Lehman et al., 1996; An et al., 2012), and two pathogenesis-related genes, *PLANT DEFENSIN1.2* (*PDF1.2*) and *BASIC CHITINASE* (*b-CHI*), induced by ERF1 (Fig. 1C; Lorenzo et al., 2003). They were significantly up-regulated in *35S*:*GLIP1* plants to the levels in ethylene-treated wild-type plants, but not in the *glip1-1* mutant, and were somewhat increased in

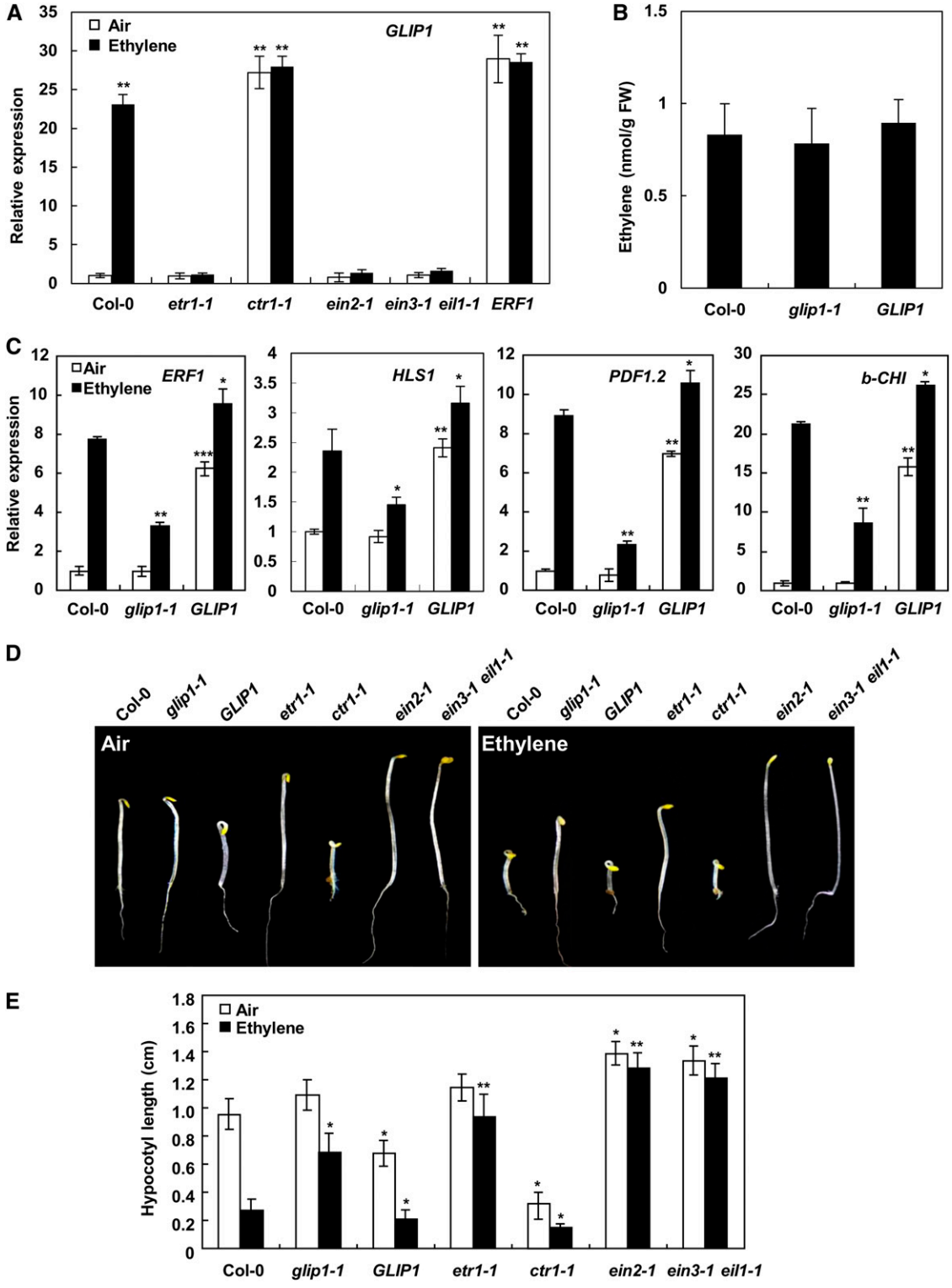

Figure 1. GLIP1 is associated with ethylene signaling. A, Expression analysis of *GLIP1* in ethylene mutants *etr1-1*, *ctr1-1*, *ein2-1*, and *ein3-1 eil1-1* and in *ERF1*-overexpressing plants. The values represent means ± SD from three independent experiments. Asterisks indicate significant differences from the air-treated Col-0 (Student's *t* test, $^{**}P < 0.01$). B, Ethylene contents of Col-0, *glip1-1*, and *35S:GLIP1* plants. Ten-day-old seedlings were used for ethylene quantification. The values are means ± SD ($n = 20$). Experiments were carried out more than five times with similar results. FW, Fresh weight. C, Expression analysis of ethylene-responsive genes *ERF1*, *HLS1*, *PDF1.2*, and *b-CHI* in Col-0, *glip1-1*, and *35S:GLIP1* plants. Four-week-old plants were treated with air or 10 μL L^{-1} ethylene for 12 h. The values represent means ± SD from three independent experiments. Asterisks indicate

glip1-1 and *35S*:*GLIP1* plants by ethylene treatment, although the increase was much smaller than that in the ethylene-treated wild type. We additionally analyzed the expression of other ethylene-responsive genes, such as the ethylene response factor gene *ERF5*, the ethylene receptor genes *ETR2* and *ERS1*, *ACO2* encoding an ACC oxidase, and *EBP* encoding an ethylene-responsive element-binding protein (Supplemental Fig. S1, B–F). *GLIP1* expression did not much affect their ethylene-inducible gene expression, although *35S*:*GLIP1* plants showed increased basal expression of *ERF5*, *ETR2*, and *ERS1*. This suggests that their expression is largely affected by other factors in ethylene signaling. According to gene expression patterns, *GLIP1* expression depends on the ethylene pathway and *GLIP1* overexpression leads to the induction of *ERF1* and the downstream effector genes, suggesting that GLIP1 may modulate ethylene signaling through a positive feedback mechanism. However, GLIP1 does not likely have a universal effect on the ethylene pathway but rather modulates a subset of ethylene responses.

To check whether *GLIP1*-induced gene expression was a result of enhanced ethylene production in *35S*:*GLIP1* plants, ethylene contents were measured in plants (Fig. 1B). Ethylene levels did not differ among wild-type, *glip1-1*, and *35S*:*GLIP1* plants, indicating that GLIP1 is related to ethylene signaling but not to ethylene biosynthesis.

GLIP1 Expression Triggers Ethylene Responses

Activation of ethylene response genes in *35S*:*GLIP1* plants prompted us to compare ethylene response phenotypes of *glip1* and *35S*:*GLIP1* plants with those of wild-type and ethylene mutant plants. Etiolated *35S*:*GLIP1* seedlings constitutively exhibited features of the triple response (increased hook curvature and shorter, thicker hypocotyl and root), although not so dramatically as *ctr1-1* (Fig. 1, D and E). In the presence of ethylene or ACC, *35S*:*GLIP1* seedlings displayed the enhanced triple response, but *glip1* mutants were less sensitive than the wild type (Fig. 1, D and E; Supplemental Figs. S2 and S3). The increased and decreased ethylene sensitivity of *35S*:*GLIP1* and *glip1* seedlings were similar to, but less marked than, those of the constitutive triple response mutant (i.e. *ctr1-1*) and ethylene-insensitive mutants (i.e. *etr1-1*, *ein2-1*, and *ein3-1 eil1-1*), respectively. Ethylene also accelerates leaf senescence (Chao et al., 1997; Yoo et al., 2008). When exposed to ACC, *35S*:*GLIP1* plants, like *ctr1-1*, had reduced chlorophyll content (Supplemental Fig. S3C). However, the leaf senescence of *glip1-1* plants was little affected by ACC treatment, as observed in *etr1-1*, *ein2-1*, and *ein3-1 eil1-1*. These results indicate that GLIP1 plays a positive role in ethylene responses, consistent with the induction of ethylene response genes in *35S*:*GLIP1* plants (Fig. 1C).

To determine whether lipase activity is required for GLIP1-mediated ethylene responses, the triple response was evaluated in wild-type plants (*35S*:*GLIP1*^{TM} [for *GLIP1* with triple mutations]) and *glip1* plants (*35S*:*GLIP1*^{TM} *glip1-1*) overexpressing *GLIP1*^{TM}, encoding an inactive GLIP1 in which residues of the catalytic triad (Ser, Asp, and His) were replaced with Ala (Supplemental Fig. S4A; Kwon et al., 2009). Transgenic lines of *35S*:*GLIP1*^{TM} and *35S*:*GLIP1*^{TM} *glip1-1* showing *GLIP1* expression similar to that of *35S*:*GLIP1* plants were used for the test (Supplemental Fig. S4B). Both *35S*:*GLIP1*^{TM} and *35S*:*GLIP1*^{TM} *glip1-1* plants were less sensitive to ethylene than wild-type and *35S*:*GLIP1* plants, implying that lipase activity is important for the activation of ethylene responses by GLIP1. Ag^{2+} ions are known to inhibit the ethylene response by replacing Cu^{2+}, an ethylene receptor cofactor (Beyer, 1976). Treatment with $AgNO_3$ effectively eliminated the ACC-induced ethylene response of wild-type, *glip1-1*, and *35S*:*GLIP1* seedlings, but not the constitutive triple response of *35S*:*GLIP1* seedlings, suggesting that GLIP1 may trigger ethylene responses independently of ethylene receptors (Supplemental Fig. S4C).

Epistasis of GLIP1 and Ethylene Pathway Genes

To further dissect how GLIP1 interacts with components of the ethylene signaling pathway, we performed an epistasis analysis by crossing *35S*:*GLIP1* plants with ethylene mutants (*etr1-1*, *ctr1-1*, *ein2-1*, and *ein3-1 eil1-1*; Supplemental Figs. S5 and S6). Homozygous crossed lines were obtained, and their growth phenotypes were observed. Ethylene has an inhibitory effect on cell elongation, leading to a reduction in leaf epidermal cell size (Guzman and Ecker., 1990; Kieber et al., 1993; Chao et al., 1997). As reported, *ein2-1* and *ein3-1 eil1-1* mutants had larger leaves, and the *ctr1-1* mutant showed a great reduction in leaf size (Supplemental Fig. S5). However, crossed lines lost the leaf size changes associated with ethylene mutants and were similar in appearance to their *35S*:*GLIP1* counterpart.

The triple response was compared in *35S*:*GLIP1*, ethylene mutants, and their crossed lines (Fig. 2A). *GLIP1* overexpression in ethylene-insensitive mutants, *etr1-1*, *ein2-1*, and *ein3-1 eil1-1*, induced the constitutive triple response as shown in *35S*:*GLIP1* seedlings, but the triple response was not further enhanced by ethylene treatment. This indicates that GLIP1 acts positively and does not require the core components of ethylene

Figure 1. (*Continued.*)
significant differences from Col-0 (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). D, Triple response of 4-d-old etiolated seedlings of Col-0, *glip1-1*, *35S*:*GLIP1*, and ethylene mutants grown in air or 10 μL L^{-1} ethylene. E, Hypocotyl lengths of the plants in D. The values are means ± SD ($n = 10$). Asterisks indicate significant differences from Col-0 (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$).

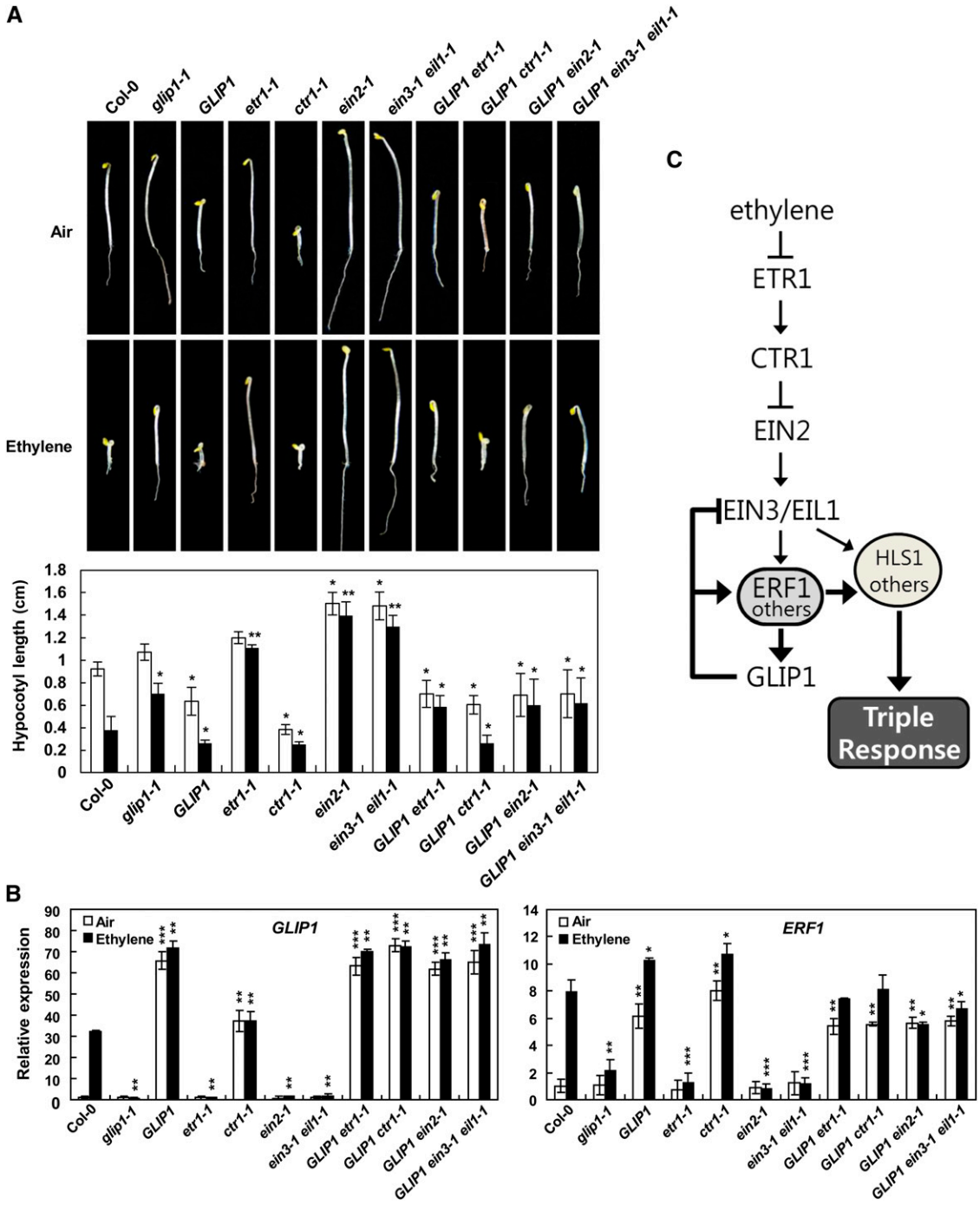

Figure 2. Effect of *GLIP1* on phenotypes and gene expression of ethylene mutants. A, Triple response phenotypes (top) and hypocotyl lengths (bottom) of 4-d-old etiolated seedlings of Col-0, *glip1-1*, *35S:GLIP1*, ethylene mutants, and crossed lines grown in air or 10 μL L^{-1} ethylene. The values are means ± SD (n = 20). Asterisks indicate significant differences from Col-0 (Student's t test, $^{*}P < 0.05$, $^{**}P < 0.01$). B, Expression analysis of *GLIP1* and *ERF1* in Col-0, *glip1-1*, *35S:GLIP1*, ethylene mutants, and crossed lines. Four-week-old plants were treated with air or 10 μL L^{-1} ethylene for 12 h. The values represent means ± SD from three independent experiments. Asterisks indicate significant differences from Col-0 (Student's t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). C, A model for the modulation of ethylene responses by GLIP1. GLIP1, which requires ethylene signaling for its expression, may form negative and positive feedback loops at EIN3 and ERF1, respectively, to regulate ethylene responses. In addition to *ERF1*, other *ERFs* are probably activated by GLIP1, leading to the expression of effector genes, such as *HLS1*, necessary for provoking ethylene responses.

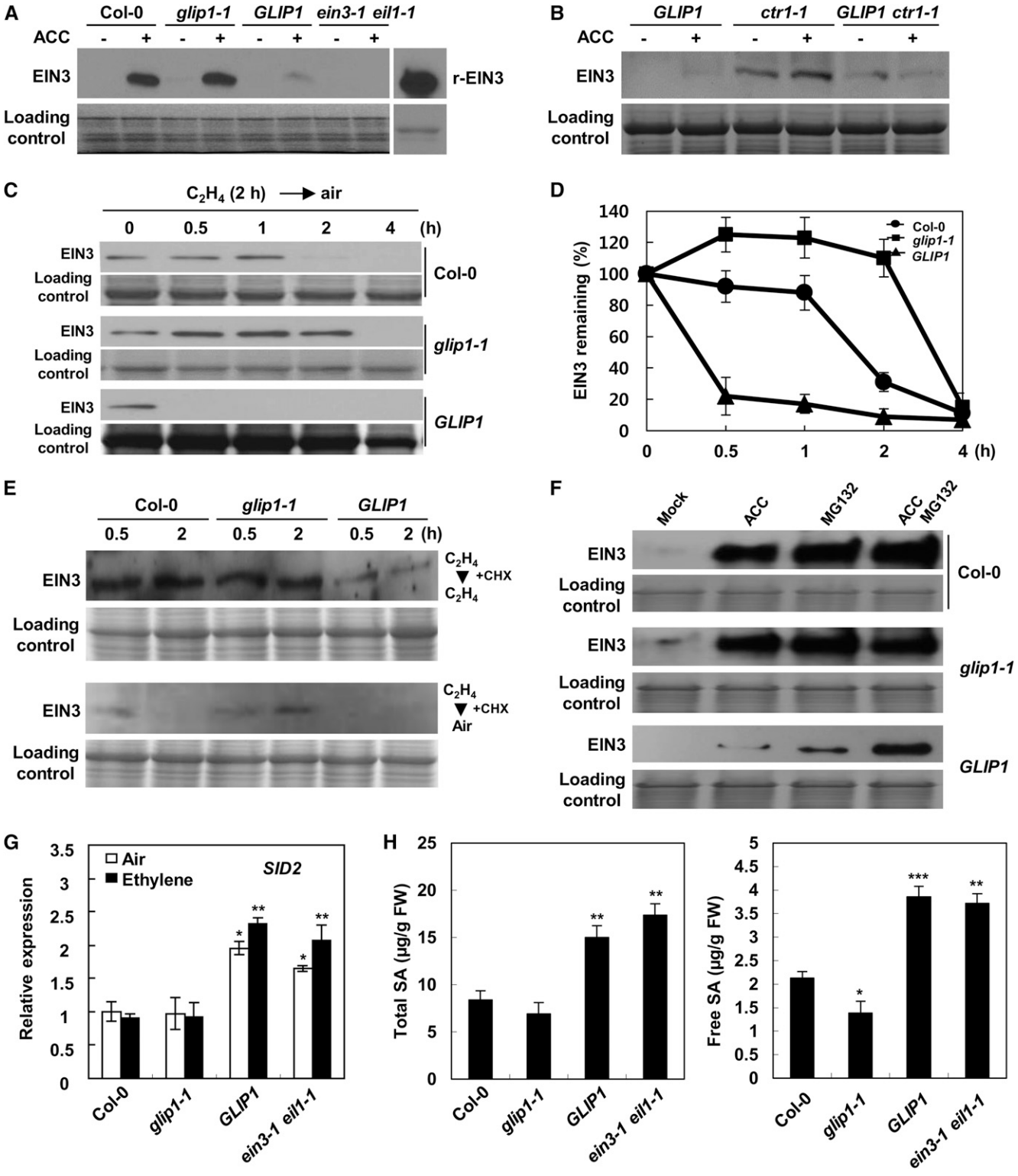

Figure 3. *GLIP1* overexpression down-regulates EIN3 but up-regulates *SID2* and SA levels. A, EIN3 levels in Col-0, *glip1-1*, *35S:GLIP1*, and *ein3-1 eil1-1* plants. r-EIN3, Recombinant EIN3 (full length). B, EIN3 levels in *35S:GLIP1*, *ctr1-1*, and *35S:GLIP1 ctr1-1* plants. C, Time course of EIN3 degradation in the absence of ethylene in Col-0, *glip1-1*, and *35S:GLIP1* plants. Four-week-old plants were treated with 10 μL L^{-1} ethylene for 2 h, moved back into air, and incubated for the indicated times. For *35S:GLIP1*, about 3-fold more proteins were loaded into lanes for a fair comparison of EIN3 degradation in different plants. D, Quantitative analysis of the data in C. EIN3 levels were assessed by densitometric measurement and calculated as the amounts of EIN3 remaining. The values represent means ± SD from five independent experiments. E, EIN3 levels in Col-0, *glip1-1*,

signaling for activation of the ethylene response, but the increased ethylene sensitivity of *35S*:*GLIP1* seedlings depends on ethylene pathway components such as ETR1, EIN2, and EIN3/EIL1. On the other hand, the phenotype that resulted from crossing *ctr1-1* with *35S*:*GLIP1* was quite unexpected: *35S*:*GLIP1 ctr1-1* seedlings lost the strong constitutive triple response of *ctr1-1* but displayed the ethylene response phenotype of *35S*:*GLIP1* seedlings (Fig. 2A). Together with the observation that the leaf size change associated with the *ctr1-1* mutant was abolished in *35S*:*GLIP1 ctr1-1* plants (Supplemental Fig. S5), this implies that GLIP1 regulates ethylene signaling in a dominant way and may play a dual role in ethylene signaling. In addition to activating *ERF1* and ethylene-related genes, GLIP1 may eliminate the constitutive triple response effect of the *ctr1-1* mutant by negatively affecting downstream components of CTR1, such as EIN2 and/or EIN3.

Gene expression was then examined in ethylene mutants and crossed lines (Fig. 2B). Whereas the ethylene-responsive genes *GLIP1* and *ERF1* were ethylene inducible in wild-type plants, *35S*:*GLIP1*, *ctr1-1*, and *35S*:*GLIP1*-crossed ethylene mutants (*35S*:*GLIP1 etr1-1*, *35S*:*GLIP1 ctr1-1*, *35S*:*GLIP1 ein2-1*, and *35S*:*GLIP1 ein3-1 eil1-1*) all showed marked constitutive expression of *GLIP1* and *ERF1*. However, strong induction of *GLIP1* and *ERF1* was abolished in ethylene-insensitive *etr1-1*, *ein2-1*, and *ein3-1 eil1-1* mutants. *HLS1*, which requires EIN3/EIL1 for ethylene-responsive expression, was also constitutively activated in *35S*:*GLIP1* and *GLIP1 ein3-1 eil1-1* plants (Supplemental Fig. S7).

GLIP1 Plays a Negative Role in Ethylene Signaling by Down-Regulating EIN3

It was assessed whether GLIP1 negatively affects the expression of EIN2 and EIN3. Because EIN2 and EIN3 are modulated at the protein level (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Yoo et al., 2008; Qiao et al., 2009; Wang et al., 2009), we determined the expression of EIN2 and EIN3 in wild-type, *glip1-1*, and *35S*:*GLIP1* plants by western-blot analysis (Fig. 3A; Supplemental Fig. S8A). EIN2 proteins (C-terminal fragments) accumulated to comparable levels in ACC-treated wild-type, *glip1-1*, and *35S*:*GLIP1* plants, although the basal level of EIN2 was higher in *glip1-1* plants than in the wild type and *35S*:*GLIP1* (Supplemental Fig. S8A). On the other hand, EIN3 expression was elevated by ACC treatment in wild-type and *glip1-1* plants, but it was significantly reduced in *35S*:*GLIP1* plants (Fig. 3A). It was also observed that the protein level of EIN3 in *35S*:*GLIP1 ctr1-1* was much lower than that in *ctr1-1* plants, suggesting that elimination of the constitutive triple response of *ctr1-1* in *35S*:*GLIP1 ctr1-1* seedlings was due to EIN3 down-regulation by GLIP1 (Fig. 3B). We monitored EIN3 levels in plants treated with ethylene for 2 h and then moved back into air (Fig. 3, C and D). In the absence of ethylene, EIN3 proteins rapidly disappeared within 30 min in *35S*:*GLIP1* plants but persisted for 2 h in *glip1-1* plants. We further checked whether this is related to EIN3 protein stability. Ethylene-exposed plants were treated with the protein synthesis inhibitor cycloheximide and kept in ethylene or moved into air (Fig. 3E). In the presence of ethylene, EIN3 proteins accumulated in the wild type and *glip1-1*, but EIN3 stability markedly decreased in *35S*:*GLIP1* plants. Treatment with the proteasome inhibitor MG132 enhanced EIN3 stability in *35S*:*GLIP1* plants as well as in wild-type and *glip1-1* plants, indicating that GLIP1-induced EIN3 degradation is proteasome dependent (Fig. 3F). There were no differences in *EIN2* and *EIN3* transcript levels among wild-type, *glip1-1*, and *35S*:*GLIP1* plants, with the exception of a slight increase in *EIN3* in untreated *35S*:*GLIP1* plants (Supplemental Fig. S8B). Expression analysis demonstrates that GLIP1 promotes the proteasome-mediated proteolysis of EIN3 proteins. Taken together, these results suggest that GLIP1 regulates ethylene signaling via both positive (i.e. *ERF1* activation) and negative (i.e. EIN3 degradation) feedback mechanisms, and this is the molecular basis of how GLIP1 affects ethylene responses (Fig. 2C).

GLIP1 Leads to Increased SID2 Expression and SA Production

We previously showed that *35S*:*GLIP1* plants are more resistant to *Pst* DC3000 than wild-type plants (Kwon et al., 2009). Moreover, it was previously reported that EIN3 and EIL1 negatively regulate plant innate immunity by repressing *SID2* expression; as a

Figure 3. (*Continued.*)
and *35S*:*GLIP1* plants treated with the protein synthesis inhibitor cycloheximide (CHX). Three-day-old seedlings grown in the presence of 10 μL L^{-1} ethylene were treated with 100 μM cycloheximide for 2 h, moved into air or ethylene, and incubated for the indicated times. F, EIN3 levels in Col-0, *glip1-1*, and *35S*:*GLIP1* plants treated with the proteasome inhibitor MG132. Three-day-old seedlings were treated with either 10 μM ACC or 50 μM MG132, or both together, for 2 h. G, Expression analysis of *SID2* in Col-0, *glip1-1*, *35S*:*GLIP1*, and *ein3-1 eil1-1* plants. Four-week-old plants were treated with air or 10 μL L^{-1} ethylene for 12 h. The values represent means ± SD from three independent experiments. Asterisks indicate significant differences from Col-0 (Student's *t* test, $*P < 0.05$, $**P < 0.01$). H, Quantification of total and free SA in Col-0, *glip1-1*, *35S*:*GLIP1*, and *ein3-1 eil1-1* plants. The values are means ± SD ($n = 20$). Asterisks indicate significant differences from Col-0 (Student's *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). The experiment was repeated three times with similar results. FW, Fresh weight. For A and B, 10-d-old seedlings were either untreated or treated with 10 μM ACC for 4 h. For A to F, protein samples were separated by SDS-gel electrophoresis and subjected to Coomassie staining (bottom) and western-blot analysis with anti-EIN3 antibody (top).

result, *ein3-1 eil1-1* mutant plants accumulate SA and exhibit enhanced resistance to *Pst* DC3000 (Chen et al., 2009). Demonstrating the negative effect of GLIP1 on EIN3 stability, we tested *glip1-1* and *35S:GLIP1* plants for *SID2* expression (Fig. 3G). *35S:GLIP1* plants showed a marked increase in *SID2* expression as in the *ein3-1 eil1-1* mutant. *SID2* expression was correlated with SA accumulation in these plants (Fig. 3H). These results suggest that GLIP1 induces SA production and, thus, SA-dependent pathogen resistance through negative regulation of EIN3.

GLIP1-Mediated Regulation of Ethylene Signaling Is Associated with Its Activity to Induce Systemic Resistance

The up-regulation of *ERF1* and *SID2* in *35S:GLIP1* plants suggests that the feedback regulation of ethylene signaling by GLIP1 may be a mechanism by which GLIP1 regulates immune responses. *35S:GLIP1* plants accumulate *PDF1.2*-inducing activity in petiole exudates, suggesting that GLIP1 elicits systemic resistance by mediating the production of a systemic signal (Kwon et al., 2009). We thus evaluated whether positive (*ERF1* activation) and negative (EIN3 degradation and *SID2* activation) regulation by GLIP1 are associated with GLIP1 activity to generate and accumulate a systemic signal(s). Petiole exudates were collected from wild-type, *glip1-1*, *35S:GLIP1*^{TM}, and *35S:GLIP1* plants (Supplemental Fig. S9) and inoculated into wild-type, *etr1-1*, and *35S:EIN3* plants to assess their effect on the stability of EIN3 proteins and the expression of *ERF1* and *SID2* (Fig. 4). Upon inoculation with collected petiole exudates, EIN3 expression was significantly decreased in ethylene-treated wild-type and *35S:EIN3* plants in response to *35S:GLIP1* exudates, although a smaller reduction in EIN3 levels was observed in ethylene-treated *35S:EIN3* plants than in untreated *35S:EIN3* plants (Fig. 4A). We further examined whether the expression of *ERF1* and *SID2* was also altered by *35S:GLIP1* exudate treatment (Fig. 4B). Significant induction of *PDF1.2*, *ERF1*, and *SID2* was detected in wild-type, *etr1-1*, and *35S:EIN3* plants when they were inoculated with *35S:GLIP1* exudates but not with exudates of other plants. These results suggest that feedback regulation of GLIP1 occurs through the production of a systemic signal(s) that is independent of upstream ethylene signaling involving ETR1 and that it is related to GLIP1-elicited induced resistance against pathogens.

GLIP1-Induced EIN3 Degradation Is EBF1/EBF2 Dependent

Since EIN3 degradation in *35S:GLIP1* plants was proteasome dependent (Fig. 3F), it led us to investigate whether GLIP1-induced EIN3 degradation occurs through

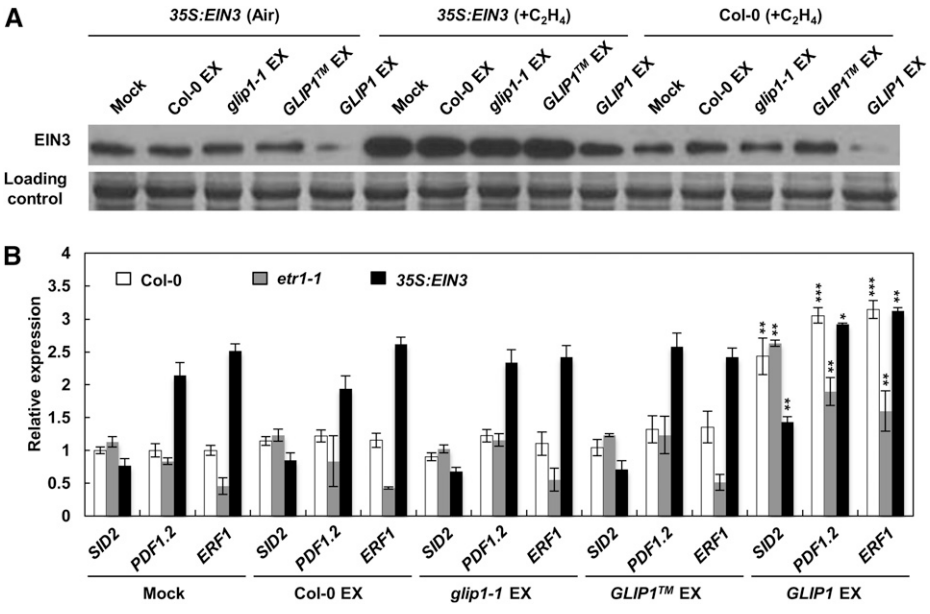

Figure 4. Petiole exudates of *35S:GLIP1* plants decrease EIN3 but induce the expression of *SID2* and *ERF1*. A, Immunoblot analysis of EIN3 in Col-0 and *35S:EIN3* plants in response to petiole exudates (EX). Four-week-old plants were infiltrated with 10 μL of exudates (0.3 μg μL^{-1}) and kept for 12 h in air or 10 μL L^{-1} ethylene. Protein samples were separated by SDS-gel electrophoresis and subjected to Coomassie staining (bottom) and western-blot analysis with anti-EIN3 antibody (top). B, Expression analysis of *SID2*, *ERF1*, and *PDF1.2* in Col-0, *etr1-1*, and *35S:EIN3* plants in response to petiole exudates. Total RNAs were extracted from 4-week-old plants infiltrated with 10 μL of exudates (0.3 μg μL^{-1}) for 24 h and used for quantitative real-time PCR analysis. The values represent means ± SD from three independent experiments. Asterisks indicate significant differences from the mock treatments (Student's *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

the action of the F-box proteins EBF1 and EBF2. We first checked for EIN3 levels in *ebf1-1* and *ebf2-1* mutant plants (Fig. 5A). Whereas EIN3 proteins underwent rapid turnover in ethylene-treated wild-type plants upon inoculation with *35S:GLIP1* exudates, EIN3 stability was remarkably increased in *ebf1-1* and *ebf2-1* mutants. In line with increased EIN3 protein levels, *SID2* expression was not induced in *ebf1-1* and *ebf2-1* mutants in response to *35S:GLIP1* exudates (Fig. 5B). Expression levels of *ERF1* and *PDF1.2* were constitutively high in *ebf1-1* and *ebf2-1* plants, reflecting the increased EIN3 accumulation (Guo and Ecker, 2004).

We next examined whether GLIP1-induced EIN3 degradation is related to changes in the levels of EBF1/EBF2 proteins and/or *EBF1/EBF2* transcripts (Fig. 5C; Supplemental Fig. S10). EBF1/EBF2 protein levels were checked in *35S:EBF1-TAP* and *35S:EBF2-TAP* plants, as previously (An et al., 2010). ACC treatment promoted EBF1/EBF2 protein degradation, and this was substantially suppressed by pretreatment of *35S:EBF1-TAP* and *35S:EBF2-TAP* seedlings with *35S:GLIP1* exudates (Fig. 5C). *EBF1/EBF2* expression was not significantly different among wild-type, *glip1-1*, and *35S:GLIP1* plants, although *EBF1/EBF2* transcript levels were slightly higher in untreated *35S:GLIP1* plants (Supplemental Fig. S10). These results suggest that GLIP1 triggers EIN3 degradation via the EBF1/EBF2-dependent proteasome pathway.

GLIP1 Interacts with Ethylene Signaling to Control Disease Resistance

We then monitored how ethylene mutants and their *35S:GLIP1*-crossed lines respond to pathogens. Because of tiny leaf size, *ctr1-1* plants were excluded from this test. *35S:GLIP1 ctr1-1* plants, however, had normal-sized leaves and were included. First, plants were inoculated with the JA/ethylene-associated necrotrophic fungus *A. brassicicola* and assessed for disease development (Fig. 6). Whereas the *glip1* mutant was highly susceptible to *A. brassicicola* infection, *35S:GLIP1* plants, like wild-type ecotype Columbia (Col-0), formed hypersensitive response-like small necrotic lesions (Kwon et al., 2009). *A. brassicicola*-inoculated *35S:GLIP1 ctr1-1* leaves did not differ from *35S:GLIP1* in resistance phenotype. Supposing that *ctr1-1* plants with high expression of *ERF1* and *GLIP1* would be resistant to *A. brassicicola*, pathogen response phenotypes of *35S:GLIP1 ctr1-1* plants were consistent with ethylene response and gene expression data. The ethylene-insensitive mutants *ein2-1*

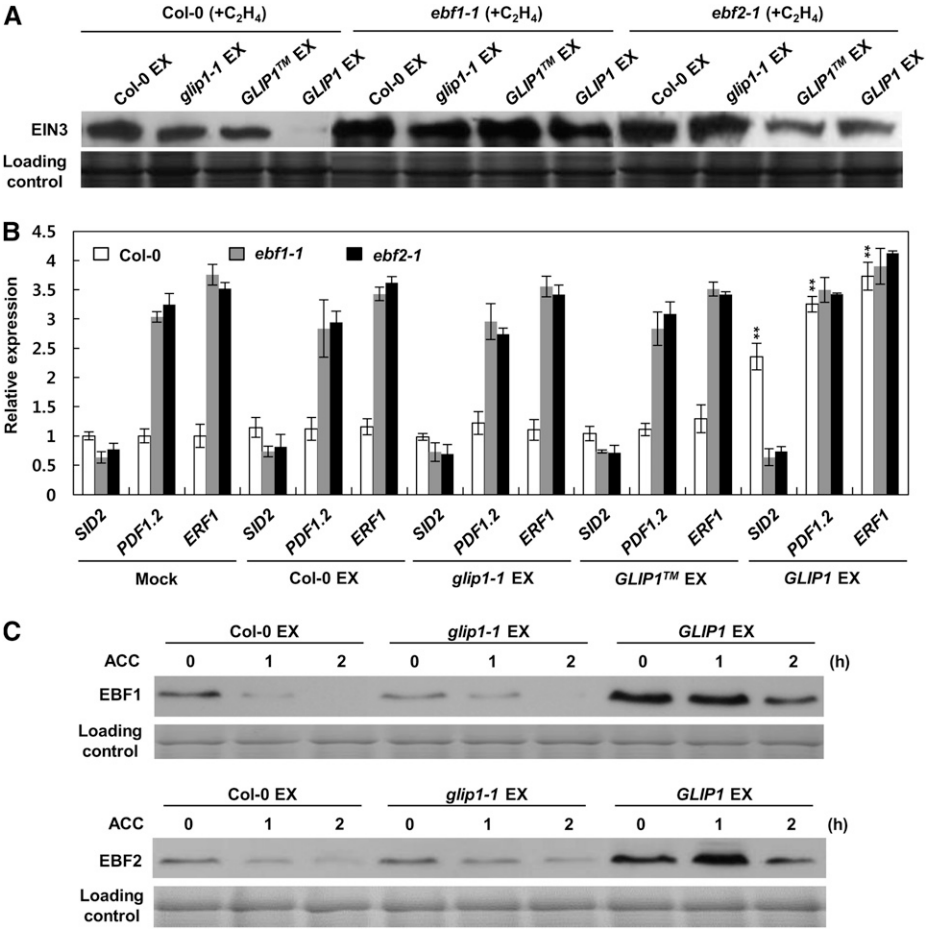

Figure 5. Petiole exudates of *35S:GLIP1* plants trigger EIN3 degradation via EBF1/EBF2. A, Immunoblot analysis of EIN3 in Col-0, *ebf1-1*, and *ebf2-1* plants in response to petiole exudates (EX). Four-day-old seedlings were treated with 10 μL of exudates (0.3 μg μL^{-1}) and kept for 12 h in 10 μL L^{-1} ethylene. B, Expression analysis of *SID2*, *ERF1*, and *PDF1.2* in Col-0, *ebf1-1*, and *ebf2-1* plants in response to petiole exudates. Total RNAs were extracted from 4-week-old plants infiltrated with 10 μL of exudates (0.3 μg μL^{-1}) for 24 h and used for quantitative real-time PCR analysis. The values represent means ± SD from three independent experiments. Asterisks indicate significant differences from the mock treatments (Student's *t* test, $^{**}P < 0.01$). C, Immunoblot analysis of EBF1/EBF2 in *35S:EBF1-TAP* and *35S:EBF2-TAP* plants in response to petiole exudates. Four-day-old seedlings were pretreated with 10 μL of exudates (0.3 μg μL^{-1}) for 4 h and then treated with 10 μM ACC for the indicated times. For A and C, protein samples were separated by SDS-gel electrophoresis and subjected to western-blot analysis with anti-EIN3 (A) or anti-MYC (C) antibody.

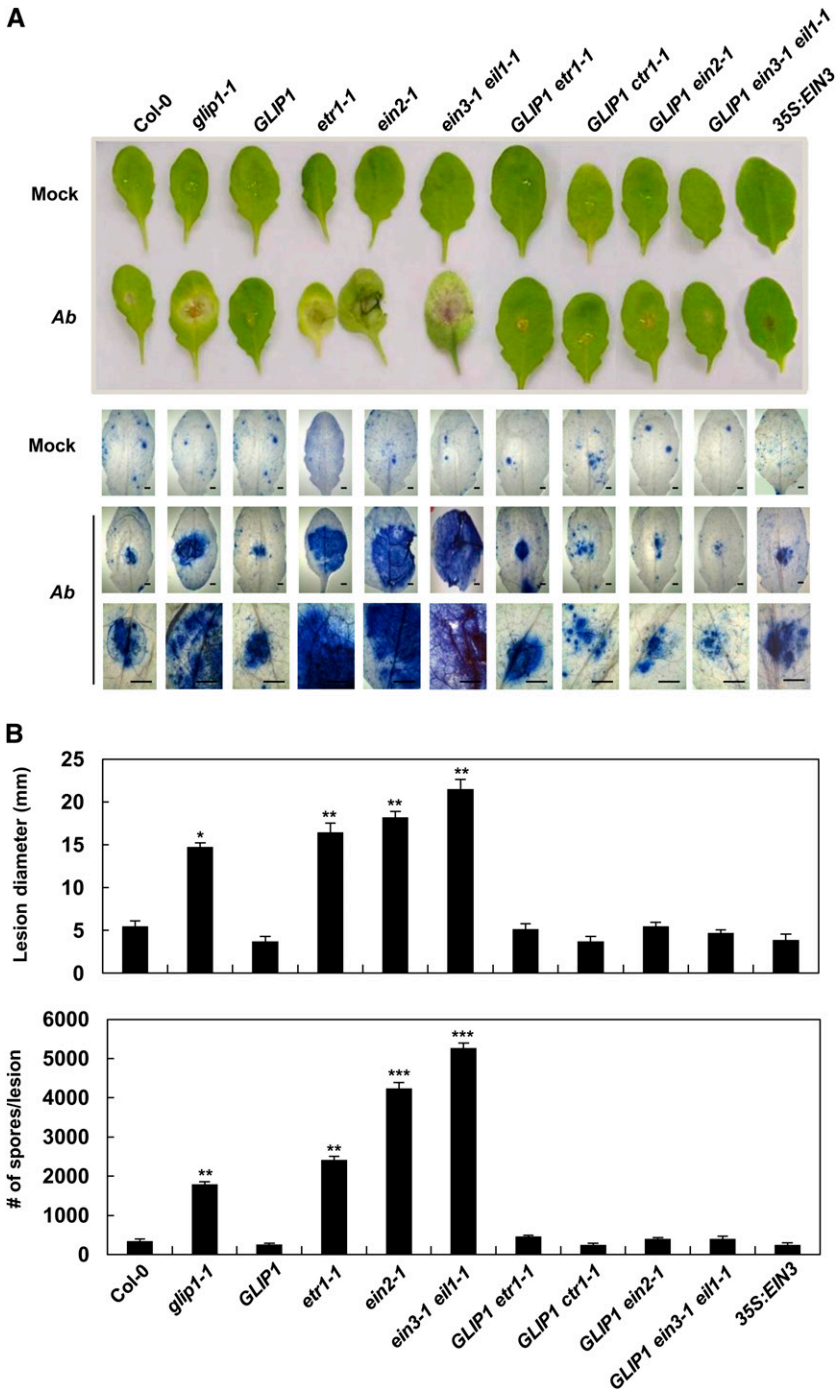

Figure 6. Functions of GLIP1 and ethylene components in resistance to *A. brassicicola*. A, Phenotypes (top) and necrotic lesions (bottom) of leaves inoculated with 10 μL of water (mock) or drops of *A. brassicicola* spore suspension (5×10^5 spores mL^{-1}). Necrotic lesions of leaves were stained with lactophenol-aniline blue. *Ab*, *A. brassicicola*. Bars = 100 μm. B, Measurement of lesion diameter (top) and number of newly formed spores (bottom) in leaves from A. The values are means ± SD ($n = 10$). Asterisks indicate significant differences from Col-0 (Student's *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). The experiment was repeated three times with similar results.

and *ein3-1 eil1-1* had susceptible phenotypes, whereas overexpression of *GLIP1* in *35S*:*GLIP1*-crossed *ein2-1* and *ein3-1 eil1-1* plants restored resistance, as in wild-type and *35S*:*GLIP1* plants. *35S*:*EIN3* plants also showed resistance phenotypes, consistent with high expression of *PDF1.2* and *ERF1* (Fig. 4B, mock treatment).

Plants were then challenged with the SA-associated hemibiotrophic bacterial pathogen *Pst* DC3000 (Fig. 7, A and B). Compared with the wild type, bacterial growth was significantly suppressed in *35S*:*GLIP1* plants but little altered in *glip1* (Kwon et al., 2009). *35S*:*GLIP1 ctr1-1* plants exhibited the bacterial resistance phenotype of *35S*:*GLIP1*, again correlating with triple response and gene expression patterns. It was previously reported that *ein2-1* and *ein3-1 eil1-1* plants have enhanced resistance to *Pst* DC3000 (Chen et al., 2009). This was supported by our observations that *ein2-1* and *ein3-1 eil1-1* plants experienced significantly lower levels of bacterial growth. Furthermore, this was maintained in their *35S*:*GLIP1*-crossed lines. Consistently, *35S*:*EIN3* plants were more susceptible to *Pst* DC3000. As observed in our previous work (Kwon

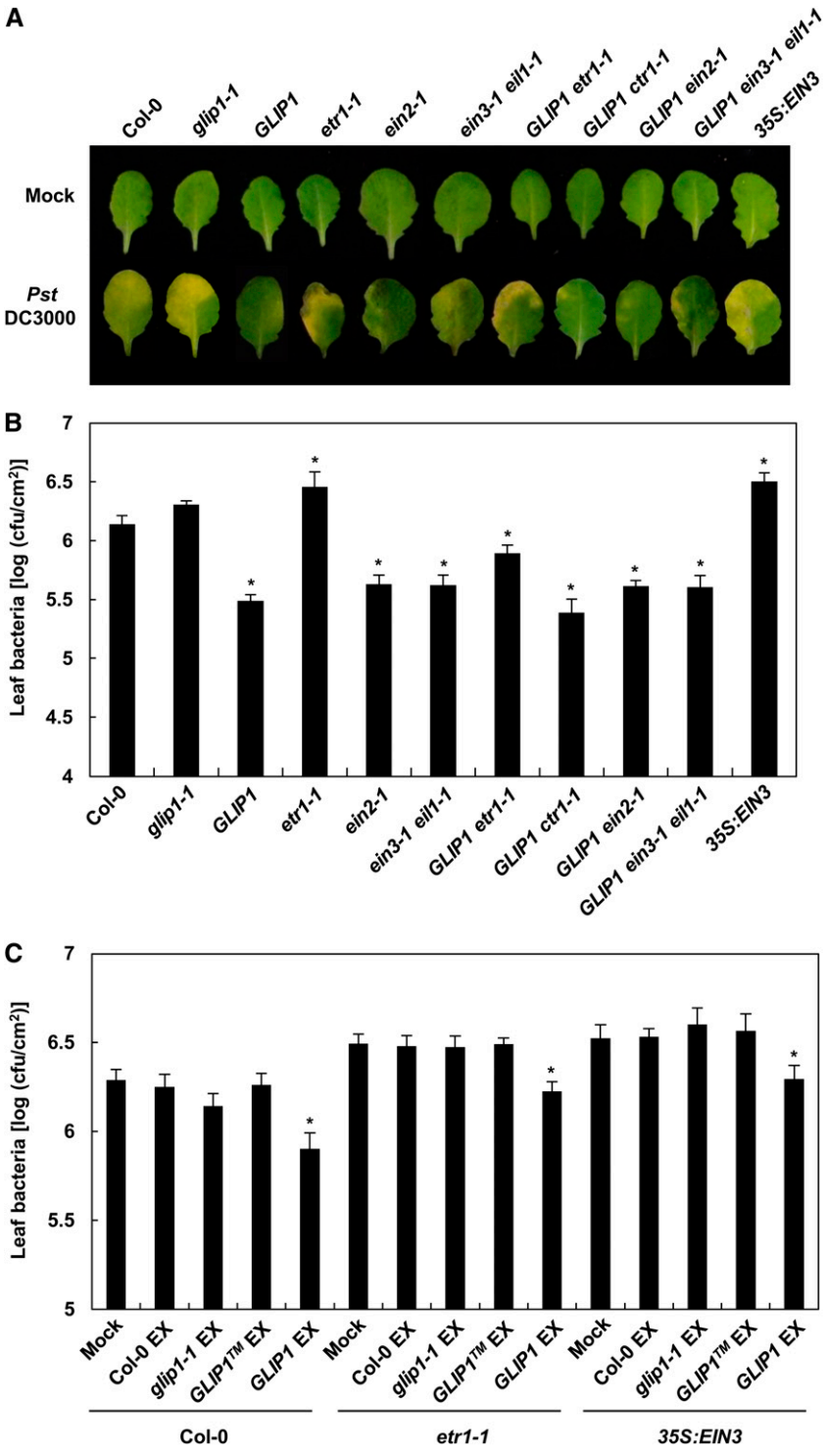

Figure 7. Functions of GLIP1 and ethylene components in resistance to *Pst* DC3000. A, Phenotypes of leaves inoculated with 10 μL of 10 mM $MgCl_2$ (mock) or aliquots of *Pst* DC3000 (10^6 colony-forming units [cfu] mL^{-1}). B, Bacterial growth in leaves inoculated with 10-μL aliquots of *Pst* DC3000 (10^5 cfu mL^{-1}). The values are means ± SD ($n = 5$). Asterisks indicate significant differences from Col-0 (Student's *t* test, $*P < 0.05$). The experiment was repeated five times with similar results. C, Effect of petiole exudates on bacterial growth. Plant leaves were pretreated with petiole exudates (EX) from Col-0, *glip1-1*, *35S:GLIP1*, and *35S:GLIP1* plants for 24 h and then infiltrated with 10-μL aliquots of *Pst* DC3000 (10^5 cfu mL^{-1}). The values are means ± SD ($n = 5$). Asterisks indicate significant differences from the mock treatments (Student's *t* test, $*P < 0.05$). The experiment was repeated three times with similar results.

et al., 2009), *etr1-1* plants had slightly increased levels of bacterial growth compared with the wild type, contrasting with the enhanced bacterial resistance in other ethylene-insensitive *ein2-1* and *ein3-1 eil1-1* mutants. We speculate that another EIN2/EIN3-independent ethylene pathway may exist downstream of ETR1 and cross talk with the SA pathway.

Since *35S:GLIP1* exudates induced EIN3 down-regulation and *SID2* activation in wild-type, *etr1-1*, and *35S:EIN3* plants (Fig. 4), we further tested whether *35S:GLIP1* exudates can lead to the suppression of bacterial growth (Fig. 7C). The growth of *Pst* DC3000 was significantly suppressed in wild-type, *etr1-1*, and *35S:EIN3* plants when they were pretreated with *35S:*

GLIP1 exudates but not with other exudates. This demonstrates that the bacterial resistance-inducing activity of *35S:GLIP1* exudates correlates with their EIN3-down-regulating and *SID2*-activating activities.

DISCUSSION

In this work, we present evidence that GLIP1 mechanistically interacts with ethylene signaling components to control plant immunity. *GLIP1* expression depends on ethylene signaling and is up-regulated by ERF1. Activated *GLIP1* increases *ERF1* expression via positive feedback. Additionally, GLIP1 forms a negative feedback loop in ethylene signaling through EBF1/EBF2-dependent proteasomal degradation of EIN3 proteins. We propose that GLIP1-mediated positive and negative feedback regulation of the ethylene signaling pathway is the mechanism underlying GLIP1-induced systemic resistance (Fig. 8).

Reciprocal Activation of GLIP1 and Ethylene Signaling

We previously showed that *GLIP1* is induced and functions in an ethylene-dependent manner (Oh et al., 2005; Kwon et al., 2009). In this study, ethylene induction of *GLIP1* was significantly reduced in *etr1-1*, *ein2-1*, and *ein3-1 eil1-1* mutants, but *GLIP1* was highly expressed in the *ctr1-1* mutant and *ERF1*-overexpressing plants.

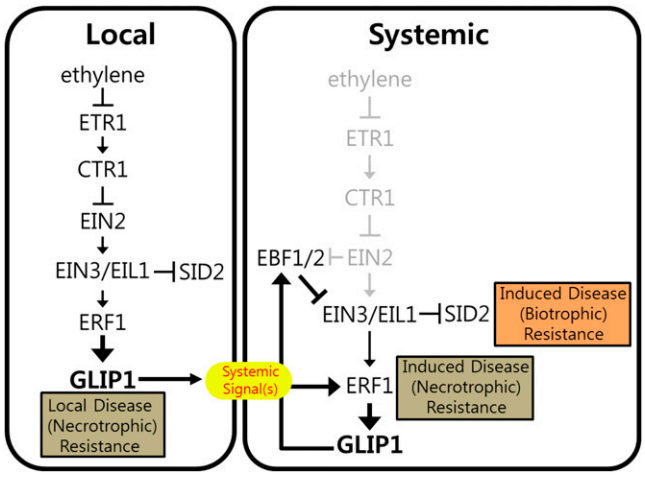

Figure 8. A model for GLIP1 function in the regulation of ethylene signaling and immunity. *GLIP1* expression depends on ethylene signaling components and positively regulates both local and systemic pathogen resistance. GLIP1 constitutes feedback regulation loops and modulates ethylene signaling in two ways: by inducing *ERF1* (positive) and by suppressing EIN3 (negative) via EBF1/EBF2. *ERF1* induction increases JA/ethylene-regulated pathogen resistance, and EIN3 down-regulation elevates *SID2* expression and SA production, resulting in the enhanced SA-regulated pathogen resistance. We propose that GLIP1-mediated feedback regulation of ethylene signaling is the underlying mechanism of GLIP1 function in induced systemic resistance to pathogens and that it operates through GLIP1-mediated production of a systemic signal(s).

This indicates that *GLIP1* is downstream of ERF1 and is regulated by ethylene signaling components. Noticeably, *GLIP1* overexpression enhanced the triple response of ACC/ethylene-treated etiolated seedlings, which correlated with elevated expression of ethylene response genes, including *ERF1* and *HLS1*. In fact, *ERF1* expression was largely dependent on *GLIP1*, as *ERF1* induction was much less in the *glip1-1* mutant. Therefore, ethylene-regulated *GLIP1* appears to affect ethylene signaling via a positive feedback mechanism. *35S:GLIP1* and *glip1-1* seedlings showed increased and decreased ethylene sensitivity, respectively, but to a lesser extent than ethylene mutants. Whereas expression levels of *GLIP1* and *ERF1* in *35S:GLIP1* plants were similar to those in *ctr1-1*, the triple response was weakly constitutive but largely inducible, unlike in *ctr1-1* plants. These data suggest that *GLIP1* and *ERF1* expression are important, but not sufficient, for ethylene responses. Other factors and/or additional posttranscriptional modifications may be necessary for and act in concert to facilitate a full ethylene response. It was previously shown that *ERF1* overexpression rescued the mutant phenotypes of *ein3* but only a subset of ethylene responses (Solano et al., 1998).

Epistatic Interaction of GLIP1 and Ethylene Signaling Genes

Supporting that GLIP1 positively acts downstream of ERF1 and ethylene signaling components, *GLIP1* overexpression in *etr1-1*, *ein2-1*, and *ein3-1 eil1-1* mutants rescued the loss of ethylene responses. Intriguingly, *35S:GLIP1 ctr1-1* displayed ethylene-response phenotypes of *35S:GLIP1* plants. Loss of the constitutive triple response of *ctr1-1* in *35S:GLIP1 ctr1-1* seedlings led us to propose that GLIP1 constitutes a negative feedback loop downstream of CTR1 and upstream of ERF1, probably at EIN2 and/or EIN3. Western-blot analysis showed that the protein level of EIN3 is negatively modulated by *GLIP1* expression, suggesting that GLIP1 contributes to the negative regulation of EIN3 in ethylene signaling. As shown by treatments with petiole exudates, GLIP1-mediated down-regulation of EIN3 depended on EBF1 and EBF2, which induce the degradation of EIN3/EIL1 (Guo and Ecker, 2003). It is known that EIN3 and EIL1 play a role in integrating other signals into the ethylene signaling pathway. Glc accelerated EIN3 degradation through the Glc sensor hexokinase and thus antagonized ethylene signaling (Zhou et al., 1998; Yanagisawa et al., 2003). Interaction between light and ethylene signaling has also been reported. EIN3/EIL1 activated *PIF3* expression by directly binding to the promoter of *PIF3*, leading to ethylene-induced hypocotyl elongation in light (Zhong et al., 2012). In addition, EIN3/EIL1 protein stability increased in light-grown seedlings (Lee et al., 2006). Apical hook development is coordinately regulated by ethylene and GAs. In a recent study, DELLA proteins, key repressors of the GA pathway, were shown to associate with the DNA-binding domains of EIN3/EIL1

and to inhibit EIN3/EIL1-induced *HLS1* expression, and GAs enhanced the hook curvature by derepressing EIN3/EIL1 (An et al., 2012). Moreover, as demonstrated both by previous work (Chen et al., 2009) and our current results, EIN3/EIL1 negatively controlled *SID2* expression and thus the SA signaling pathway in plant immunity. EIN3 and EIL1, as key modulators of ethylene signaling, may serve as a molecular link connecting distinct signaling pathways to the ethylene pathway in order to coordinate plant growth and development and environmental responses.

GLIP1 and Ethylene Signaling in the Control of Pathogen Resistance

Here, we tested ethylene mutants and their *35S:GLIP1*-crossed lines for resistance responses to both necrotrophic *A. brassicicola* and hemibiotrophic *Pst* DC3000. Consistent with the epistatic interactions of *GLIP1* and ethylene signaling genes, *GLIP1* overexpression restored resistance to *A. brassicicola* in the ethylene mutants *etr1-1*, *ein2-1*, and *ein3-1 eil1-1*. *35S:GLIP1* plants displayed strong induction of *ERF1*, which was previously shown to be a key factor for the regulation of defense response genes (Lorenzo et al., 2003). Constitutive expression of *ERF1* in Arabidopsis conferred resistance to several fungal pathogens, such as *B. cinerea*, *Plectosphaerella cucumerina*, and *Fusarium oxysporum* (Berrocal-Lobo et al., 2002). This indicates that *ERF1* expression may be necessary for GLIP1-induced resistance to necrotrophic pathogens, meaning that GLIP1-mediated feedback regulation of *ERF1* accumulation is critical for disease resistance responses in plants. On the other hand, enhanced resistance of *ein2-1* and *ein3-1 eil1-1* to *Pst* DC3000 was previously shown to be related to *SID2* induction and SA accumulation (Chen et al., 2009), and this was further confirmed in our work here. We showed that the EIN3 protein level was decreased and that *SID2* expression, repressed by EIN3 and EIL1, was increased in *35S:GLIP1* plants. These results suggest that GLIP1-induced resistance to *Pst* DC3000 may be regulated by GLIP1-mediated feedback suppression of EIN3. We propose that positive (i.e. *ERF1* induction) and negative (i.e. EIN3 destabilization) feedback regulation of ethylene signaling by GLIP1 is an underlying mechanism for GLIP1 functions in plant immunity, specifically in induced systemic resistance (Fig. 8).

Inoculation of plants with petiole exudates from *35S:GLIP1* plants led to significant reduction of EIN3 and induction of *SID2* and *ERF1* and also suppressed the growth of *Pst* DC3000 in wild-type, *etr1-1*, and *35S:EIN3* plants. These results suggest that ETR1, and its ethylene binding, are required for *GLIP1* expression, but that once it is activated, *GLIP1* elicits induced resistance through the feedback regulation of ethylene signaling. This further supports our proposed model for GLIP1, in which GLIP1-mediated feedback regulation operates through a systemic signal(s), probably generated by catalytic processes involving GLIP1. However, our previous results demonstrated that, unlike *35S:GLIP1* exudates, GLIP1 proteins failed to induce systemic resistance upon inoculation into *etr1-1* (Kwon et al., 2009). These differing effects of *35S:GLIP1* exudates and GLIP1 proteins suggest that GLIP1 proteins, unlike the systemic signals in petiole exudates, may not be sufficient for signal amplification and propagation in the *etr1-1* mutant background.

There have been numerous reports about both antagonistic and synergistic interactions between ethylene/JA and SA pathways (Kunkel and Brooks, 2002; Glazebrook et al., 2003; Broekaert et al., 2006). The activation of local SA- and JA/ethylene-dependent resistance appears to be mutually exclusive. Upon attack by necrotrophic pathogens, plants may suppress the SA pathway via EIN3/EIL1-mediated SID2 suppression; this antagonistic effect of ethylene signaling allows plants to prioritize the ethylene/JA signaling pathway. On the other hand, two types of induced resistance, SAR and ISR, which require SA and JA/ethylene, respectively, both depend on NPR1 and seem to act additively (Pieterse et al., 1998; Ryu et al., 2004). In fact, positive interactions between ethylene, JA, and SA have previously been observed for induced resistance (Kwon et al., 2009). In the case of induced resistance, hormone pathways may act in concert to evoke resistance against multiple types of pathogens that plants often encounter in the natural environment. For this, ethylene signaling can positively affect the SA pathway through GLIP1-mediated EIN3 downregulation. Further studies will hopefully allow us to elucidate how cross talk between hormone pathways is fine-tuned in the complex signaling networks that control plant immune responses.

MATERIALS AND METHODS

Plant Materials

Wild-type, mutant, and transgenic Arabidopsis (*Arabidopsis thaliana* Col-0) plants were grown at 23°C under long-day conditions in a 16-h-light/8-h-dark cycle. The following plants were used in this study: *glip1-1* (Oh et al., 2005), *35S:GLIP1(3-2)* (Kwon et al., 2009), *etr1-1* (Hua et al., 1998), *ctr1-1* (Kieber et al., 1993), *ein2-1* (Roman et al., 1995), *ein3-1 eil1-1* (Alonso et al., 2003), *ebf1-1* (Guo and Ecker, 2003), *ebf2-1* (Guo and Ecker, 2003), *35S:EBF1-TAP* (An et al., 2010), *35S:EBF2-TAP* (An et al., 2010), *35S:EIN3* (Chao et al., 1997), and *35S:ERF1* (Lorenzo et al., 2003). *35S:ERF1* (CS6142) seeds were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). Mutation and insertion sites were verified by sequencing, and homozygous lines were selected. *35S:GLIP1* was crossed to *etr1-1*, *ctr1-1*, *ein2-1*, and *ein3-1 eil1-1*, and homozygous lines were confirmed by PCR and sequence analysis using gene-specific primers (Supplemental Table S1) for ethylene mutants and by segregation analysis and PCR using *35S* primers for *35S:GLIP1*.

Preparation of Petiole Exudates

Petiole exudates were prepared as described previously (Kwon et al., 2009). Petioles of wild-type, *glip1-1*, *35S:GLIP1*^{TM}, and *35S:GLIP1* plants were cut above the stem. The cut surface was briefly sterilized in a solution containing 50% (v/v) ethanol and 0.0006% (w/v) sodium hypochlorite and then rinsed in sterile distilled water. Exudates were collected in distilled water for 2 d. Prior to use, exudates were syringe filtered and tested on medium plates for bacterial contamination.

Chemical Treatments

Sterilized seeds were plated on Murashige and Skoog (MS)-Suc (2% [w/v]) agar medium alone or supplemented with ACC (0.5–10 μM) or $AgNO_3$ (100 μM). For the triple response, plates were wrapped in foil and kept for 4 d at 23°C, as described (Yoo et al., 2008). For ethylene treatment, seedlings grown on MS plates in a 500-mL container for 2 weeks were supplemented with 10 μL L^{-1} ethylene in hydrocarbon-free air, wrapped in foil, and kept for 4 d (triple response) or 12 h (gene expression) at 23°C (Kieber et al., 1993). For ethephon treatment, 4-week-old plants were sprayed with water (mock) or with ethephon (1.5 mM) dissolved in water. For ACC treatment, 4-week-old plants were sprayed with water (mock) or with ACC (10 μM) dissolved in water. The treated plants were maintained at 100% humidity for the indicated times and then harvested. For inhibitor treatments, 3-d-old seedlings germinated on Whatman 3MM filter paper placed on MS plates were treated with 0.1% (v/v) dimethyl sulfoxide (mock), MG132 (50 μM), or cycloheximide (100 μM) for 2 h.

Pathogen Infection

For pathogen infection, 4-week-old plants grown under short-day conditions in an 8-h-light/16-h-dark cycle were used. Treatment with *Alternaria brassicicola* was performed by applying 10 μL of water or drops of spore suspension (5×10^5 spores mL^{-1}) to each plant leaf, as described (Oh et al., 2005). The number of spores was counted, and the lesions and fungal hyphae were visualized by staining the infected leaves with lactophenol-aniline blue, as described (Oh et al., 2005). Treatment with *Pseudomonas syringae* was performed as described (Oh et al., 2005). Three other leaves of each plant were infiltrated at one site with 10 μL of $MgCl_2$ (10 mM) or aliquots of *P. syringae* pv *tomato* DC3000 (10^5–10^6 colony-forming units mL^{-1}). Inoculated plants were kept in a growth chamber at 100% humidity for 4 d as indicated. This experiment was designed as a randomized complete block with five replications and one plant per replication and was repeated at least three times.

Immunoblot Analysis

The coding regions corresponding to residues 800 to 1,294 of EIN2 and full-length EIN3 were cloned into the pDEST17 Gateway vector (Invitrogen) and expressed in *Escherichia coli* as described (Guo and Ecker, 2003). His-tagged recombinant EIN2 and EIN3 proteins were purified using nickel-nitrilotriacetic acid agarose columns (Qiagen) according to the manufacturer's instructions and used to produce polyclonal antibodies in rats and rabbits (Komabiotech). Western-blot analysis was performed as described (Oh et al., 2005). Total proteins were extracted and separated by SDS-gel electrophoresis for immunoblotting. The blots were probed with anti-EIN2 and anti-EIN3 antisera for EIN2 and EIN3 immunoblots or with anti-MYC antibody (AbChem) for EBF1/EBF2-TAP immunoblots. Antibody-bound proteins were detected following incubation with secondary antibody conjugated to horseradish peroxidase using the ECL system (Amersham Biosciences).

Ethylene Quantification

Ethylene production in plants was measured by gas chromatography (GC 7890; Agilent) as described (Tamaoki et al., 2008). Seedlings (100 mg) grown on MS-Suc (2% [w/v]) agar medium for 10 d under long-day conditions were enclosed in 60-mL vials and incubated in light for 6 h. Ethylene standards with different concentrations were made by diluting ethylene in 1-L Tedlar sample bags. Gas-phase samples (5 mL) from the vials were injected into an HP-PLOT Q column (30 m × 0.53 mm, 40 μm; Agilent) with column and detector temperatures of 75°C and 250°C, respectively. The amount of ethylene from the plants was determined from ethylene peak area based on comparison with ethylene standards.

SA Quantification

Total and free SA were quantified as described (Segarra et al., 2006; Garcion et al., 2008). Leaf tissues (250 mg), together with an internal standard (1 μg of *o*-anisic acid), were ground in liquid nitrogen and extracted twice with 90% methanol. After methanol was evaporated from extracts, total SA was further extracted by acid hydrolysis with 4 N HCl at 95°C for 1 h. Liquid chromatography analysis was performed on an Optima Pak C_{18} column (250 × 4.6 mm, 5 μm; RS Tech). The injection volume was 50 μL, and elution was performed with a binary solvent system consisting of 0.05% (v/v) HOAc in water (solvent A) and 0.05% (v/v) HOAc in acetonitrile (solvent B) at a constant flow rate of 1 mL min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent B was applied (time [min], percentage B): (0, 15), (20, 50), (30, 100), (32, 100), (33, 15), and (45, 15) with 5 min for reequilibration. Fluorescence was recorded with excitation/emission wavelengths of 305/365 nm and 305/407 nm for *o*-anisic acid and SA, respectively.

Gene Expression Analysis

Quantitative real-time reverse transcription-PCR was performed using KAPA SYBR FAST qPCR master mix in a LightCycler 480 system (Roche). PCR was performed with gene-specific primers (Supplemental Table S1) according to the manufacturer's protocol. The expression levels of the tested genes were standardized to the constitutive expression level of *ACTIN1* and calculated using the geNorm program (Vandesompele et al., 2002). The experiments were repeated at least three times with biologically independent samples.

Determination of Chlorophyll Content

To measure chlorophyll content, leaves were submerged in 95% ethanol and incubated for 20 min at 80°C (Kwon et al., 2007). Absorbance was monitored at 648 nm (A_{648}) and 665 nm (A_{664}), and chlorophyll content was calculated according to the following formula: μg chlorophyll = $[(13.36A_{664}) - (5.19A_{648})] + [(27.43A_{648}) - (8.12A_{664})]$ (Lichtenthaler, 1987).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_104918 (ACO2), M38240 (b-CHI), NM_180429 (CTR1), NM_112550 (EBP), NM_128106 (EBF1), NM_122444 (EBF2), NM_120406 (EIL1), NM_112968 (EIN3), NM_113225 (ERF1), NM_124094 (ERF5), NM_129658 (ERS1), NM_105305 (ETR1), NM_113216 (ETR2), NM_123464 (GLIP1), NM_119922 (HLS1), NM_123809 (PDF1.2), and NM_106129 (SID2).

Supplemental Data

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

Supplemental Figure S1. Expression analysis of ethylene-responsive genes in Col-0, *glip1-1*, and *35S:GLIP1* plants.

Supplemental Figure S2. Triple response of *glip1* mutants and homozygous T3 lines of *35S:GLIP1*.

Supplemental Figure S3. Positive regulation of ethylene responses by GLIP1.

Supplemental Figure S4. Effects of the catalytic mutation of *GLIP1* and Ag^{2+} on the triple response of *glip1-1* and *35S:GLIP1* seedlings.

Supplemental Figure S5. Genetic crosses between *35S:GLIP1* and ethylene mutants.

Supplemental Figure S6. Genomic DNA analysis of crossed lines.

Supplemental Figure S7. Expression analysis of *HLS1* in Col-0, *glip1-1*, *35S:GLIP1*, *ein3-1 eil1-1*, and *35S:GLIP1 ein3-1 eil1-1* plants.

Supplemental Figure S8. Expression analysis of EIN2 proteins and *EIN2* and *EIN3* transcripts in Col-0, *glip1-1*, and *35S:GLIP1* plants.

Supplemental Figure S9. Proteins isolated from petiole exudates of Col-0, *glip1-1*, *35S:GLIP1*^{TM}, and *35S:GLIP1* plants.

Supplemental Figure S10. Expression analysis of *EBF1* and *EBF2* in Col-0, *glip1-1*, and *35S:GLIP1* plants.

Supplemental Table S1. List of primers used for PCR and quantitative reverse transcription-PCR.

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