Secretome Analysis Reveals an *Arabidopsis* Lipase Involved in Defense against *Alternaria brassicicola* [W]

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The *Arabidopsis thaliana* secretome was analyzed by the proteomic approach, which led to the identification of secreted proteins implicated in many aspects of cell biology. We then investigated the change in the *Arabidopsis* secretome in response to salicylic acid and identified several proteins involved in pathogen response. One of these, a secreted lipase with a GDSL-like motif designated GDSL LIPASE1 (GLIP1), was further characterized for its function in disease resistance. *glip1* plants were markedly more susceptible to infection by the necrotrophic fungus *Alternaria brassicicola* compared with the parental wild-type plants. The recombinant GLIP1 protein possessed lipase and antimicrobial activities that directly disrupt fungal spore integrity. Furthermore, GLIP1 appeared to trigger systemic resistance signaling in plants when challenged with *A. brassicicola*, because pretreatment of the *glip1* mutant with recombinant GLIP1 protein inhibited *A. brassicicola*–induced cell death in both peripheral and distal leaves. Moreover, *glip1* showed altered expression of defense- and ethylene-related genes. *GLIP1* transcription was increased by ethephon, the ethylene releaser, but not by salicylic acid or jasmonic acid. These results suggest that GLIP1, in association with ethylene signaling, may be a critical component in plant resistance to *A. brassicicola*.

INTRODUCTION

The cell wall or extracellular matrix (ECM) is a dynamic zone harboring active components that regulate cell–cell interactions. The ECM contains secreted proteins that play crucial roles in a variety of physiological processes, such as growth, development, and defense responses (Showalter, 1993; Pennell, 1998). The most abundant and well-studied cell wall proteins are structural proteins, including extensins, Gly-rich proteins, Pro-rich proteins, solanaceous lectins, and arabinogalactan proteins (Showalter, 1993). However, the ECM also contains a large number of nonstructural proteins, including peroxidases, invertases, proteases, mannosidases, galactosidases, and glucanases.

ECM peptide or protein participation in the plant defense process has been reported previously. For example, plants have been found to contain families of antimicrobial peptides (Garcia-Olmedo et al., 1998), which include the thionins, defensins, lipid transfer proteins, hevein- and knottin-like peptides, maize (*Zea mays*) basic peptide 1, *Impatiens balsamina* antimicrobial peptide, snakins, and systemins. Systemins are a unique class of 18–amino acid peptides derived from the precursor polypeptide prosystemin and have been functionally well defined (Ryan et al., 2002). They activate the defense signaling process by interacting with the plasma membrane receptor kinase SR160. Plants also bear polygalacturonase-inhibiting proteins, which are ubiquitous cell wall proteins that protect plants from fungal invasion (De Lorenzo and Ferrari, 2002). Polygalacturonase-inhibiting proteins directly inhibit the polygalacturonases secreted by pathogenic fungi and at the same time enhance the accumulation of oligogalacturonides, which in turn activates the defense response.

Proteomics is the global analysis of gene products in various tissues and physiological states of cells (Pandey and Mann, 2000) and has become very important in the functional genomics field now that several genomes have been sequenced completely and analytical methods for protein characterization have been developed. Progress in plant proteomics has largely been made possible by two-dimensional (2D) gel electrophoresis–based proteomic approaches (van Wijk, 2001; Kersten et al., 2002), and several major plant proteomics studies have focused on subcellular proteomes and protein complexes (Rouquie et al., 1997; Peltier et al., 2000; Prime et al., 2000; Kruft et al., 2001; Millar et al., 2001; Chivasa et al., 2002; Bae et al., 2003).

Despite technical difficulties in preparing pure cell walls, the proteomic approach has also been used to study plant cell wall proteins (Okushima et al., 2000; Chivasa et al., 2002; Ndimba et al., 2003). In one of these studies, isolated cell walls were subjected to sequential protein extractions using $CaCl_2$ and a urea buffer, resulting in fractions that included classical cell wall

proteins such as glucanases, expansins, glucosidases, pectinesterases, and peroxidases (Chivasa et al., 2002). In the cell wall proteomics study of Okushima et al. (2000), cultured BY2 cells were used to examine the extracellular proteins of tobacco (*Nicotiana tabacum*) cells. Six proteins were found by N-terminal amino acid sequencing to be similar to wheat (*Triticum aestivum*) chemically induced 5, ascorbate oxidase, peroxidase, and exoglucanase. In the proteomics study of Ndimba et al. (2003), *Arabidopsis thaliana* cell suspension cultures were investigated for the proteomic changes induced by fungal elicitors, chitosan, and extracts of *Fusarium moniliforme*. Proteins whose abundance was changed by these treatments were identified in the cell wall fraction and in the culture filtrate.

In this work, we conducted a comprehensive analysis of the secretome of cultured *Arabidopsis* cells. We collected proteins secreted into the culture medium and analyzed them by 2D gel electrophoresis and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). To identify the secreted proteins involved in the plant pathogen response, we evaluated salicylic acid (SA)–induced changes in the isolated secretome. 2D gel analysis revealed that 18 protein spots corresponding to 13 different protein species were either upregulated or downregulated by SA treatment. Of these, GDSL LIPASE1 (GLIP1), a secreted lipase with a GDSL motif, was selected for further characterization. We found that GLIP1 plays critical roles in plant resistance to the fungus *Alternaria brassicicola*. This disease resistance is regulated by two different mechanisms: direct disruption of fungal spores, and systemic resistance signaling associated with ethylene. As a result, we conclude that the proteomics approach can be an effective means of identifying novel proteins implicated in signaling processes.

RESULTS

Isolation of Secreted Proteins from *Arabidopsis* Cell Cultures

Cell cultures derived from *Arabidopsis* ecotype Columbia (Col-0) were established to isolate proteins secreted into the medium. The culture medium was harvested on d 4, at which point the cells were actively proliferating at the mid-log phase. The isolated secreted proteins were tested for contamination with nonsecretory proteins by protein gel blot analysis using antibodies specific for the cell wall protein myrosinase (Husebye et al., 2002), the cytosol proteins glutathione *S*-transferase (GST) and cyclin D, the cytosol and membrane protein annexin1 (AnnAt1), and the chloroplast protein oxygen-evolving enhancer protein2 (OEE2) (Figure 1). The extracellular protein myrosinase was found mostly in the secreted fraction, whereas the others were detected exclusively in the cultured cells (i.e., the nonsecreted fraction).

To confirm the lack of contamination of the culture medium, we estimated cytosolic marker enzyme activities, namely, those of alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (G6PDH), in the separated medium and callus fractions (Table 1). The enzyme activities were high in the cells but not detectable in the medium. These data suggest that the secre-
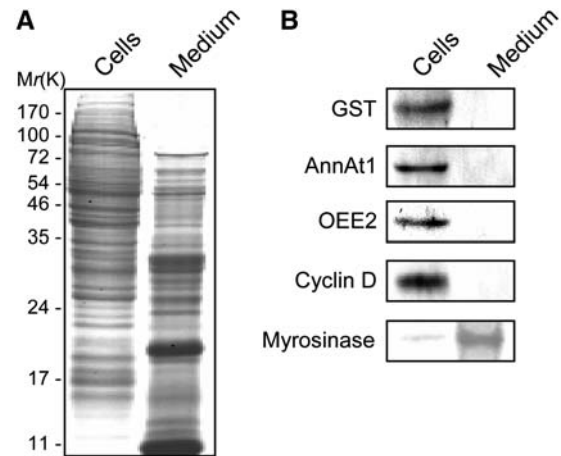

Figure 1. Isolation of the Secreted Proteins from *Arabidopsis* Cell Cultures.

Arabidopsis suspension cultures were separated into cell and culture medium fractions and subjected to gel electrophoresis. Each lane was loaded with the same amount of protein (10 μg).
(A) Fractionated proteins separated by SDS-gel electrophoresis and visualized by Coomassie blue staining.
(B) Protein gel blot analysis of fractionated proteins with anti-GST, anti-AnnAt1, anti-OEE2, anti-cyclin D, and anti-myrosinase antibodies.

tome prepared from the culture medium was essentially free of contamination by nonsecreted proteins. However, we cannot rule out the possibility that some proteins in the secretome were leaking from dead or damaged cells. The instability of contaminating cytosolic marker proteins accumulating in the culture medium may explain this.

Identification of the *Arabidopsis* Secreted Proteins

For proteomic analysis, the isolated secreted proteins were subjected to 2D gel electrophoresis (Figure 2). Approximately 400 protein spots were detected on a silver-stained 2D gel. The secreted proteins were relatively small and mostly distributed in the 10- to 100-kD range. Analysis of the protein spots by MALDI-TOF MS led to the identification of 107 spots that correspond to 91 different proteins (Figure 2; see Supplemental Table 1 online).

Of the proteins identified, 60 (66%) have known functions or sequences similar to those of known proteins, whereas 31 (34%) are novel and have not been assigned any functions. All proteins were categorized into classes based on their functions. The largest class consisted of the novel or uncharacterized proteins (34%). The next largest class contained proteins involved in signaling and regulation (29.6%). The other functions included catalysis (17.6%), protein degradation (6.7%), protein protection (5.5%), structure (3.3%), and retrotransposition (3.3%). These results show that secreted proteins are implicated in a variety of cellular functions.

Secreted proteins are known to bear a short stretch of amino acids called a signal sequence at their N terminus (Nielsen et al.,

Table 1. Cytosolic Marker Enzyme Activities in the Cell and Culture Medium Fractions

Fraction	ADH Activity (units/mg)	G6PDH Activity (units/mg)
None[a]	0.002 ± 0.003	0.001 ± 0.002
Medium	0.002 ± 0.004	0.001 ± 0.003
Cells	2.193 ± 0.047	0.033 ± 0.004

One unit of activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NAD+ or NADP+ per minute under the specific assay conditions described in Methods. The values shown are means ± SD ($n = 10$).
[a] No fractions added.

1997). To determine whether the secreted proteins we identified contain such a signal sequence, we analyzed them with SignalP software (http://www.cbs.dtu.dk/services/SignalP), which searches for signal sequences and their cleavage sites (Nielsen et al., 1997). Of the proteins analyzed, 49 (54%) appear to contain

signal sequences with mean S scores > 0.48 (see Supplemental Table 1 online).

Identification of SA-Responsive Secreted Proteins

To identify secreted proteins involved in the pathogen response, we analyzed the change in the *Arabidopsis* secretome in response to SA, a plant hormone that regulates defense signaling (Dürner et al., 1997). Thus, *Arabidopsis* cell cultures were left untreated or were treated with 0.5 mM SA for 2 and 6 h, after which the culture medium was collected and analyzed by comparative 2D gel electrophoresis. Protein spots whose intensity in the SA-treated medium was altered relative to the untreated control medium were quantitatively analyzed. We performed three independent experiments and selected only those spots whose abundance varied reproducibly by greater than twofold. Eighteen protein spots were selected and subjected to MALDI-TOF MS analysis for protein identification (Table 2).
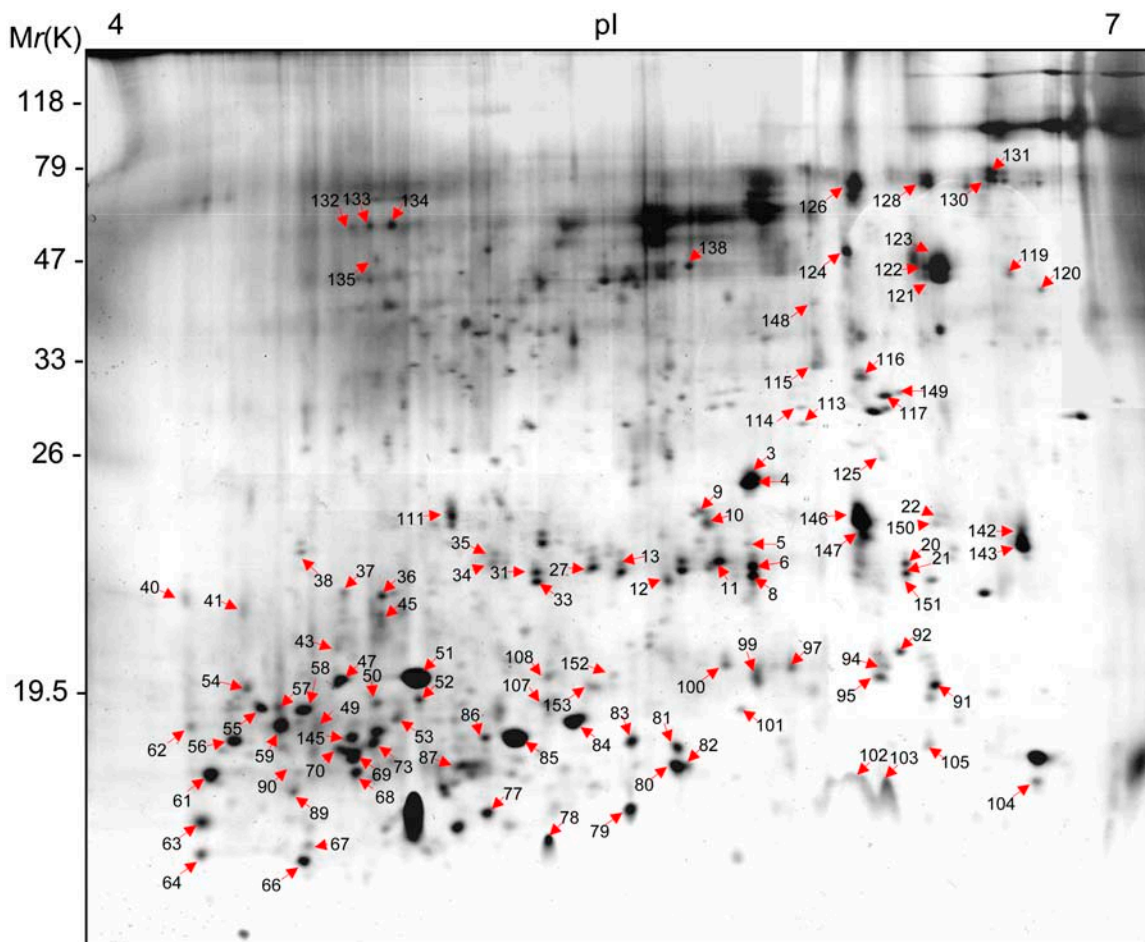
Figure 2. Silver-Stained 2D Gel of Secreted Proteins.

Isolated secreted proteins were resolved in the range of pH 4 to 7. Protein spots identified by MALDI-TOF MS are numbered and listed in Supplemental Table 1 online.

Table 2. Identification of SA-Responsive Proteins Secreted by *Arabidopsis* Cell Cultures

Spot No.[a]	Match MM (kD)/pI[b]	Accession No.[c]	Protein Name[d]	Induction Factor[e]	
				2 h	6 h
6	17.15/5.8	18403909	Ubiquitin-like UBQ7/AtRUB2	0.63	0.21
8	17.15/5.8	18403909	Ubiquitin-like UBQ7/AtRUB2	0.82	0.43
9	34.05/5.3	26452648	Jacalin-related protein	2.91	2.51
10	34.05/5.3	26452648	Jacalin-related protein	1.56	2.10
20	22.83/6.4	24417448	Leu-rich repeat, plant specific	0.81	0.50
21	22.83/6.4	24417448	Leu-rich repeat, plant specific	0.90	0.50
151	22.83/6.4	24417448	Leu-rich repeat, plant specific	0.88	0.37
92	19.03/5.6	11288845	Hypothetical protein	0.38	0.29
100	23.43/5.7	26451897	Unknown protein	0.18	0.11
116	27.22/5.8	15221583	Expressed protein	2.82	2.77
117	32.43/6.3	21553514	Unknown protein	2.25	3.54
119	41.71/6.6	15237530	GDSL motif lipase/hydrolase–like protein	3.04	3.03
143	20.81/5.7	15226149	Athila retroelement open reading frame1–like protein	2.04	1.06
148	37.74/6.1	15228497	Esterase/lipase/thioesterase, active site	1.79	2.00
149	32.32/5.4	15234160	Expressed protein	1.34	2.19
150	25.50/6.0	15239633	Unknown protein	3.59	2.31
152	18.56/5.4	15223328	Hypothetical protein	0.36	0.49
153	18.56/5.4	15223328	Hypothetical protein	0.18	0.11

[a] Number of the spot.
[b] Predicted molecular mass (MM) and pI of the matched sequence.
[c] Accession number in the National Center for Biotechnology Information nonredundant database.
[d] Entry name according to the National Center for Biotechnology Information database.
[e] Fold change in protein expression upon SA treatment as calculated by ImageMaster analysis software.

The identified protein spots corresponded to 13 different proteins, as some were present in multiple spots, which suggests that they undergo posttranslational modifications. Of the 13 proteins, 3 had protein names or known functions but 10 were novel or unknown proteins in the *Arabidopsis* genome databases. These unknown proteins were then scanned against Prosite (http://ca.expasy.org/tools/#pattern), a database of protein families and domains, and additionally assigned functional motifs (i.e., a jacalin-related protein, a Leu-rich repeat, and the active site of esterase/lipase/thioesterase; Table 2).

Sequence Comparison of GLIP1 with Other GLIPs

A GDSL motif lipase/hydrolase–like protein (spot 119), designated GLIP1, was selected for further analysis because plant-derived secreted lipases have not been implicated previously in the pathogen response. In a BLAST search of the *Arabidopsis* genome database, six additional *GLIP* genes were identified and designated *GLIP2* to *GLIP7* (Figure 3). A comparison of the amino acid sequences reveals that GLIP1 is 68 to 78% identical to GLIP2, GLIP3, and GLIP4, whereas its similarity to the other GLIPs is lower (32 to 52% identical to GLIP5, GLIP6, and GLIP7). The GLIPs represent a novel lipase gene family with a GDSL-like motif (Brick et al., 1995) and a signal peptide as predicted by SignalP (Nielsen et al., 1997). All seven GLIPs contain a catalytic triad of Ser, Asp, and His conserved among the lipase and esterase families. Another characteristic feature of the GLIPs is that the classical lipase signature sequence GxSxG is replaced by GxSxxxxG, which overlaps with the GDSL motif (Brick et al., 1995).

Subcellular Localization of GLIP1

To investigate the subcellular localization of GLIP1, we fused soluble, modified green fluorescent protein (smGFP) to GLIP1 expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The smGFP fusion constructs of full-length GLIP1, GLIP1 lacking the signal peptide (GLIP1ΔSP), and the signal peptide alone (SP) were introduced into onion (*Allium cepa*) epidermal cells. After incubation for 24 h, fluorescence from smGFP was examined by fluorescence microscopy (Figure 4). The smGFP control and GLIP1ΔSP were expressed in the cytosol. However, GLIP1 and SP fluorescence signals were clearly located in the intercellular space, indicating that the signal sequence leads to the secretion of GLIP1. The fluorescence of GLIP1 appeared to be confined in the intercellular space, unlike the diffuse pattern of SP. The intercellular localization was clarified in the presence of mannitol, which induces plasmolysis, pulling the cytosol away from the cell wall, and thus allowing the two to be distinguished from one another (Figure 4E). These results are consistent with the proteomic data demonstrating that GLIP1 is secreted into the cell wall or extracellular space.

Isolation of *GLIP1* T-DNA Insertion Mutants

To assess the in vivo function of GLIP1, we searched the Salk Institute insertion sequence database for T-DNA insertion mutants and obtained two alleles, *glip1-1* and *glip1-2*, in which the T-DNA inserts are in the fifth exon and second intron, respectively (Figure 5A). The mutant seeds were originally heterozygous

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GLIP1   --------MENSQL--VSITFLAYTIIISIGSINCIDNNN--LVTNQSALFVFGDSVFDA    48
GLIP2   --------MENSRS--TLIIFFAYTTIILIGSINCRDNNNNNLVTNQSALFVFGDSVFDA    50
GLIP3   ----------MVRL--VLIIFFVYTIILSIGSINCIDNNN--LVTNQAALFVFGDSLFDA    46
GLIP4   --------MASPRFNSIIIILFICTISLSIVSISCKEDLK----TNQAALFAFGDSLFEA    48
GLIP5   MRESTLMEKVTRRTISSFIFFIVSSTILFLAGKSSAKISHN-GDNNVTALFLFGDSELDA    59
GLIP6   ------------MSSSSSMDLLMCLLLLISPVVLAKSSS-----TVPAIFTFGDSIFDA    42
GLIP7   --------------MKSLLICLVLLELVWLGNGQSRDHQP-----LAPAFFVFGDSIVDS    41
                         :     : :       .       .  .*:*.****..::

GLIP1   GNNNYIDTLSSVRSNYWPYGQTTFKSPTGRVSDGRLIPDFIAEYAWLPLIPPNLQP----   104
GLIP2   GNNNYIDTLPSFRSNYWPYGQTTFKFPTGRVSDGRTIPDFIAEYAWLPLIPAYLQP----   106
GLIP3   GNNNYINTVSSFRSNIWPYGQTNFKFPTGRLSDG-------PEKAWLPSIPPNLQP----    95
GLIP4   GNNNYFDSISSFRSNFWPYGKTTFKFPTGRVSDGRIMIDFIAEYAWLPLIPPNLQP----   104
GLIP5   GNNNYINTTTLDQANFPPYGQTFFGLPTGRFSDGRLISDFIAEYANLPLIPPFLEP----   115
GLIP6   GNNHYNKN-CTAQADFPPYGSSFFHRPTGRFTNGRTVADFISEFVGLPLQKPFLELQIQI   101
GLIP7   GNNNYIP--TLARANYFPYG-IDFGFPTGRFCNGRTVVDYGATYLGLPLVPPYLSP----    94
        ***:*   .   :::  ***.  *  ****. :*     .    .  **    . *.

GLIP1   FNGNSQFAYGVNFASGGAGALVGTFSGLVIN----LRTQLNNFKKVEEMLRSKLGD--AE   158
GLIP2   SNGKNQFPYGVSFASAGAGALVGTFPGMVIN----LKSQLNNFKKVEKLLRSTLGE--AQ   160
GLIP3   NNGNNQFTYGVSFASAGAGALAESFLGMVIN----LGTQLNNFKDVEKSLRSELGD--AE   149
GLIP4   GYSNSQLTYGLNFATTAAGVFAGTFPGSVTNLSKDLGTQLNNFKNVEKTLRSNLGD--AE   162
GLIP5   GNSQKKL-YGVNFASAGAGALVETFQGSVIN----LRTQLDHYKKVERLWRTNFGK--EE   168
GLIP6   LNGTSNFSNGINFASAGSGLLLDTNK--FMG-VTPIQTQLQQFQTLVEQNLI--------   150
GLIP7   LSIGQNALRGVNYASAAAGILDETGR--HYGARTTFNGQISQFEITIELRLRRFFQNPAD   152
          .:    *:.:*: ..:*  :   :   .    .    :   *:.:::    .     . .

GLIP1   GKRVISRAVYLFHIGLNDYQYPFTTNSS--LFQSISNEKYVDYVVGNMTDVFKEVYNLGG   216
GLIP2   GKMVISRAVYLFHIGVNDYQYPFSTNSS--IFQSSPQEIYVDFVVGNTTAVIKEVYKIGG   218
GLIP3   TKRVFSRAVYLFHIGANDYFYPFSANSS--TFKSNSKEKFVDFVIGNITFVIEEVYKMGG   207
GLIP4   ARRVISKAVYLFHIGANDYQYPFFANTS--TFSNTTKERFIDFVIGNTTTVIEELYKLGA   220
GLIP5   SKKRISRAVYLISIGSNDYSSIFLTNQS--LPISMS--QHVDIVIGNLTTFIHEIYKIGG   224
GLIP6   EKSIIQESLFLLETGSNDIFN-YFLPFR---APTLSPDAYVNAMLDQVNKTIDQIYKLGA   206
GLIP7   LRKYLAKSIIGINIGSNDYINNYLMPERYSTSQTYSGEDYADLLIKTLSAQISRLYNLGA   212
         :    :  .::    :  *  **     :           . . . .  :  ::    .   : ..:*::*.

GLIP1   RKFGILNTGPYDCAP-ASLVIDQTKIRSCFQPVTELINMHNEKLLNGLRRLNHELSGFKY   275
GLIP2   RKFGFLNMGAYDCAP-ASLIIDQTKIGTCFKPVTELINLHNEKLESGLRRLERELSGFKY   277
GLIP3   RKFGFLNVGPYECSP-NSLIRDRTKIGSCFKPVAELIDMHNKKFPDVLRRLQRQLSGFRY   266
GLIP4   RKFGFLSLGPFGCTP-SALIINSTKIGSCFEPVTELINLHNQEFPKVLRRLERRLSGFKY   279
GLIP5   RKFGFLNVPDLGCFP-ALRILQPKNDDSCLRDASRLASMHNRALTNLLFQMQRQVKGFKF   283
GLIP6   RRIAFFSLGPVGCVP-ARAMLPNAPTNKCFGKMNVMAKMYNKRLEDIVNIIPTKYPGAIA   265
GLIP7   RKMVIAGSGPLGCIPSQLSMVTGNNTSGCVTKINNMVSMFNSRLKDLANTLNTTLPGSFF   272
         *:: :   .      * *    :           *.     : .:.*   :.     :     *

GLIP1   ALHDYHTSLSERMNDPSKYGFKEGKKACCGSGPLRGINTCGGRMGLSQSYELCENVTDYL   335
GLIP2   ALHDYHTSLSVRMNNPSKYGFKEGKMACCGTGPLRGINTCGGRMGVSQSYELCEKVTDYL   337
GLIP3   ALHDYHTSLSERINSPSKYGFKEGKKACCGSGPLRGINTCGNRIGPSQGYGLCENVTDYL   326
GLIP4   ALHDFHTSLSQRINNPSRYGFKEGEMACCGSGPLRGINTCGFRNGPSQGYKLCENADDYV   339
GLIP5   SLFDMNKSLRLRMQHPSKFGFKEGEEACCGTGKWRGVFSCGGK-RIVKEYQLCENPKDYI   342
GLIP6   VFGAVYGITHRFQTYPARYGFSDVSNACCGNGTLGGLMQCGRE-----GYKICNNPNEFL   320
GLIP7   VYQNVFDLFHDMVVNPSRYGLVVSNEACCGNGRYGGALTCLP------LQQPCLDRNQYV   326
                 *:::*:   . ****.*    *   *         .   * . :::

GLIP1   FFDPFHLTEKANRQIAELIWSGPTN----------ITGPY--------------------   365
GLIP2   FFDHFHLTEKAHQQIAELIWSGPTNGLRKGIRHVCGTGPLRGINTCGGRVSAQVQGYELC   397
GLIP3   FYDSSHLTEKAHRQIAELIWNGPPN----------VTRPY--------------------   356
GLIP4   FFDPSHLTETAHQQIAELIWSGPPN----------VTAPY--------------------   369
GLIP5   FWDSLHLTQNTYNQFANLIWNGGHMS------DSLVVGPY--------------------   376
GLIP6   FWDFYHPTEHTYRLMSKALWNGNKN----HIRPF-------------------------   350
GLIP7   FWDAFHPTETANKIIAHNTFSKSAN----YSYPI-------------------------   356
        *:*  * *:  : . ::.  :.                .

GLIP1   -NLKALFELN-------------------------   374
GLIP2   ENVADYLFLNSFHLIEKAHRQTTDRRDYLERTGQCYRTL   436
GLIP3   -NLKALFELRLT--------------------------   367
GLIP4   -NLKTLFRL-----------------------------   377
GLIP5   -NINNLFQIP----------------------------   385
GLIP6   -NLMALATNKITF-------------------------   362
GLIP7   -SVYELAKL-----------------------------   364
         .:
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Figure 3. Alignment of the Deduced Amino Acid Sequence of *GLIP1* with Those of Other *GLIP*s.

Sequences were aligned using ClustalW. The signal sequences are shown in boldface at the N termini, and the residues of the catalytic triad (Ser, Asp, and His) are indicated in boldface and by arrows. The lipase signature sequence is shown by the solid line, and the GDSL-like motif is boxed. Consensus symbols under the alignment are as follows: asterisks indicate identical residues in all sequences; colons and periods indicate conserved and semiconserved substitutions, respectively.
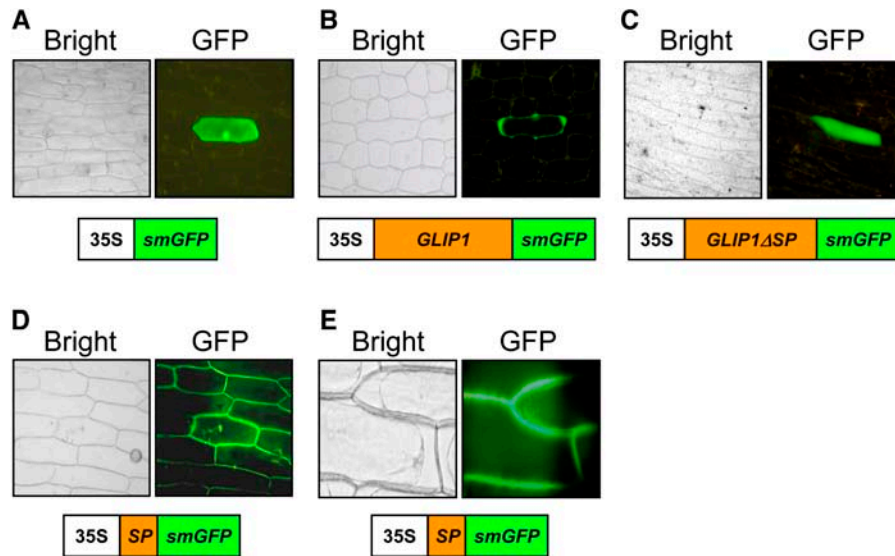
Figure 4. Subcellular Localization of GLIP1 Proteins.

GFP fusion constructs were introduced into onion epidermal cells by particle bombardment. The expression of the fusion proteins was driven by the CaMV 35S promoter. Bright field (left) and fluorescence (right) images are shown. Schemes of the fusion constructs are shown at the bottom of each panel: 35S, CaMV 35S promoter.
(A) Expression of a GFP control.
(B) Expression of the full-length GLIP1-GFP fusion protein.
(C) Expression of the GLIP1ΔSP-GFP fusion protein.
(D) Expression of the SP-GFP fusion protein.
(E) Expression of SP-GFP in the presence of 1 M mannitol. Plasmolysis was induced in cells that express GFP fusion proteins.

for a single T-DNA insertion with a 3:1 segregation ratio. Homozygous lines were identified in the next generation. To determine the exact position of the T-DNA insertion, genomic DNA fragments of the *GLIP1* gene and T-DNA junction were amplified and sequenced (Figure 5A). DNA gel blot analysis determined that these lines contained a single T-DNA insertion (Figure 5B). It was also demonstrated that the homozygous mutants did not express *GLIP1* transcripts (Figure 5C). Under normal growth conditions, *glip1* plants showed growth similar to Col-0 (data not shown).

Function of *GLIP1* in Disease Resistance

Because GLIP1 was isolated in a screen for SA-responsive secreted proteins, it was first assessed whether *glip1* mutants exhibit altered responses to infection by the bacterial pathogen *Pseudomonas syringae*, which is closely associated with SA signaling (Crute et al., 1994). The bacterial growth and disease symptom development after inoculation with virulent *Pseudomonas syringae* pv *tomato* DC3000 (Pst DC3000) did not differ significantly between Col-0 and *glip1* plants (Figure 6D). The development of systemic acquired resistance (SAR) was also observed in both Col-0 and *glip1* plants, as examined by treatment of primary leaves with avirulent Pst DC3000 carrying *avrRpt2* [Pst DC3000 (*avrRpt2*)] before challenge of secondary leaves with Pst DC3000 (Figure 6D). These results

suggest that GLIP1 is not involved in resistance to *P. syringae* in *Arabidopsis*.

We then examined the resistance phenotypes of *glip1-1*, *glip1-2*, Col-0, and a different Columbia ecotype, Col-6, after inoculation with the necrotrophic fungus *A. brassicicola*. In the infection cycle, spore germination is followed by the extension of the germ tube over the leaf surface. At the tip of hyphae, fungi develop appressoria for direct penetration into the host and eventually colonize the tissue (McRoberts and Lennard, 1996). Col-0 and Col-6 were used as natural wild-type ecotypes that are resistant and susceptible, respectively, to *A. brassicicola* (Kagan and Hammerschmidt, 2002). It was previously shown that inoculation of Col-0 wild-type plants with *A. brassicicola* induces the hypersensitive response, resulting in small necrotic lesions restricted to the infection sites (Thomma et al., 1998, 1999). We also observed that Col-0 inoculated with *A. brassicicola* formed small brown necrotic lesions (Figure 6A). By contrast, the *glip1-1*, *glip1-2*, and Col-6 plants developed spreading lesions upon inoculation (Figures 6A and 6C). As visualized by lactophenol–aniline blue staining, those spreading lesions were heavily colonized by fungal hyphae (Figure 6B). Furthermore, determination of spore number produced de novo at the inoculation site revealed that new spores were abundantly produced on *glip1* and Col-6 leaves, unlike Col-0, on which few, if any, new spores were formed (Figure 6C). No difference between *glip1-1* and *glip1-2* was observed in disease resistance, indicating that the
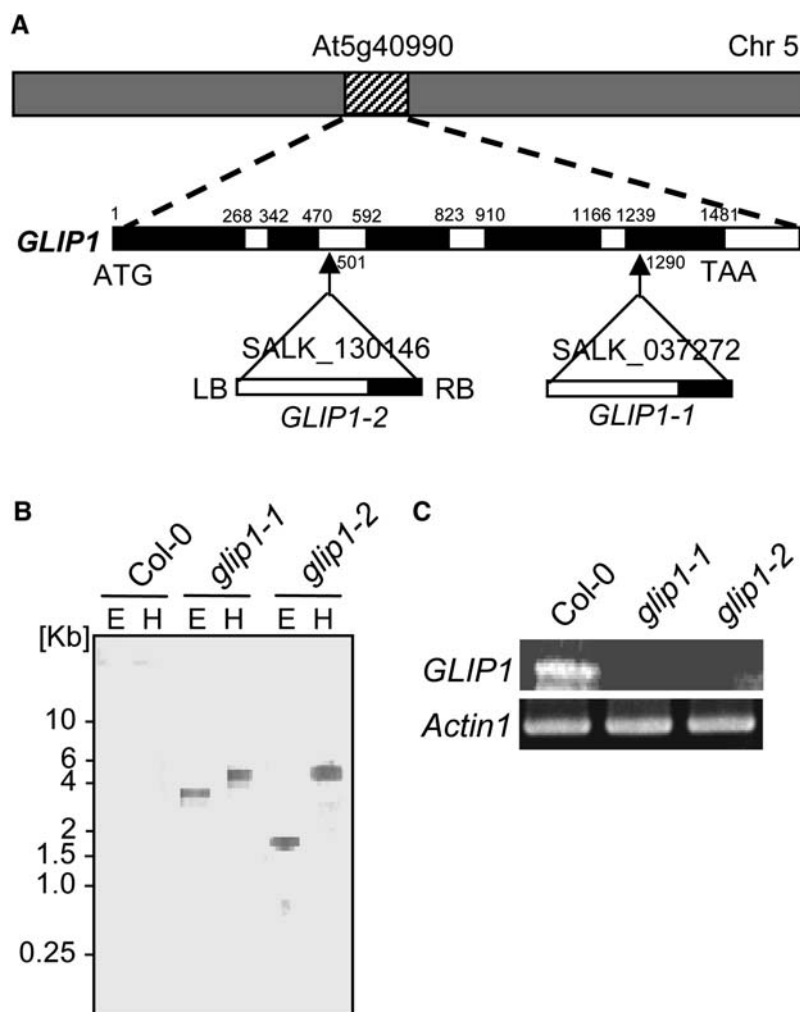
Figure 5. T-DNA Insertion Mutants of *GLIP1*.

(A) Genomic organization of *GLIP1* and location of the T-DNA insertion in the *glip1* mutants. The arrows indicate the positions of the T-DNA insertions (triangles). Genomic *GLIP1* DNA is represented by exons (black) and introns (white). The T-DNA orientation is indicated by left (LB) and right (RB) borders. The numbers refer to nucleotides in the genomic *GLIP1* DNA. Chr 5, chromosome 5.
(B) DNA gel blot analysis of the T-DNA insertion in Col-0 and *glip1* plants. The genomic DNA was digested with *Eco*RI (E) and *Hin*dIII (H), and the blot was probed with the T-DNA left border–specific DNA.
(C) RNA analysis of *GLIP1* gene expression in Col-0 and *glip1* plants. *GLIP1* expression was analyzed by RT-PCR. Total RNA (0.1 μg) was used to detect *GLIP1* and *Actin1* (loading control) expression.

observed phenotypes are the consequence of a T-DNA insertion into the *GLIP1* gene.

Antimicrobial Activity of Recombinant GLIP1 Proteins

To determine whether GLIP1 has a lipase activity that is required for resistance to *A. brassicicola*, the recombinant GLIP1 protein was expressed in *Escherichia coli* (Figure 7A). Along with wild-type protein, we prepared a GLIP1 mutant whose Ser-44 of the catalytic triad was replaced by Ala (S44A). These proteins were assayed for lipase activity using *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate as substrates (Figure 7B). The wild-type GLIP1 exhibited high activity, but the GLIP1(S44A) mutant did

not, demonstrating that GLIP1 is a lipase and that Ser-44 in the catalytic triad participates in the hydrolytic activity.

Because GLIP1 is a secreted protein, it is conceivable that GLIP1 directly inhibits the pathogenic activity of *A. brassicicola*. The antimicrobial activity of GLIP1 against *A. brassicicola* was thus assessed. When fungal spores were incubated with recombinant wild-type and mutant proteins, spore germination was inhibited by the active form of GLIP1 (Figures 8A and 8B). GLIP1(S44A) had little effect on the fungal growth. Furthermore, scanning electron microscopy showed that GLIP1 caused severe morphological changes in the fungal spores (Figure 8C). These results suggest that GLIP1 may directly disrupt the structure of the fungal cell wall and/or membrane in a specific manner.
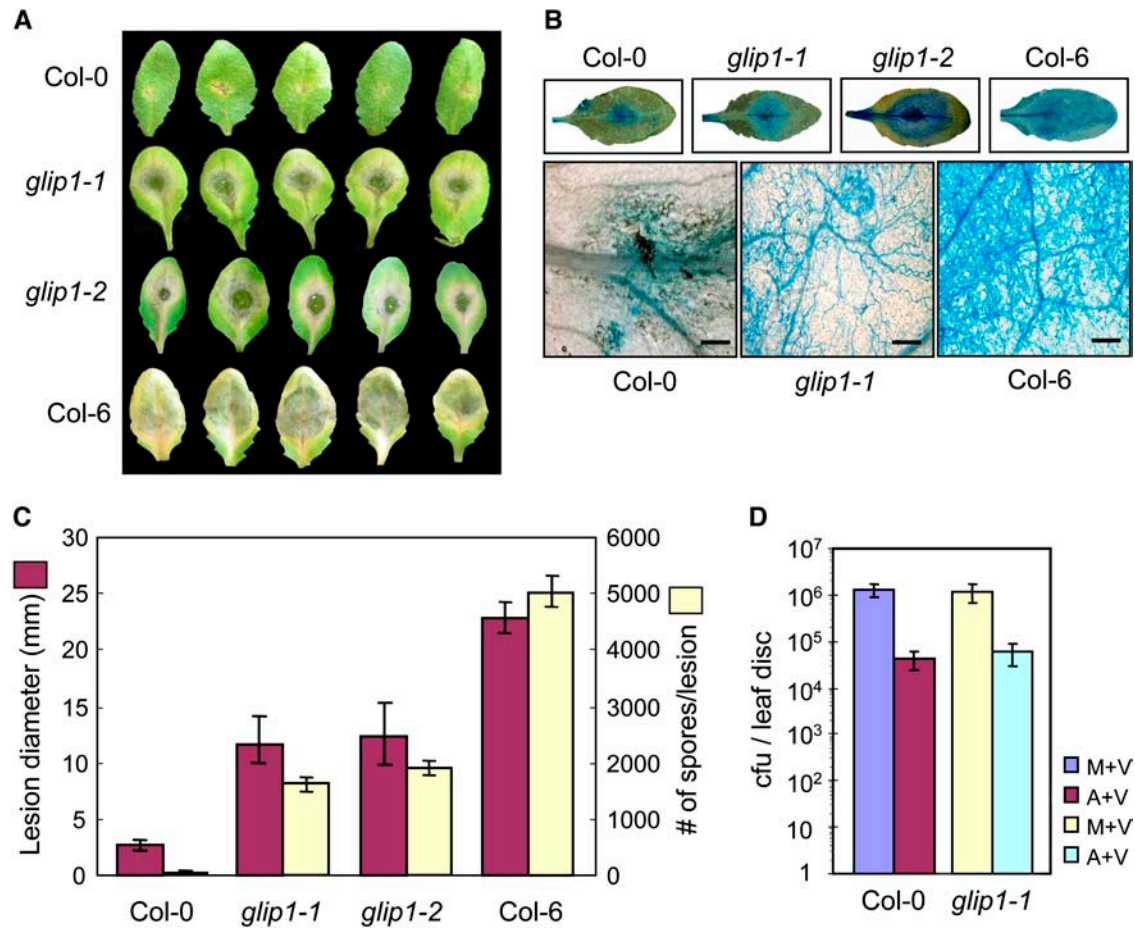
Figure 6. Disease Development in *glip1* Mutants Inoculated with *P. syringae* and *A. brassicicola*.

Four-week-old plants were challenged with either 20 μL of *P. syringae* bacterial suspensions (10^6 colony-forming units [cfu]/mL) for both primary and secondary inoculations **(D)** or 10 μL of fungal spore suspensions (5×10^5 spores/mL) (**[A]** to **[C]**). The inoculated leaves were detached 4 d after inoculation for analysis. This experiment was repeated three times with similar results.

(A) Necrotic lesion phenotypes.
(B) Necrotic lesions stained with lactophenol–aniline blue. The formation of fungal mycelium on *glip1* and Col-6 leaves was visualized with a microscope. Bars = 25 μm.
(C) Measurement of lesion diameter and number of newly formed spores. The bars and error bars represent averages and SD, respectively, of three independent experiments each with 10 leaves.
(D) Bacterial growth determined in secondary leaves challenged with Pst DC3000 1 d after the inoculation of primary leaves with either 10 mM MgCl$_2$ or Pst DC3000 (*avrRpt2*). M+V, primary and secondary inoculations with MgCl$_2$ and Pst DC3000, respectively; A+V, primary and secondary inoculations with Pst DC3000 (*avrRpt2*) and Pst DC3000, respectively. The bars and error bars represent averages and SD, respectively, of three independent experiments.

Function of GLIP1 in Systemic Defense Response

When inoculated with *A. brassicicola*, Col-0 activates the SAR response that leads to the induction of defense-related genes such as *PDF1.2*, *PR-3*, and *PR-4* (Penninckx et al., 1996; Thomma et al., 1998). We thus investigated whether GLIP1 is implicated in local and systemic defense responses that are induced in plants by pathogen challenge. When the leaves of *A. brassicicola*–challenged plants were stained with trypan blue, extensive cell death associated with the systemic spreading of this fungus was observed in both the peripheral and distant leaves in *glip1-1* but not in Col-0 (Figure 9A). In Col-0 with SAR

induced, cell death was restricted to the inoculated site. Therefore, the expression of SAR could be determined by monitoring the presence of trypan blue–stained cells. The *glip1-1* plants were pretreated with recombinant GLIP1 protein, followed by localized *A. brassicicola* inoculation (1°p) (Figure 9B). This localized inoculation on the pretreated leaves (1°p) was then followed by the application of *A. brassicicola* to the other distal leaves (1°). There was a significant decrease in both local (1°p) and systemic (1° and 2°) cell death, reflecting the induction of SAR in leaves remote to the infection (Figure 9B). It was noticeable that pretreatment of *glip1-1* with GLIP1 protein in local
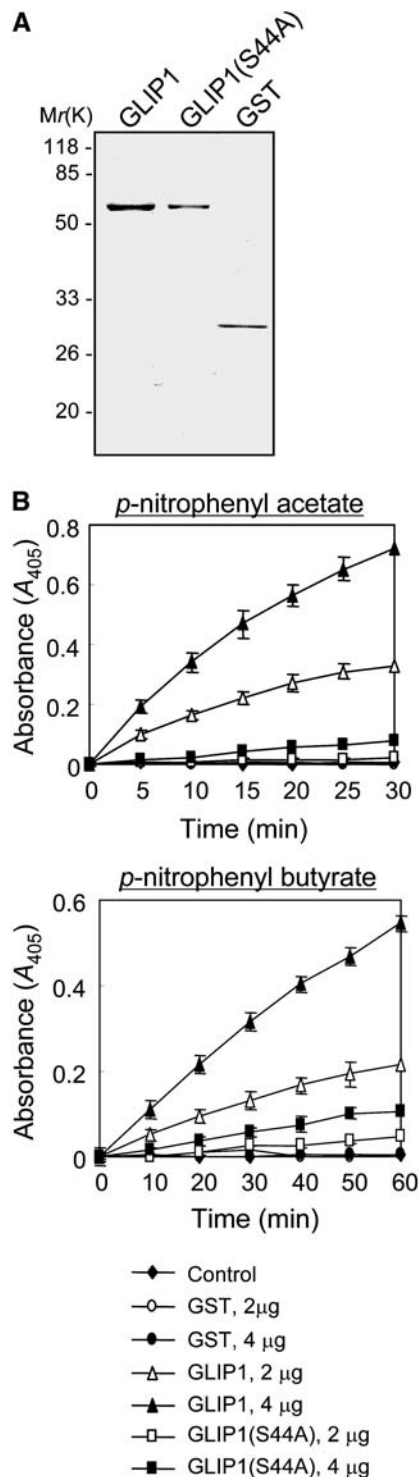
Figure 7. Lipase Activity of Recombinant GLIP1 Proteins.

(A) Coomassie blue–stained gel of purified recombinant GST-GLIP1 proteins.

(B) Lipase activities of the recombinant GLIP1 and GLIP1(S44A) proteins and the GST control. In the assays, 2 μg (open symbols) and 4 μg (closed symbols) of proteins were incubated with two substrates, p-nitrophenyl acetate and p-nitrophenyl butyrate. Each data point represents the average and SD of five independent measurements.

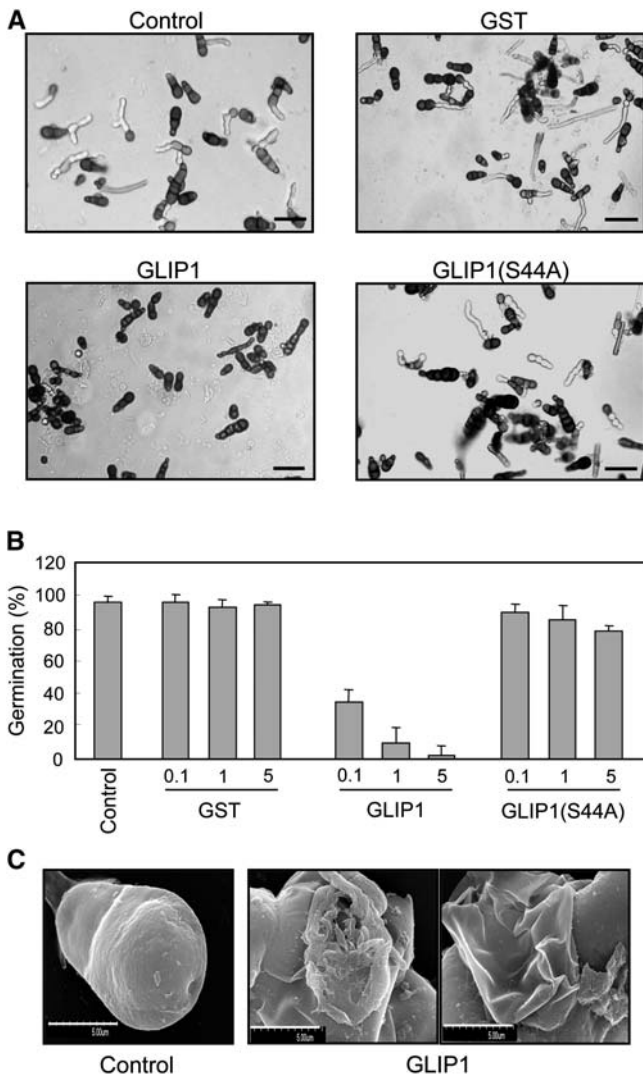leaves (1°p) systemically inhibited cell death in distant leaves (1°) inoculated with *A. brassicicola*, in which cell death would be otherwise strongly induced. By contrast, pretreatment with GLIP1 in the absence of localized *A. brassicicola* inoculation (p) failed to inhibit cell death in both 1° and 2° leaves (Figure 9B, right). Here, primary (1°) and secondary (2°) indicate inoculated and uninoculated leaves, respectively. These data suggest that GLIP1 activates systemic resistance signaling only when challenged with *A. brassicicola*.

Signaling molecules such as SA, jasmonic acid (JA), and ethylene accumulate during defense and, in turn, trigger the coordinated expression of defense genes. Resistance to *A. brassicicola* involves the cooperative actions of JA and ethylene (Dong, 1998; Penninckx et al., 1998). *PDF1.2*, *PR-3*, and *PR-4* require JA and ethylene for activation, whereas *PR-1*, *PR-2*, and *PR-5* are induced by SA (Kunkel and Brooks, 2002). In addition, many genes (e.g., *ERF1*, *EIN2*, and *EIN3*) have been identified to function in ethylene signaling (Wang et al., 2002). We thus analyzed the expression levels of these genes, including *GLIP1*, in Col-0 and *glip1-1* upon *A. brassicicola* treatment. *GLIP1* transcription was strongly induced in Col-0 (Figure 9C). Whereas *PR* gene expression did not differ, *PDF1.2* was less induced in *glip1-1*. Furthermore, the basal RNA levels of the SA-dependent *PR* genes, *PR-1*, *PR-2*, and *PR-5*, were much higher in *glip1-1* than in Col-0. Of the ethylene-related genes, *EIN2* and *EIN3* also exhibited altered expression in *glip1-1*. The expression of *EIN2* was repressed in Col-0 but somewhat induced in *glip1-1* in response to *A. brassicicola*. An increase of *EIN3* in Col-0 was reversed in *glip1-1* with reduced expression. These results suggest that *glip1* may be defective in ethylene signaling. In agreement with this, the high basal expression level of SA-related *PR* genes in *glip1-1* is consistent with the suggestions that the SA and ethylene pathways interact in an antagonistic manner (Wang et al., 2002). Although *A. brassicicola* infection induces *PR-1*, it has been shown that the resistance to this pathogen is dependent on the JA/ethylene signaling pathway (Penninckx et al., 1996). Noticeably, *GLIP1* was strongly induced by ethephon over 24 h (Figure 9D). A slight increase in *GLIP1* expression was observed upon treatment with SA and JA. These results demonstrate that the increase of GLIP1 protein in the secretome is regulated posttranslationally (i.e., by secretion) and/or transcriptionally. Together, these results suggest that GLIP1 may be a critical component in the resistance response in association with ethylene signaling.

DISCUSSION

In this work, we present a comprehensive analysis of the *Arabidopsis* secretome in the medium of cultured cells. Subsequently, to identify secreted proteins that participate in plant defense, we measured increased or decreased protein accumulation in the secretome in response to SA treatment. This revealed several intriguing proteins. In particular, our studies

Proteomic Analysis of Secreted Proteins

In our analysis of the secretome, we focused on the culture medium, which would contain diffusible nonsticky proteins in the cell wall. We were able to isolate and identify proteins involved in a variety of cellular functions, such as signaling and regulation, catalysis, protein degradation, protein protection, and structure. These results indicate that complicated biological events take place in the extracellular environment. The purity of the isolated secretome was evaluated by immunoblotting with antibodies for proteins in different subcellular compartments. Two cytoplasmic proteins, GST and cyclin D, the chloroplast protein OEE2, and the cytosol and membrane protein AnnAt1 were not detected in the secretome, although they were found in the medium-free callus. By contrast, the secreted protein myrosinase was mostly detected in the secretome. The cytoplasmic contamination of the secreted fraction was also assessed by enzyme assays. These data together indicated that the secretome prepared from the culture medium was enriched in secreted proteins with no detectable contamination.

SignalP analysis predicted that 54% of the secreted proteins possess a signal peptide that would target them for secretion. The rest of the proteins lack an identifiable signal peptide, suggesting the presence of nonclassical endoplasmic reticulum– and Golgi-independent secretory pathways. Consistent with this finding, several proteins lacking a signal sequence in bacteria and yeast are secreted through an alternative secretory machinery such as ATP binding cassette transporters (Kuchler, 1993). Accumulating evidence also suggests that eukaryotic cells secrete proteins lacking signal peptides, including interleukins (MacKenzie et al., 2001), fibroblast growth factors (Bryckaert et al., 2000), annexin II (Siever and Erickson, 1997), and galectins (Barondes et al., 1994). These proteins are extracellularly trans- located by the alternative secretory pathways, which may also involve ATP binding cassette transporters (Lottaz et al., 2001). Moreover, research in the last few years has revealed the cell-to-cell movement of plant regulatory proteins through plasmo- desmata as a novel mechanism that controls developmental processes (Heinlein, 2002; Oparka, 2004). Those non-cell- autonomous proteins include transcription factors such as LEAFY (Sessions et al., 2000), KNOTTED1 (Lucas et al., 1995), and SHORTROOT (Nakajima et al., 2001). In addition, a wide range of molecules are released into the phloem through plasmodesmata for long-distance transport, suggesting the apoplastic movement of these molecules (Oparka and Cruz, 2000). Such alternative secretory processes may facilitate the extracellular secretion of proteins that lack a classical signal sequence.

ECM Proteins in Defense Response

In our search for secreted proteins involved in the defense process, we isolated 13 proteins whose levels are changed in the secretome by SA treatment. Of the eight SA-induced proteins, four share sequences with known proteins. Jacalin-related proteins are known to be inducible defense proteins (Mann et al., 2001). They share the domain structure of plant lectins and are upregulated by several low molecular mass regulators, such
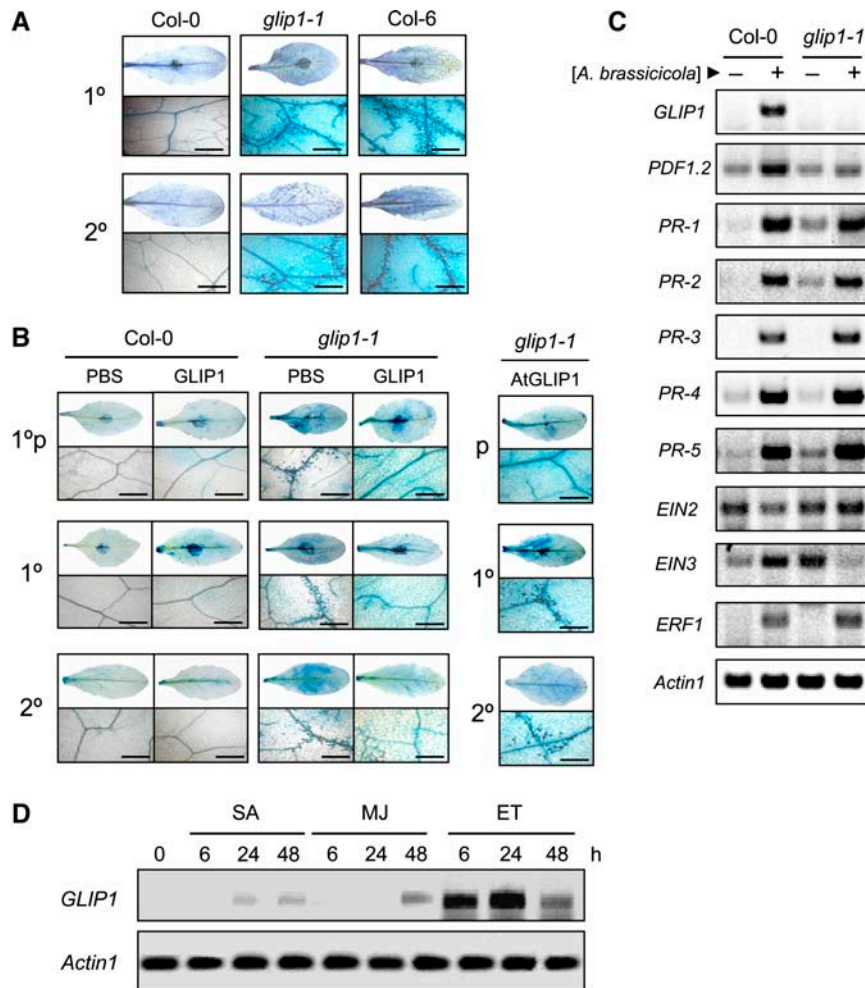

Figure 8. Antimicrobial Activity of Recombinant GLIP1 Proteins against *A. brassicicola*.

Recombinant proteins were added to 10 μL of spore suspension (5 × 10⁵ spores/mL). The samples were incubated for 24 h at 23°C and observed with a microscope. PBS was used as a mock control.
(A) The inhibitory effect of recombinant proteins (5 μg) on the germination of fungal spores as observed by light microscopy. Bars = 50 μm.
(B) Quantitative analysis of germination rates. The percentage of germinated spores shown in **(A)** was determined by light microscopy. Bars and error bars represent averages and SD, respectively, of three independent experiments.
(C) Scanning electron microscopy images of fungal spores incubated with GLIP1 proteins (5 μg). Five spores for each condition were observed with similar results. PBS was used as a mock control. Bars = 500 μm.

highlight the functional importance of a secreted lipase, GLIP1, in the defense response against *A. brassicicola*. These results suggest that GLIP1 protects plants from *A. brassicicola* attack in two distinct ways: by directly disrupting fungal spore integrity, and by activating defense signaling in plants.

Figure 9. Function of GLIP1 in the Systemic Defense Response.

Four-week-old plants were inoculated with a 10-μL drop of *A. brassicicola* spore suspension (5 × 10^5 spores/mL). In **(A)** and **(B)**, primary (1°) and secondary (2°) indicate inoculated and uninoculated leaves, respectively. The term 1°p indicates local leaves that were pretreated with PBS or GLIP1 proteins and then inoculated with *A. brassicicola*. Bars = 50 μm. High magnification (×400) micrographs at the bottom show cell death at a distance from the inoculation site in the case of the primary leaves (1°p and 1°). This experiment (**[A]** to **[D]**) was repeated more than three times with similar results.

(A) Cell death, as shown by staining with lactophenol–trypan blue. The staining of primary and secondary leaves was performed 4 d after the primary leaves were treated with *A. brassicicola*.

(B) Inhibition of the *A. brassicicola*–induced local and systemic cell death in the *glip1-1* mutant by pretreatment with GLIP1 proteins. For the pretreatment, plants were infiltrated into one site on the abaxial surface of each leaf with PBS (control) or GLIP1 proteins (1 μg) as indicated, incubated for 1 d, and then inoculated with *A. brassicicola*. This localized inoculation on the pretreated leaves (1°p) was followed by the application of *A. brassicicola* to the other distal leaves 1 d later. As a control (right), leaves were only pretreated with GLIP1 proteins (p) without local inoculation of *A. brassicicola*. Cell death was monitored by trypan blue staining over 4 d.

(C) Induction of pathogenesis- and ethylene-related genes in Col-0 and *glip1-1* plants infected with *A. brassicicola*. Four-week-old plants were inoculated with a 10-μL drop of spore suspension (5 × 10^5 spores/mL), harvested 1 d after treatment, and used to extract total RNA. RNA levels were determined by RT-PCR analysis. *Actin1* was used as a control.

(D) RNA analysis of the induction of *GLIP1* in response to hormone treatments. SA (1 mM), methyl jasmonate (MJ; 50 μM), and the ethylene releaser ethephon (ET; 1.5 mM) were dissolved in water and applied to 4-week-old Col-0 plants. Total RNAs were isolated at the indicated times after treatment, and RNA levels were determined by RT-PCR analysis. *Actin1* was used as a control.

as JA, SA, and ethylene, or by altered ambient conditions, such as salt stress (Zhang et al., 2000). Another enhanced secreted protein is an Athila retroelement open reading frame1 (ORF1)–like protein. The Athila retroelement is a Ty3-*gypsy* group retrotransposon with an *envelope*-like ORF (Wright and Voytas, 1998). Plant retrotransposons are activated by stresses, including wounding, pathogen attack, freezing, UV light, JA, and SA (Kimura et al., 2001). This may be either a survival strategy or a stress-induced generator of genomic diversity (Wessler, 1996).

The other two secreted proteins with increased expression have sequence similarity to lipases or esterases but belong to different gene families, such as the GDSL motif lipase and Ser hydrolase families (eukaryotic lipases and esterases). That lipases are implicated in disease resistance in plants has been reported previously. Mutational analyses of *Arabidopsis* to screen for genes required for *R* gene–mediated resistance or SAR identified two lipase-encoding genes, enhanced disease susceptibility1 (*EDS1*) and phytoalexin deficient4 (*PAD4*), that have been well characterized (Falk et al., 1999; Jirage et al., 1999). Mutations in both *EDS1* and *PAD4* suppressed disease resistance conferred by R proteins. EDS1 functions upstream of SA-dependent *PR1* mRNA accumulation and is not required for JA-induced *PDF1.2* expression (Falk et al., 1999). PAD4 also participates in a positive regulatory loop that increases SA levels, thus activating SA-dependent defense responses (Jirage et al., 1999). A recent study demonstrated that SA binding protein2 (SABP2) in tobacco binds SA with high affinity and belongs to the α/β-Ser hydrolase superfamily (Kumar and Klessig, 2003). SABP2 retains SA-stimulated lipase activity and is required for full local and systemic resistance to pathogen infection. In a differential screening study, a different lipase gene family of *Arabidopsis* was also identified (Jakab et al., 2003). It contains nine genes encoding pathogenesis-related lipase-like proteins (PRLIP1 to PRLIP9), which are induced by treatment with SA, JA, or ethylene. All of these lipases, including EDS1 and PAD4 in *Arabidopsis* and tobacco SABP2, belong to the α/β-Ser hydrolase superfamily. They share little sequence homology but contain the catalytic triad (Ser, Asp, His) and the lipase signature sequence GxSxG. The two lipases that we isolated in this work also contain the catalytic triad but have different features (i.e., the GxSxxxxG signature sequence instead of GxSxG and a signal sequence at the N terminus). Thus, these two proteins are members of distinct and unique families of secreted lipases.

Secreted Lipases in Pathogen Response

Of the two putative secreted lipases we isolated, we further characterized the GDSL motif lipase/hydrolase GLIP1. It possesses a typical signal peptide at its N terminus, which allowed secretion to the cell wall, as verified by expression analysis. Analysis of the *Arabidopsis* genomic database revealed six more proteins that share 50 to 85% similarity (32 to 78% identity) to the sequence of GLIP1, and all have a signal sequence as predicted by SignalP.

The extracellular localization of GLIP1 led to the notion that it may directly inhibit fungal infection and/or produce a lipid-derived defense signaling molecule. Indeed, we found that fungal spores were disrupted when treated with recombinant GLIP1 protein, suggesting that GLIP1 directly interferes with the fungal infection process. It has been reported previously that fungal plant pathogens secrete hydrolyzing enzymes such as cutinases or esterases to degrade components of the plant cell wall during their invasion of the host. Moreover, extracellular lipases have been considered to be virulence factors of pathogenic bacterial and fungal species, including *A. brassicicola* (Berto et al., 1999; Eddine et al., 2001). We show here that the fungal spores may actually be counterattacked by the plant-derived lipase GLIP1. Fungal cells have significant internal turgor pressure and thus undergo lysis when their cell walls are even slightly perturbed (Selitrennikoff, 2001). The lipase activity of GLIP1 was required for its function, because the alteration of a Ser residue at the active site (S44A) eliminated both the enzymatic activity of GLIP1 and its ability to disrupt the fungal spores. Thus, we have demonstrated here a plant-derived secreted lipase that can directly disrupt invading fungi. Notably, GLIP1 appears to have substrate specificity toward fungal cell walls that are composed of components different from those of plant hosts (Selitrennikoff, 2001).

It was previously shown that inoculation of Col-0 wild-type plants with *A. brassicicola* induced a hypersensitive response resulting in small necrotic lesions restricted to the infection sites (Thomma et al., 1998, 1999). This initial response activated a SAR response that led to the induction of defense-related genes such as *PDF1.2*, *PR-3*, and *PR-4* (Penninckx et al., 1996; Thomma et al., 1998). Based on our observation, GLIP1 may play dual roles in the defense response, as it not only directly interacts with the fungal pathogen but may also influence the defense signaling in the plant itself. This possibility is suggested by the results that the expression of defense- and ethylene-related genes was partially defective in *glip1* plants and that pretreatment of *glip1* with GLIP1 recombinant protein systemically inhibited the cell death induced by fungal infection. The defense signaling may be activated by a lipid-derived molecule generated by the lipid-hydrolyzing activity of GLIP1. Alternatively, materials or chemicals released by the disrupted fungal spores may function as elicitors that trigger the systemic resistance signaling. The existence of a lipid-derived mobile signal that promotes long-distance signaling has been proposed previously (Maldonado et al., 2002). It was shown that *defective in induced resistance1* (*dir1*) was defective in developing SAR to virulent *Pseudomonas* or *Peronospora parasitica*. DIR1 is an apoplastic lipid transfer protein (LTP) and thus may function in the generation of an essential mobile signal or its transmission from the induced leaf. It is conceivable that a GLIP1-derived mobile signal moves around as an LTP-bound form, which remains to be examined. We found that GLIP1 may be implicated in both short- and long-distance defense responses, because *glip1* failed to develop local and systemic resistance responses to *A. brassicicola*, but these responses were recovered upon pretreatment with recombinant GLIP1.

We originally identified GLIP1 as an SA-stimulated secreted protein. However, it turned out that GLIP1 is actually involved in ethylene signaling, unlike the previously characterized SA-dependent lipases, such as EDS1 and PAD4. Whereas SA has been recognized as a key modulator for the activation of both the hypersensitive response and SAR, its role in the defense response triggered by necrotrophic fungal pathogens is not yet clear (Schenk et al., 2003). Accumulating data indicate that

resistance to *A. brassicicola* is induced via the activation of the JA and ethylene signaling pathways (Penninckx et al., 1998; Pieterse and van Loon, 1999). Furthermore, the ethylene and JA pathways cooperate with each other, as supported by the fact that the induction of *PDF1.2* requires both JA and ethylene (Wang et al., 2002). Here, we propose that GLIP1 is a critical component for both local and systemic resistance responses in the incompatible interaction with *A. brassicicola* in the ethylene-dependent pathway.

METHODS

Plant Materials, Growth Conditions, and Treatments

Arabidopsis thaliana plants were grown in a growth room at 23°C under long-day conditions (16-h light/8-h dark cycle). Plants were also grown under short-day conditions (8-h light/16-h dark cycle) for infection with *Pseudomonas syringae*, because *Arabidopsis* displays age-related resistance to *P. syringae* (Kus et al., 2002). *Arabidopsis* ecotype Col-0 (catalog no. WT-02) was purchased from Lehle Seeds. *Arabidopsis* ecotype Col-6 (catalog no. CS8155) and the T-DNA insertion mutants *glip1-1* (SALK_037272) and *glip1-2* (SALK_130146) were obtained from the ABRC.

Callus was generated from the cotyledons of young seedlings and induced on induction medium (Murashige and Skoog [MS] medium, pH 5.8, 3% sucrose, 0.8% agar, and 2 mg/L 2,4-D). A cell suspension culture was initiated by inoculating 1 to 2 g of callus into 50 mL of MS medium supplemented with 3% sucrose and 2 mg/L 2,4-D as described (May and Leaver, 1993). The suspension cells (10 mL) were subcultured into 50 mL of fresh medium once every week and maintained with gentle agitation (120 rpm).

For hormone treatments, SA (1 mM), methyl jasmonate (50 μM), and ethephon (1.5 mM) were dissolved in water and applied as a spray to 4-week-old plants. The treated plants were kept at 100% RH and then harvested at the indicated times.

Infection with *P. syringae* was performed as described (Alvarez et al., 1998). Pst DC3000 (*avrRpt2*) and Pst DC3000 were grown overnight in King's B medium containing 100 μg/mL rifampicin with and without 50 μg/mL kanamycin, respectively, washed twice, and resuspended in 10 mM $MgCl_2$. Four-week-old plants were infiltrated at two sites of two primary leaves with 20-μL aliquots of 10 mM $MgCl_2$ or Pst DC3000 (*avrRpt2*) (10^6 colony-forming units/mL), which was followed by infiltration at two sites of secondary leaves with 20-μL aliquots of Pst DC3000 (10^6 colony-forming units/mL) the next day.

Inoculation with *Alternaria brassicicola* was performed by applying a 10-μL drop of spore suspension (5×10^5 spores/mL) on each leaf of 4-week-old plants. For pretreatment with GLIP1 proteins, plants were infiltrated into one site on the abaxial surface of each leaf with PBS (control) or GLIP1 proteins (1 μg) in 10-μL aliquots and incubated for 24 h before pathogen inoculation. Inoculated plants were kept at 100% RH in a growth chamber for the indicated times. To count the number of spores, 10 leaves were collected and vigorously shaken in 5 mL of 0.1% Tween 20 in a test tube. Leaves were removed, and the spore-containing suspension was centrifuged at 5000*g* for 15 min. The spores were resuspended in 200 μL of 0.1% Tween 20, serially diluted, and counted with a microscope.

Preparation of Secreted Proteins

The suspension cultures were left untreated or treated with 0.5 mM SA 4 d after subculture. At the indicated times, the cultures were passed twice through a membrane filter (0.2 μm) in a vacuum apparatus to remove the cells from the culture medium. The collected medium (secreted fraction)

was then freeze-dried in a vacuum lyophilizer and dialyzed against buffer (10 mM MES, pH 5.8). The prepared secreted proteins were stored at −70°C.

2D Gel Electrophoresis

The 2D gel electrophoresis and gel analysis were performed as described (Lee et al., 2004). Secreted proteins were resuspended in isoelectric focusing sample buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamido propyl)-dimethylammonio]-1-propane sulfate, 1% carrier ampholytes, 20 mM DTT, and 0.001% bromophenol blue) and used to rehydrate immobilized pH gradient strips (240 mm, pH 4 to 7; Amersham Biosciences). Isoelectric focusing was performed for a total of 320,000 to 350,000 voltage hours on the IPGphor system (Amersham Biosciences). To run gels in the second dimension, 12% SDS polyacrylamide gels were used. The 2D gels were stained with silver nitrate according to the manufacturer's instructions (Amersham Biosciences). To create the reference map, 2D gels were scanned and analyzed by ImageMaster 2D Elite software (Amersham Biosciences). For each condition analyzed, three to five gels were prepared from three different protein extractions. The volumes of silver-stained spots were normalized according to the volumes of internal standards. The SA-induced changes were subjected to statistical analysis with Student's *t* test, and those spots with P < 0.05 were considered for identification by MALDI-TOF MS.

MALDI-TOF MS and Database Searching

Peptide samples were prepared as described previously (Lee et al., 2004). Peptide masses were measured on a MALDI-TOF MS system (Voyager-DE STR; Applied Biosystems). Peptide mass fingerprint data were matched to the National Center for Biotechnology Information nonredundant database entries using the MS-Fit program (http://prospector. ucsf.edu/ucsfhtml4.0/msfit.htm). The following search parameters were applied. Mass tolerance was set to 50 ppm [[experimental mass − theoretical mass]/theoretical mass in daltons], and one incomplete cleavage was allowed. Acetylation of the N terminus, alkylation of Cys by carbamidomethylation, oxidation of Met, and pyroGlu formation of N-terminal Gln were set as possible modifications. Molecular mass and pI ranges were set to 10 to 200 kD and 4 to 7, respectively. The database search disclosed matching proteins ranked according to peptide number matches, sequence coverage, and the Molecular Weight Search (MOWSE) score. Although the candidate ranked at the top was considered a positive identification, protein identification was assigned when the following criteria were met: at least five matching peptides, >15% sequence coverage, and a MOWSE score > 10^3.

Protein Analysis

For immunoblotting, proteins were separated on 12% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were incubated with anti-GST, anti-AnnAt1 (Lee et al., 2004), anti-OEE2 (Yang et al., 2003), anti-cyclin D (Cho et al., 2004), and anti-myrosinase (Husebye et al., 2002) antibodies. Anti-myrosinase antibody was provided by Atle Bones, and the other antibodies were prepared in the Kumho Life and Environmental Science Laboratory. Antibody-bound proteins were detected by incubation with secondary antibodies conjugated to horseradish peroxidase using the enhanced chemiluminescence system (Amersham Biosciences).

DNA and RNA Analysis

The genomic DNA was digested, separated, and blotted onto a Hybond N$^+$ nylon membrane (Amersham Biosciences). The T-DNA–specific probe for DNA analysis contained the partial sequence of the left border

of T-DNA and was generated by PCR using primers 5′-GCTGC-CTGTATCGAGTGGTGATTTTGT-3′ and 5′-TCCTTTCGCTTTCTTCCCT-TCCTTTCTC-3′. Hybridization was performed for 16 to 24 h at 60°C. Membranes were then washed with 1× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)/0.1% SDS, then with 0.5× SSC/0.1% SDS, and finally with 0.1% SSC/0.1% SDS. The bands were visualized by autoradiography.

RT-PCR was performed with 0.1 µg of total RNA using the Access RT-PCR system (Promega). The primers used in the RT-PCR are as follows: for *GLIP1*, 5′-CGATTGTGCACCAGCCTCATTGGTT-3′ and 5′-CAGCG-CTTTGAGATTATAGGGTCC-3′; for *PR-1*, 5′-TCGTCTTTGTAGCTCTTG-TAGGTG-3′ and 5′-TAGATTCTCGTAATCTCAGCTCT-3′; for *PR-2*, 5′-CGTTGTGGCTCTTTACAAACAACAAAAC-3′ and 5′-GAAATTAACTT-CATACTTAGACTGTCGAT-3′; for *PR-3*, 5′-CGGTGGTACTCCTCCTG-GACCCACCGGC-3′ and 5′-CGGCGGCACGGTCGGCGTCTGAAGG-CTG-3′; for *PR-4*, 5′-GACAACAATGCGGTCGTCAAGG-3′ and 5′-AGC-ATGTTTCTGGAATCAGGCTGCC-3′; for *PR-5*, 5′-ATGGCAAATATCTC-CAGTATTCACA-3′ and 5′-ATGTCGGGGCAAGCCGCGTTGAGG-3′; for *PDF1.2*, 5′-GCTAAGTTTGCTTCCATCATCACCCTT-3′ and 5′-AACAT-GGGACGTAACAGATACACTTGTG-3′; for *EIN2*, 5′-TGCAGCTCGCA-TAAGCGTTGTGACTGGTA-3′ and 5′-CGCTCTCTCCATTTAACCGAG-TTAACAC-3′; for *EIN3*, 5′-GATGTTGATGAATTGGAGAGGAGGATG-3′ and 5′-ACGTCTCTGAGGAGGATCACAGTGT-3′; for *ERF1*, 5′-CGGC-TTCTCACCGGAATATTCTATCG-3′ and 5′-TCTCCGAAAGCGACTCTT-GAACTCTCT-3′; and for *Actin1*, 5′-GGCGATGAAGCTCAATCCAA-ACG-3′ and 5′-GGTCACGACCAGCAAGATCAAGACG-3′.

Assays of Endogenous Enzyme Activities

Endogenous ADH and G6PDH activities were measured as described (Ranieri et al., 1996; Chung and Ferl, 1999). Proteins were extracted in extraction buffer (50 mM Tris, pH 8.0, and 15 mM DTT) and centrifuged at 12,000*g* for 15 min at 4°C. The reaction mixture for ADH contained 50 mM Tris, pH 9.0, 0.867 mM NAD^+, 20% ethanol, and aliquots of protein extracts. The reaction for G6PDH was performed in 86.3 mM triethanolamine, pH 7.6, 6.7 mM $MgCl_2$, 0.37 M $NADP^+$, 12 mM glucose-6-phosphate, and aliquots of protein extracts. The increase in absorbance at 340 nm was monitored every 15 s for 60 s. The activities were expressed as micromoles of NAD^+ or $NADP^+$ reduced per minute per milligram of protein.

Subcellular Localization of GLIP1

The DNAs of *GLIP1*, *GLIP1ΔSP*, and *SP* were generated by PCR using the following primers with the sequences for cloning sites *Xba*I and *Xho*I (underlined): for *GLIP1*, 5′-GATCTCTAGAATGGAAAACTCTCAATTAG-3′ and 5′-GATCCTCGAGATTAAGTTCAAACAGCGC-3′; for *GLIP1ΔSP*, 5′-GATCTCTAGAATGGACAACAATAATCTTGTA-3′ and 5′-GATCCTC-GAGATTAAGTTCAAACAGCGC-3′; and for *SP*, 5′-GATCTCTAGAAT-GGAAAACTCTCAATTAG-3′ and 5′-CCGCTCGAGGACACTAGAGAGA-GTATC-3′. The amplified DNAs were cloned into the *Xba*I and *Xho*I sites of the pBI221 vector (Clontech) so that *smGFP* was fused in frame to the C termini of the DNA products. The resulting constructs were introduced into onion (*Allium cepa*) epidermal cells using a helium biolistic particle delivery system (Bio-Rad) as described by Shieh et al. (1993). After incubation for 12 to 48 h at 23°C, the subcellular distribution of the GFP fusion proteins was examined with a fluorescence microscope (Olympus BX-51TRF).

Purification of GLIP1 and Lipase Assay

The full-length coding region of *GLIP1* was cloned into the pGEX 6P-1 vector (Amersham Biosciences). The GLIP1(S44A) mutant was generated by mutagenic PCR using the following primers with one mismatch (lowercase): for sense, 5′-TCGTGTTTGGAGATgCTGTGTTCGATGCT-GGA-3′; for antisense, 5′-TCCAGCATCGAACACAGcATCTCCAAACA-CGA-3′. *Escherichia coli* BL21(DE3) cells transformed with constructs were cultured, and the expression of the recombinant proteins was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside for 30 min. The GST-GLIP1 fusion proteins were purified according to the manufacturer's instructions, dialyzed against PBS, and stored at –70°C before use. Lipase activity was measured as described (Baudouin et al., 1997). The enzyme reaction mixture contained 0.5 M HEPES, pH 6.5, 1 mM substrate (*p*-nitrophenyl acetate or *p*-nitrophenyl butyrate), and aliquots of recombinant proteins (2 to 4 µg) and was incubated for 60 min at 30°C. Absorbance was measured at 405 nm every 5 min for 60 min.

Microscopic Analyses

The lesions and fungal hyphae were visualized by staining the infected leaves according to a previously described method (Kumar et al., 2004). Leaves detached 4 d after inoculation were stained with lactophenol–aniline blue. Lactophenol was prepared by mixing phenol, acetic acid, distilled water, and glycerol at a ratio of 1:1:1:2. Aniline blue (0.1%) and lactophenol were then mixed at a ratio of 1:3. Cell death was detected by lactophenol–trypan blue staining followed by destaining in saturated chloral hydrate as described (Koch and Slusarenko, 1990). Stained samples were mounted in 60% glycerol and examined using a light microscope (Olympus BX-51TRF).

The antimicrobial activity of GLIP1 was determined by adding the recombinant proteins (0.1 to 5 µg) to 10 µL of spore suspension (5×10^5 spores/mL). The samples were placed on glass slides, incubated for 24 h at 23°C, and observed by light microscopy. Fungi samples were prepared for scanning electron microscopy by fixation in fixing solution (0.1 M sodium phosphate, pH 7.0, 1% sucrose, 3% paraformaldehyde, and 0.2% glutaraldehyde) under vacuum for 6 h, dehydration through a graded ethanol series, and critical point drying. The samples were sputter-coated with gold and viewed using a S-2500 scanning electron microscope (Hitachi).

Accession Numbers

Arabidopsis Genome Initiative locus numbers for the genes described in this work are as follows: *GLIP1* (At5g40990), *GLIP2* (At1g53940), *GLIP3* (At1g53990), *GLIP4* (At3g14225), *GLIP5* (At1g53920), *GLIP6* (At1g71120), and *GLIP7* (At5g15720). Other accession numbers are listed in Table 2.

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