The Pepper Mannose-Binding Lectin Gene *CaMBL1* Is Required to Regulate Cell Death and Defense Responses to Microbial Pathogens[1][C][W][OA]

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Plant mannose-binding lectins (MBLs) are crucial for plant defense signaling during pathogen attack by recognizing specific carbohydrates on pathogen surfaces. In this study, we isolated and functionally characterized a novel pepper (*Capsicum annuum*) MBL gene, *CaMBL1*, from pepper leaves infected with *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). The *CaMBL1* gene contains a predicted *Galanthus nivalis* agglutinin-related lectin domain responsible for the recognition of high-mannose *N*-glycans but lacks a middle *S*-locus glycoprotein domain and a carboxyl-terminal PAN-Apple domain. The CaMBL1 protein exhibits binding specificity for mannose and is mainly localized to the plasma membrane. Immunoblotting using a CaMBL1-specific antibody revealed that CaMBL1 is strongly expressed and accumulates in pepper leaves during avirulent *Xcv* infection. The transient expression of *CaMBL1* induces the accumulation of salicylic acid (SA), the activation of defense-related genes, and the cell death phenotype in pepper. The *G. nivalis* agglutinin-related lectin domain of *CaMBL1* is responsible for cell death induction. *CaMBL1*-silenced pepper plants are more susceptible to virulent or avirulent *Xcv* infection compared with unsilenced control plants, a phenotype that is accompanied by lowered reactive oxygen species accumulation, reduced expression of downstream SA target genes, and a concomitant decrease in SA accumulation. In contrast, *CaMBL1* overexpression in Arabidopsis (*Arabidopsis thaliana*) confers enhanced resistance to *Pseudomonas syringae* pv *tomato* and *Alternaria brassicicola* infection. Together, these data suggest that *CaMBL1* plays a key role in the regulation of plant cell death and defense responses through the induction of downstream defense-related genes and SA accumulation after the recognition of microbial pathogens.

Plants protect themselves against a wide range of pathogens, such as bacteria, fungi, and viruses, with numerous defense mechanisms, including a complicated innate immune system against pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). Plants can distinguish between self and nonself or detect specific pathogens by recognizing pathogen-associated molecular patterns mostly generated or secreted from pathogens. Defense responses mediated via signal transduction pathways lead to the reinforcement of plant cell walls, the production of antimicrobial metabolites (phytoalexins) and pathogenesis-related (PR) proteins, and the hypersensitive response (HR), a form of programmed cell death at infection sites that limits pathogen development (Dangl and Jones, 2001; Mur et al., 2008). Signaling molecules, such as reactive oxygen species (ROS), ethylene, salicylic acid (SA), and jasmonic acid, play important roles in the complex signaling networks that activate defense mechanisms (Hammond-Kosack and Parker, 2003). Although the regulation and execution of cell death associated with the HR are not fully understood, the production of ROS, ion fluxes, defense-related gene activation, and the induction of signaling molecules have been proposed to be implicated in the cell death process (Torres and Dangl, 2005).

Plant cell walls are dynamic structures whose composition and architecture change during growth, development, and defense responses. Preformed physical and chemical barriers, such as cell walls, and constitutively produced antimicrobial compounds protect plants against invading pathogens. Proteins embedded in the cell wall and plasma membrane are involved in a monitoring system that is required for the recognition and transduction of environmental, developmental, and defense-associated signals (Garcia-Brugger et al.,

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2006). These proteins are released from plants or pathogens, and pathogens can trigger cell wall-mediated defense responses. Disruption of cell wall-plasma membrane adhesion during pathogen penetration into host cells may lead to a reduction of cell wall-associated defense responses, thereby making the plant more susceptible to disease (Mellersh and Heath, 2001).

Carbohydrate-binding proteins, commonly referred to as lectins or agglutinins, are ubiquitous in many plant species (Peumans and Van Damme, 1995) and function in defense responses to pathogen invaders. The most abundant structural proteins in plant cell walls, Hyp-rich glycoproteins, are also induced in disease resistance responses, especially during incompatible plant-pathogen interactions (Davies et al., 1997). There is convincing evidence that Hyp-rich glycoproteins act as impenetrable physical barriers against pathogen ingress (Deepak et al., 2007). While the function of lectins in plants is believed to be the binding of glycoproteins on cell surfaces, their role in animals also includes the binding of soluble extracellular and intercellular glycoproteins. Plant lectins are involved in specific protein-carbohydrate interactions within the cytoplasmic and/or nuclear compartments (Van Damme et al., 2004). The broad spectrum of the carbohydrate-binding specificity of lectins can be interpreted as the successful recognition by plant cells of different types of sugar-containing receptors. Lectins bind to the glycans of glycoproteins, glycolipids, or polysaccharides with high affinity, and they are the only plant proteins that recognize the glycoconjugates present on the surfaces of microorganisms such as bacteria and fungi. For example, chitin-binding plant lectins recognize a carbohydrate that is a typical constituent of fungal cell walls (Broekaert et al., 1989). Several plant lectins play a role in the defense against bacteria through an indirect mechanism, based on an interaction with cell wall carbohydrates or extracellular glycans. The potato (*Solanum tuberosum*) lectin immobilizes avirulent strains of *Pseudomonas solanacearum* on the plant cell wall; however, a virulent strain is not recognized by lectins (Sequeira and Graham, 1977).

Recent studies demonstrate that all known plant lectins can be classified into 12 lectin families of structurally and evolutionarily related proteins (Van Damme et al., 2008). There are the amaranthins, Cucurbitaceae phloem lectins (now called the Nictaba lectins), lectins with hevein domains, jacalin-related lectins, legume lectins, monocot Man-binding lectins (now called the GNA-related lectins [for *Galanthus nivalis* agglutinin]), and type II ribosome-inactivating proteins (also known as the Ricin-B family) in plants (Van Damme et al., 2007b). More recently, galectins (previously called the *S*-type lectins, having a strong affinity for β-galactosides) and the calnexin/calreticulin lectin families, *Agaricus bisporus* agglutinins, class V chitinase homologs with lectin activity, EEA, LysM family, and cyanovirins, are newly identified and/or regrouped (Shridhar et al., 2009). However, the physiological role of lectins is poorly understood (Sequeira and Graham, 1977; Van Damme et al., 2008; Michiels et al., 2010).

Man-binding proteins that constitute one or two domains equivalent to the GNA purified from the bulbs of snowdrop are believed to play an important role in defense against microbial pathogens by recognizing Man-type glycans of foreign microorganisms (Van Damme et al., 1987, 1998, 2007b; Barre et al., 2002). GNA was originally considered a Man-specific lectin that possesses three similar Man-binding sites per subunit (Van Damme et al., 2008). Many of them interact only weakly with Man but exhibit a strong affinity toward oligomannosides and high-Man *N*-glycans. A monomeric Man/Glc-binding lectin inhibits the germination of *Aspergillus flavus* and *Fusarium moniliforme* spores and hyphal growth in red cluster pepper (*Capsicum frutescens*) seed (Ngai and Ng, 2007). Some lectin-like receptor-like kinases (LecRLKs) have been implicated in plant defense, senescence, and wounding (Riou et al., 2002). RLKs containing an extracellular Man-binding lectin (MBL) have also been reported in rice (*Oryza sativa*; Chen et al., 2006). The MBL domain in a Ser/Thr RLK gene, *Pi-d2*, is required for *R* gene-mediated resistance to rice blast. However, the function of plant carbohydrate-binding proteins containing this domain is not fully understood. Although several R genes that encode RLKs have been cloned and characterized, none has been shown to have an extracellular lectin domain. There is convincing in vitro evidence that some plant MBLs exert a deleterious effect on fungal pathogens. Gastrodianin, which is a monomeric Man-binding protein homolog in *Gastrodia elata* and *Epipactis helleborine*, displays in vitro antifungal activity against *Rhizoctonia solani* and *Phytophthora nicotianae* (Wang et al., 2001). Gastrodianin-like proteins also inhibit the mycelial growth of *Botrytis cinerea*, *Gibberella zeae*, *Ganoderma lucidum*, *R. solani*, and *Valsa ambients* (Cox et al., 2006). *Dendrobium findleyanum* agglutinin inhibits the growth of *Alternaria alternata* (Sudmoon et al., 2008).

In this study, we isolated genes differentially expressed in pepper (*Capsicum annuum* 'Nokwang') during the incompatible interaction with *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). Among them, the novel pepper pathogen-responsive gene *CaMBL1* encodes a putative glycoprotein with a GNA-related lectin domain that localizes to the plasma membrane. To our knowledge, this is the first report suggesting that glycoproteins with the GNA-related lectin domain play a crucial role for cell death and defense responses and the expression of defense-related genes in pepper plants. Here, we show that the *CaMBL1* gene is involved in suppressing disease development in pepper plants during *Xcv* infection. We also report that overexpression of *CaMBL1* in Arabidopsis (*Arabidopsis thaliana*) enhances innate immunity against *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) and *Alternaria brassicicola*.

RESULTS

Isolation and Identification of *CaMBL1* cDNA

In a previous study, we constructed a cDNA library using RNA isolated from pepper leaves inoculated with the *Xcv* avirulent strain BV5-4a (Jung and Hwang, 2000). The full-length cDNA clone, designated *CaMBL1* and containing glycoprotein-homologous sequences, was isolated from this pepper cDNA library. The cloned *CaMBL1* cDNA had an insert of 1,160 bp containing an 894-bp coding region encoding a protein of 298 amino acids (Supplemental Fig. S1). The *CaMBL1* cDNA sequence was analyzed using the BLASTx program (http://www.ncbi.nlm.nih.gov/BLAST/), the ExPASy Proteomics Server (http://www.expasy.org), and the PLecDom program (http://www.nipgr.res.in/plecdom.html). CaMBL1 shares 47% to 55% amino acid sequence identities with an unknown grape (*Vitis vinifera*) protein (accession no. CAN75015), the sugar beet (*Beta vulgaris*) SIEP1L protein (accession no. CAA61158), the carrot (*Daucus carota*) cell attachment protein (accession no. BAD24818), the Arabidopsis curculin-like (Man-binding) lectin family protein (CLLFP; At1g78860; accession no. NP_178007), the flax (*Linum usitatissimum*) secreted glycoprotein (accession no. AAO15899), and another Arabidopsis CLLFP (At1g78830; accession no. NP_565191; Supplemental Fig. S2). A database search revealed that CaMBL1 and related plant proteins have the GNA-related lectin domain commonly present in the plant lectin family. After the identification of GNA, similar lectins were isolated and characterized from many other plant species (Van Damme et al., 2007a). Previously, GNA and related lectins were classified into the so called "monocot Man-binding lectins." Therefore, the term bulb-type MBL domain can no longer be used and has been replaced by GNA domain (Van Damme et al., 2007a, 2008). Based on the BLAST search with CaMBL1, we revealed that CaMBL1 contains the putative GNA-related lectin domain but lacks a middle *S*-locus glycoprotein domain (SLP) and a C-terminal PAN-Apple domain that are widespread in plant lectin proteins (Van Damme et al., 2008). Moreover, the GNA-SLP-PAN-type protein itself corresponds to the N-terminal half of the so-called receptor kinases, which constitute a transmembrane helix and a C-terminal protein kinase domain next to the GNA-SLP-PAN module. Notably, some *S*-locus receptor kinases that constitute these four domains were demonstrated in Brassicaceae species (Naithani et al., 2007). Thus, CaMBL1 containing only the GNA-related lectin domain may be a truncated homolog of the GNA-SLP-PAN-type protein in pepper plants.

A phylogenetic tree was constructed with CaMBL1 and homologous proteins retrieved from the GenBank database and the PLecDom program (Supplemental Fig. S3), in which MBL family members are divided into two groups containing GNA-related lectin domain or kinase domain. MBL proteins in the first group contain the GNA-related lectin domain alone, and those in the second group contain GNA-related lectin and kinase domains. Pepper GNA-related lectins including CaMBL1 were constructed in the phylogenetic tree with the GNA-related lectin members from sugar beet, grape, carrot, flax, and Arabidopsis (Supplemental Fig. S3). Some GNA-related lectins were identified in pepper, although the role of the GNA-related lectins remains unknown. CaMBL1 was closely related to the pepper GNA-related lectin (TC7720) rather than other pepper GNA-related lectins.

Expression of the *CaMBL1* Gene in Pepper

To determine the expression profiles of *CaMBL1*, we performed RNA gel-blot and immunoblot analyses of pepper plant tissues (Fig. 1). The expression of *CaMBL1* was undetected or nearly so in leaves, stems, roots, flowers, and green and red fruits during normal growth and development (Fig. 1A). Northern analysis was also performed to determine whether *CaMBL1* expression is induced by the virulent (compatible) Ds1 and avirulent (incompatible) Bv5-4a strains of *Xcv*. *CaMBL1* transcript accumulation in pepper leaves began 5 h after inoculation with the avirulent Bv5-4a strain, and it reached maximal levels from 5 to 25 h (Fig. 1A). Mock inoculation and virulent *Xcv* infection induced *CaMBL1* in pepper leaves in a similar manner. This indicates that infiltration by itself is sufficient to induce *CaMBL1*. Furthermore, infiltration may prime for enhanced expression of *CaMBL1* upon inoculation with avirulent *Xcv*. However, avirulent *Xcv* infection led to high levels of CaMBL1 protein, as detected by western blotting. Treatments with SA, but not with methyl jasmonate, also significantly induced the *CaMBL1* gene in pepper leaves 5 to 25 h after exposure (Fig. 1B). After SA treatment, transcripts were detected within 5 h, reached their highest levels between 10 and 15 h, and gradually decreased by 20 h. A high level of CaMBL1 protein expression was also detected 24 h after SA treatment. In contrast, treatment with methyl jasmonate did not induce CaMBL1 protein expression. We tested whether the anti-CaMBL1 antibody used in this study is specific to the corresponding CaMBL1 protein (Fig. 1C). The anti-CaMBL1 antibody raised in rabbits against a synthetic peptide that corresponds to C-terminal residues 284 to 297 of CaMBL1 specifically bound to His-tagged CaMBL1 proteins that were expressed in *Escherichia coli*.

Binding of CaMBL1 to D-Man

CaMBL1 was predicted to contain the GNA-related lectin domain. To determine whether it has an essential function as an MBL, we generated a series of CaMBL1 deletion mutants (Fig. 2A) and expressed them in *E. coli* as fusion proteins with a 6×His tag at the N terminus (Fig. 2B, left panel). As a result, His-CaMBL1-1 that lacks the putative signal peptide region, His-CaMBL1-2 that contains a GNA-related

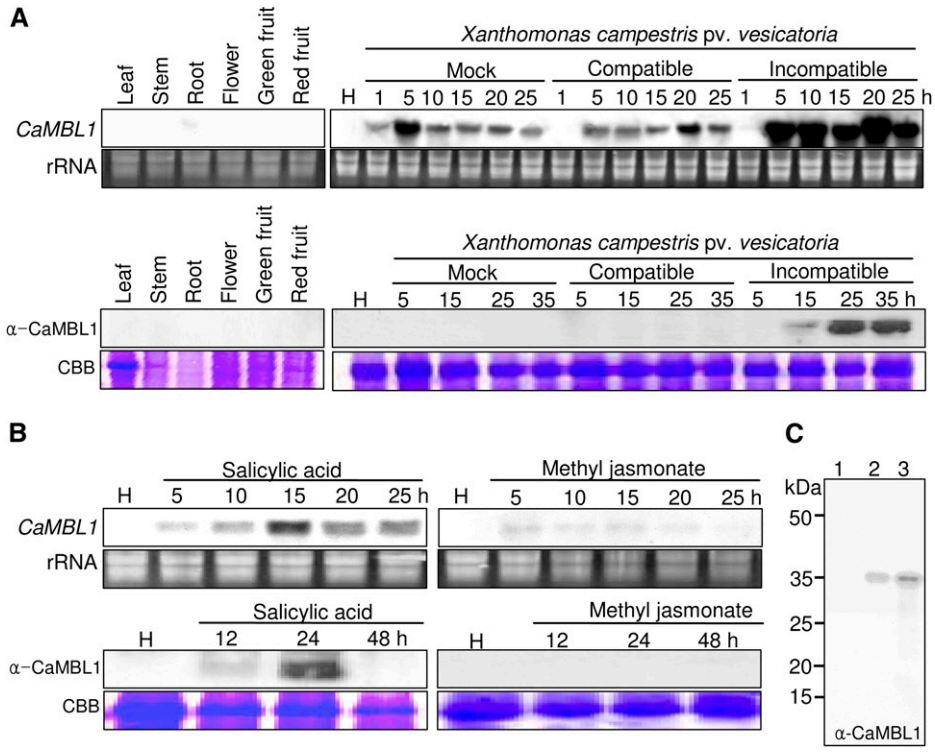

Figure 1. RNA gel- and western-blot analyses of the expression of *CaMBL1* in pepper plants. A, Expression of the *CaMBL1* gene and CaMBL1 protein in healthy organs of pepper plants and in pepper leaves at various times after inoculation with the virulent strain Ds1 and the avirulent strain Bv5-4a of *Xcv*. B, Expression of the *CaMBL1* gene and CaMBL1 protein in pepper leaves at various times after treatment with SA (5 mM) and methyl jasmonate (100 μM). Equal loadings (10 μg) of RNA were verified by visualizing rRNA on gels stained with ethidium bromide. Coomassie Brilliant Blue (CBB) staining is shown for the 60-kD region of protein extracts. C, Immunoblot of His-tagged CaMBL1 protein using the anti-CaMBL1 antiserum raised in rabbits against a synthetic peptide corresponding to C-terminal residues 284 to 297 of CaMBL1. His-tagged CaMBL1 proteins expressed in *E. coli* BL21 was used. Lane 1, Uninduced *E. coli* BL21 cell extracts (1 μg of protein); lane 2, 0.1 μg of His-tagged CaMBL1 protein expressed in *E. coli* BL21 cells after isopropylthio-β-galactoside induction; lane 3, 1 μg of His-tagged CaMBL1 protein expressed in *E. coli* BL21 cells after isopropylthio-β-galactoside induction. [See online article for color version of this figure.]

lectin domain, and His-CaMBL1-3 that contains only the C-terminal region were distinctly expressed in *E. coli*, followed by their purification using D-Man-agarose columns. However, full-length 6×His-tagged CaMBL1 was not expressed in *E. coli* (no arrowhead), even with the use of a GNA-related lectin domain and glutathione *S*-transferase-tagged ones (data not shown). This indicated that the presence of the signal peptide may block the expression of CaMBL1 in *E. coli*. Immunoblot analysis of the purified CaMBL1 proteins using the anti-His antibody showed that protein bands from CaMBL1 deletion mutants that lack the signal peptide region but contain the GNA-related lectin domain region could be immunodetected by this antibody (Fig. 2B, right panel). These immunoblotting data indicate that the GNA-related lectin domain of CaMBL1 is essential for its binding to D-Man. The glycan array screening of CaMBL1 was performed by the Consortium for Functional Glycomics at the School of Medicine, Emory University. Binding levels of CaMBL1 to the various glycans on the arrays were measured by fluorescence scanning (Supplemental Fig. S5; Supplemental Table S1). These glycan array data indicate that CaMBL1 has affinity toward Manα and/or Manβ and GalNAc residues.

Subcellular Localization of CaMBL1

To identify the subcellular location of the CaMBL1 protein, and to determine whether it depends on the GNA-related lectin domain, soluble modified GFP (smGFP) was fused to the full-length CaMBL1 protein (CaMBL1:smGFP), the GNA-related lectin domain region (GNA domain:smGFP), and the C-terminal region (CaMBL1^c:smGFP; Fig. 3A). The subcellular localization of these fusion proteins was visualized in onion (*Allium cepa*) epidermal cells by confocal microscopy. The merging of fluorescence and bright-field images revealed that both the full-length and GNA-related lectin domain fusion proteins localized to the plasma membrane, whereas the C-terminal region of CaMBL1 was expressed only in the cytosol (Fig. 3B). In contrast, the control smGFP was uniformly distributed throughout the cell. Onion epidermis was treated with

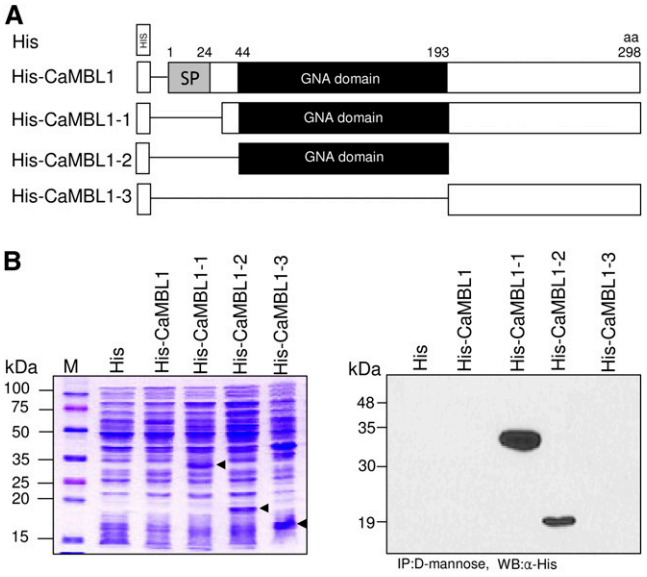

Figure 2. Binding of CaMBL1 to D-Man. A, Schematic representation of CaMBL1 and CaMBL1 deletion constructs. B, Immunoblot analysis of CaMBL1 using the anti-His antibody. His-tagged CaMBL1 and CaMBL1 deletion constructs were expressed in *E. coli* (left panel) and purified using D-Man-agarose columns. Arrowheads indicate expressed His-tagged recombinant proteins. aa, Amino acids; CaMBL1-1, signal peptide deleted; CaMBL1-2, GNA-related lectin domain only; CaMBL1-3, C-terminal region only; GNA domain, GNA-related lectin domain; His, His tag; SP, signal peptide region; α-His, anti-His antibody; IP, immunoprecipitation; M, molecular mass markers; WB, western blotting. [See online article for color version of this figure.]

1 M NaCl to induce plasmolysis. The effect of plasmolysis on onion cells expressing full-length and GNA-related lectin domain fusion proteins also confirmed that CaMBL1 is localized to plasma membrane and that the GNA-related lectin domain is required for its localization (Fig. 3C). We also tested the subcellular localization of CaMBL1 in pepper leaves. Total proteins were extracted from the pepper leaves during *Xcv* infection. To separate soluble and microsomal fractions, total proteins were centrifuged at 92,000*g* for 90 min. Immunoblotting data with anti-CaMBL1 antibody indicated that CaMBL1 was enriched in the microsomal fraction including the plasma membrane or the endoplasmic reticulum membrane from the leaves infected by the avirulent (incompatible) strain Bv5-4a of *Xcv*. However, CaMBL1 was slightly detected in the soluble fraction including secretory proteins (Fig. 3D). Collectively, these results indicate that CaMBL1 is a membrane-associated protein and requires the GNA-related lectin domain to localize to the plasma membrane.

Induction of Cell Death by Transient Expression of *CaMBL1*

To further test whether the *CaMBL1* gene and the *CaMBL1*-mediated signaling pathway affect the induction of cell death, we transiently expressed *CaMBL1* in pepper leaves by infiltration with *Agrobacterium tumefaciens* carrying 35S:*00* (empty vector) or 35S:*CaMBL1* constructs. As shown in Figure 4A, a distinct necrotic phenotype was observed in pepper leaves 4 d after infiltration with different concentrations of *Agrobacterium* carrying 35S:*CaMBL1*. Whitish necrotic symp-

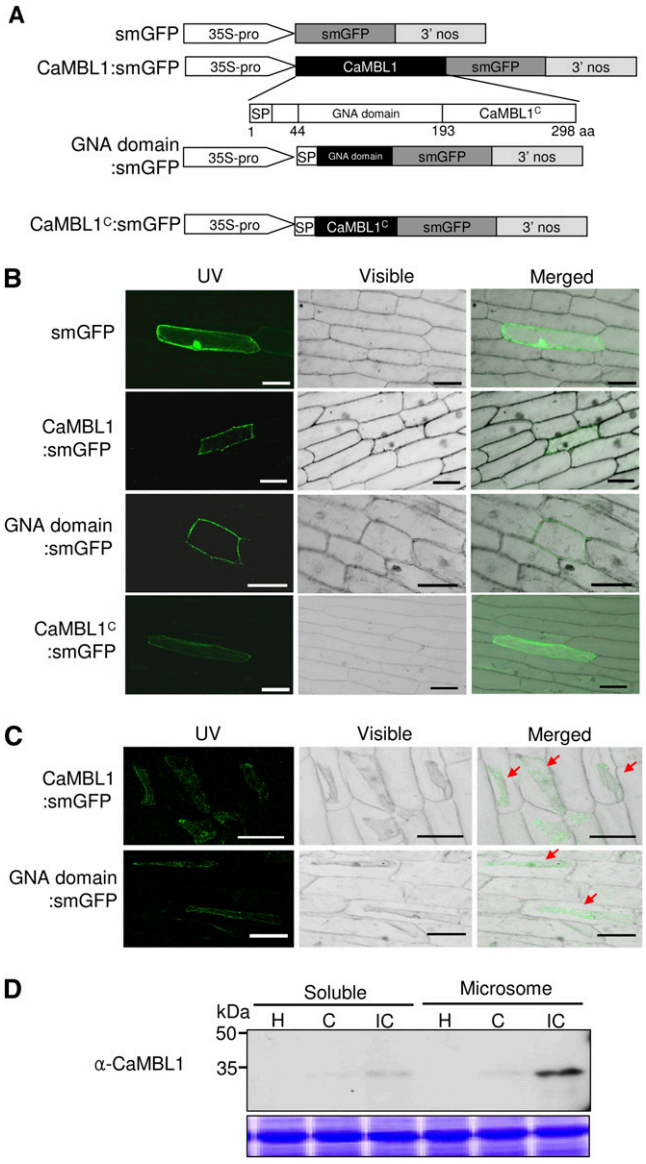

Figure 3. Subcellular localization of CaMBL1. A, Schematic representation of CaMBL1 constructs. B, Bright-field images of subcellular localization of CaMBL1:smGFP, GNA domain:smGFP, and CaMBL1^{C}:smGFP proteins in onion epidermal cells. GFP images were observed by confocal laser scanning fluorescence microscopy. Bars = 0.1 mm. C, Subcellular localization of CaMBL1:smGFP and GNA domain:smGFP in onion epidermal cells after plasmolysis with 1 M NaCl. Red arrows indicate plasmolyzed plasma membrane. Bars = 0.1 mm. D, Immunodetection of CaMBL1 with anti-CaMBL1 antibody in soluble and microsomal fractions from pepper leaves infected by *Xcv*. H, Healthy leaves; C, compatible; IC, incompatible.

toms appeared in pepper leaves 48 h after agroinfiltration. In contrast, infiltration with the *Agrobacterium* empty vector control did not induce a cell death response. Plant cells undergoing HR-like cell death accumulated autofluorescent compounds, which may contain cross-linked phenolics that could serve to strengthen the cell wall (Dixon and Paiva, 1995). As observed under UV illumination, autofluorescence was detected in pepper leaves as early as 2 d after infiltration with *Agrobacterium* (35S:*CaMBL1*). This result indicates that *CaMBL1* transient expression triggers the accumulation of fluorescent phenolic compounds as a cell death-related defense reaction. An increased expression of c-Myc-tagged CaMBL1 was detected in leaf tissues 18, 36, and 60 h after infiltration with *Agrobacterium* (35S:*CaMBL1*), as shown by western-blot analysis (Fig. 4B). However, the protein expressed by 35S:*c-Myc* was not transiently induced in empty vector control leaves. Large clusters of dead cells that stained with trypan blue were seen in the *Agrobacterium* (35S:*CaMBL1*)-infiltrated leaf areas but not in leaves transfected with the empty vector (Fig. 4C). The severity of cell necrosis caused by membrane damage in leaves was estimated by measuring electrolyte leakage from leaf tissues. Pepper leaves that transiently expressed CaMBL1 exhibited drastic electrolyte leakage compared with empty vector control leaves 36 h after infiltration, when the cell death phenotype appeared (Fig. 4D). We used real-time reverse transcription (RT)-PCR to determine whether the HR-like cell death in leaves transiently expressing *CaMBL1* is accompanied by the expression of defense-related genes (Fig. 4E). *CaBPR1* (for basic pathogenesis-related gene) was selected as a marker gene responsible for the activation of plant defense responses. The transcript level of *CaBPR1* increased significantly during the transient expression of *CaMBL1*, indicating that induction by *CaMBL1* con-

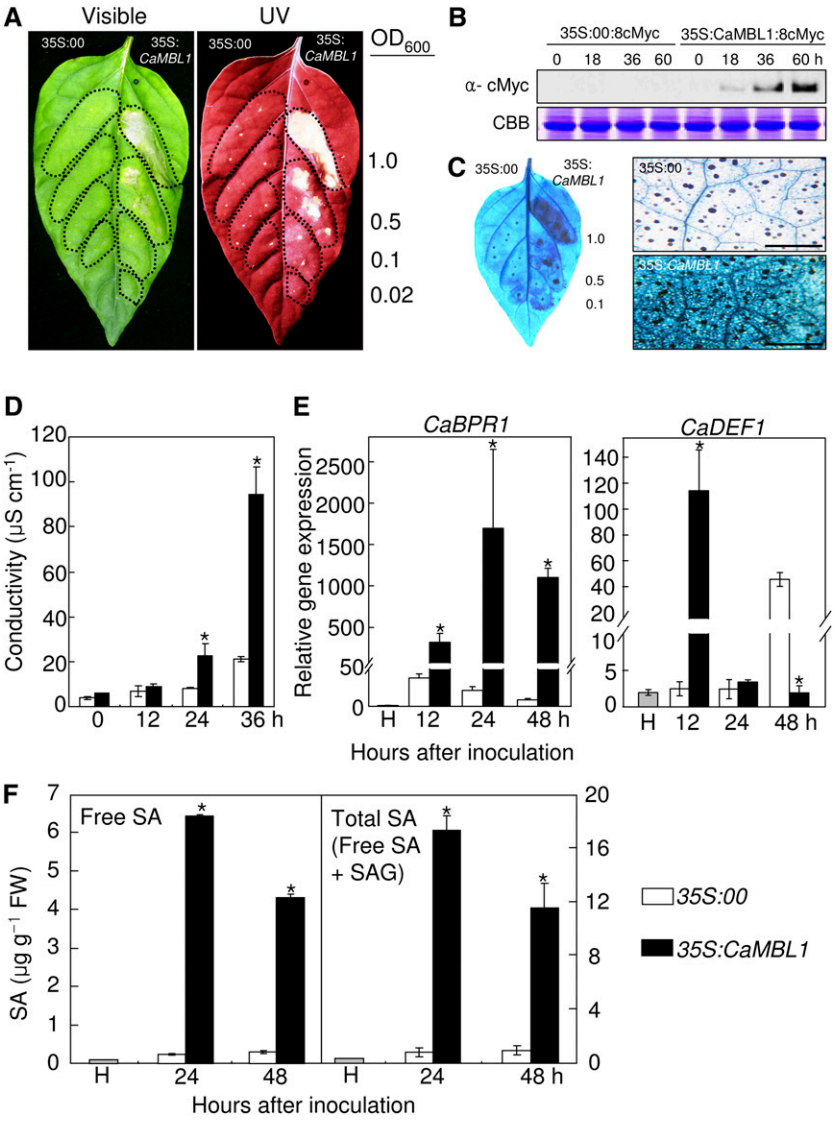

Figure 4. Transient expression of cell death in pepper leaves infiltrated with *Agrobacterium* GV3101 carrying the 35S:00 (empty vector) or 35S:*CaMBL1* construct. A, Visible and UV light-illuminated phenotypes of *CaMBL1*-transiently expressing leaves 4 d after infiltration with different bacterial concentrations (OD_{600}). B, Expression of CaMBL1 protein in empty vector control leaves and 35S:*CaMBL1* leaves as detected by immunoblotting. Coomassie Brilliant Blue (CBB) staining is shown for the 60-kD regions of protein extracts. C, Staining with trypan blue of leaf tissues 1 d (right) and 2 d (left) after infiltration. Bars = 0.5 mm. D, Electrolyte leakage assay of *CaMBL1*-transiently expressing leaves at different times after agroinfiltration. E, Real-time quantitative PCR analysis of *CaBPR1* and *CaDEF1* in *CaMBL1*-transiently expressing leaves. Transcript levels were normalized to the expression of pepper 18S ribosomal RNA measured in the same samples. F, Levels of SA in *CaMBL1*-transiently expressing leaves. All experiments were repeated three times with similar results. Values are presented as means ± SD. Asterisks indicate significant differences between the means, as determined by Student's *t* test ($P < 0.05$). FW, Fresh weight; H, healthy plants. [See online article for color version of this figure.]

tributes to the defense response in plants. However, the transcript level of *CaDEF1* (for defensin) was not affected by *CaMBL1* transient expression, except for that at the 12-h time point. As shown in Figure 4F, increased *CaMBL1* expression led to the accumulation of free SA and total SA (free SA plus Glc-conjugated SA) in pepper leaves. In particular, SA levels 24 and 48 h after infiltration were significantly higher in leaves transiently expressing *CaMBL1* than those in leaves transfected with the empty vector control. Increased SA levels in *CaMBL1* transiently expressed leaves 24 h after agroinfiltration were higher than those 48 h after agroinfiltration. These increased SA levels correlated with the increased *CaBPR1* expression levels during the transient expression of *CaMBL1*. Overall, these results indicate that the transient expression of *CaMBL1* induces cell death in pepper leaves, which is supported by the expression of the defense-related gene *CaBPR1* and SA accumulation.

The GNA-Related Lectin Domain of *CaMBL1* Is Responsible for Cell Death Induction

Induction of *CaMBL1* by *Agrobacterium*-mediated transient expression led to cell death, which was accompanied by defense responses, including the induction of defense-related genes and SA accumulation (Fig. 4). Within 36 to 48 h after agroinfiltration, hypersensitive cell death was seen in the infiltrated area of pepper leaves, which collapsed 3 to 4 d later (data not shown). To determine which region of the *CaMBL1* gene is responsible for the induction of cell death in pepper leaves, we transformed the constructs pBIN:*CaMBL1*, pBIN:*GNA* domain, and pBIN:*CaMBL1*^{c}, which contains only the C-terminal region, into *Agrobacterium* strain GV3101. As expected, the transient expression of 35S:*CaMBL1* and of 35S:*GNA* domain induced much more hypersensitive cell death 4 d after agroinfiltration compared with that induced by 35S:*CaMBL1*^{c} (Fig. 5A). All three proteins from these constructs were detected in leaf protein extracts by immunoblotting using an anti-cMyc antibody (Fig. 5B), showing that the constructs are transiently expressed in pepper leaves. The hypersensitive cell death responses were also well supported by ion conductivity data (Fig. 5C). Distinct electrolyte leakage, a measure of membrane damage, began within 12 h after agroinfiltration. Leaves transiently expressing 35S:*CaMBL1* and 35S:*GNA* domain exhibited significantly enhanced electrolyte leakage compared with empty vector and *CaMBL1*^{c}-expressing leaves 12, 24, and 36 h after agroinfiltration (Fig. 5C). Collectively, these data indicate that the GNA-related lectin domain of the *CaMBL1* gene contributes to *CaMBL1*-specific cell death induction.

Enhanced Susceptibility of *CaMBL1*-Silenced Pepper to *Xcv* Infection

To characterize the loss of function of *CaMBL1* in pepper plants, we used the virus-induced gene silencing (VIGS) system with tobacco rattle virus (TRV)-based vectors to knock down the expression of *CaMBL1*. *CaMBL1*-silenced pepper plants (TRV:*CaMBL1*) were

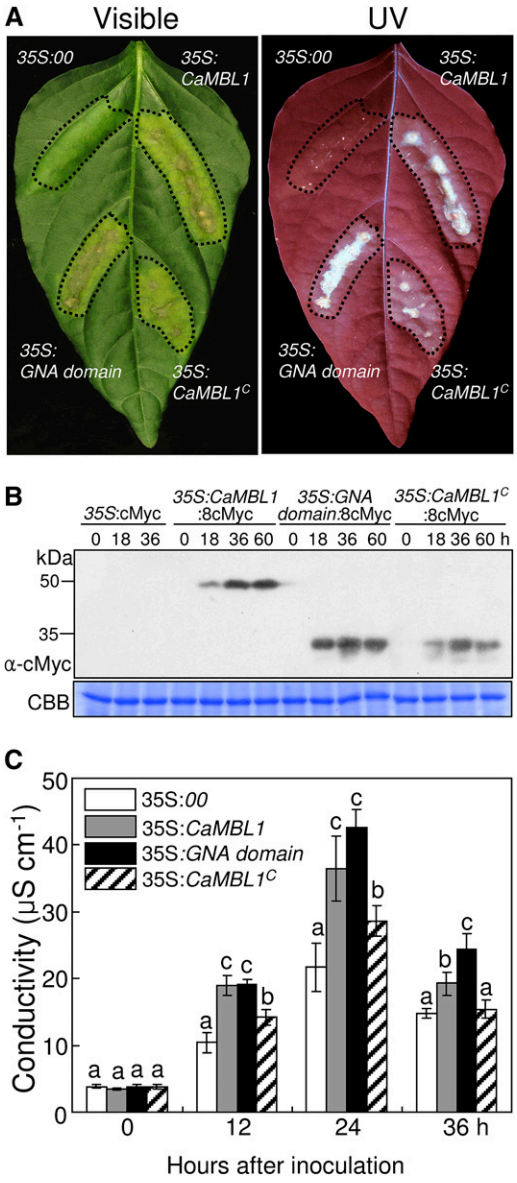

Figure 5. *Agrobacterium*-mediated transient expression of 35S:*00* (empty vector), 35S:*CaMBL1*, 35S:*GNA domain*, and 35S:*CaMBL1*^{c} in pepper leaves. A, Visible and UV light-illuminated phenotypes of the 35S:00 (empty vector), 35S:*CaMBL1*, 35S:*GNA domain*, and 35S:*CaMBL1*^{c}-transiently expressing pepper leaves 4 d after agroinfiltration. B, Immunoblot analysis of 35S:8cMyc, 35S:CaMBL1:8cMyc, 35S:GNA domain:8cMyc, and 35S:CaMBL1^{c}:8cMyc protein expression in pepper leaves using an anti-cMyc antibody. Coomassie Brilliant Blue (CBB) staining is shown for the 60-kD regions of protein extracts. C, Electrolyte leakage assay of 35S:00 (empty vector), 35S:*CaMBL1*, 35S:*GNA domain*, and 35S:*CaMBL1*^{c}-transiently expressing pepper leaves at different times after agroinfiltration. All experiments were repeated three times with similar results. Values are presented as means ± SD. Different letters indicate significant differences as determined by Fisher's LSD test ($P < 0.05$).

morphologically comparable to empty vector control plants (TRV:00). We examined the levels of resistance to *Xcv* infection in *CaMBL1*-silenced plants. Three and 4 weeks after VIGS, empty vector control and silenced plants were challenged with the virulent strain Ds1 (compatible) and the avirulent strain Bv5-4a (incompatible) of *Xcv*. Disease symptoms on pepper plants were monitored 7 d after inoculation (Fig. 6A). In leaves infected with the virulent strain Ds1, silencing of *CaMBL1* resulted in severe disease symptoms accompanied by increased bacterial growth (Fig. 6, A and B). The virulent *Xcv* strain Ds1 grew less in empty vector control (TRV:00) leaves than in *CaMBL1*-silenced leaves during infection, especially 3 d after inoculation with 5×10^4 colony-forming units (cfu) mL^{-1} (Fig. 6A). In the compatible interaction with *Xcv* Ds1, neither the empty vector control leaves nor *CaMBL1*-silenced leaves exhibited high levels of hydrogen peroxide (H_2O_2; Fig. 6C) or hypersensitive cell death. In the incompatible interaction with the *Xcv* avirulent strain Bv5-4a, silencing of the *CaMBL1* gene enhanced bacterial growth in pepper leaves (Fig. 6B), which was accompanied by susceptible disease symptoms. We further measured H_2O_2 formation using the xylenol orange assay and also hypersensitive cell death by monitoring electrolyte leakage. Silencing of *CaMBL1* compromised the induction of H_2O_2 and electrolyte leakage in pepper leaves infected by either virulent or avirulent *Xcv* strains (Fig. 6, C and D). These data indicate that the *CaMBL1* gene is involved in the early events of cell death and defense responses of pepper plants.

We next examined the expression profiles of *CaMBL1* and defense-related genes in empty vector control (TRV:00) and silenced (TRV:*CaMBL1*) pepper leaves in compatible and incompatible interactions with *Xcv*. The *CaMBL1* gene was almost uninduced in *CaMBL1*-silenced leaves during *Xcv* infection, indicating that *CaMBL1* silencing was successfully achieved. More importantly, expression of the *CaBPR1* (a SA molecular marker), *CaDEF1*, *CaSAR82A* (for systemic acquired resistance [SAR] 8.2), and *CaPOA1* (for ascorbic peroxidase) genes was significantly reduced in *CaMBL1*-silenced plants during *Xcv* infection (Fig. 7A). Immunoblot analysis using the anti-CaMBL1 antibody identified a strong expression of the CaMBL1 protein during incompatible interactions, peaking at 36 h after *Xcv* inoculation in empty vector control plants (Fig. 7B). However, CaMBL1 proteins were not induced in empty vector control leaves during *Xcv* virulent (compatible) infection. In contrast, induction of CaMBL1 was compromised in silenced pepper leaves during avirulent *Xcv* infection. To test whether defense responses were compromised in *CaMBL1*-silenced plants, SA levels in empty vector control and *CaMBL1*-silenced leaves were measured using HPLC (Fig. 7C). *CaMBL1*-silenced leaves exhibited significantly lower levels of free SA and total SA (free SA plus Glc-conjugated SA) than empty vector control leaves during compatible and incompatible interactions with *Xcv*.

Enhanced Resistance of *CaMBL1*-Overexpression Arabidopsis to *P. syringae* pv *tomato* Infection

Because stable transformation of pepper plants is difficult, we constructed transgenic Arabidopsis plants constitutively expressing *CaMBL1* under the control of the cauliflower mosaic virus *35S* constitutive promoter to study the gain-of-function phenotype of *CaMBL1* in heterologous plants during pathogen infection. When detected with the CaMBL1-specific antibody by western blotting, at least 10 independent transgenic lines were found to constitutively express the CaMBL1 protein, which was not detected in wild-type plants. Three transgenic lines with a single insertion of the *CaMBL1* transgene, lines 2, 4, and 5, were selected for this study (Fig. 8). Expression of the pathogenesis-related genes *PR1* and *PDF* was constitutively up-regulated in the three *CaMBL1*-overexpression (OX) transgenic lines compared with that in wild-type plants, although the levels of expression differed among these lines (Fig. 8A). *CaMBL1*-OX transgenic plants did not exhibit any apparent phenotypic abnormality compared with wild-type plants (data not shown). The three *CaMBL1* transgenic Arabidopsis lines were tested for resistance against *Pst* DC3000 and the *Pst* DC3000 strain carrying *avrRpm1* (*Pst* DC3000 *avrRpm1*). Five days after inoculation, the leaves of *CaMBL1*-OX plants exhibited mild chlorotic lesions compared with wild-type leaves (Fig. 8B). Overexpression of *CaMBL1* significantly inhibited the growth of *Pst* DC3000 and *Pst* DC3000 *avrRpm1* in Arabidopsis leaves (Fig. 8C). Reduction of disease symptoms was intimately correlated with the bacterial growth suppressed in the leaves of *CaMBL1*-OX plants.

We next examined the necrotizing process at the microscopic level by assessing H_2O_2 production after 3,3′-diaminobenzidine (DAB) staining (dark brown) and HR-like cell death after trypan blue staining (dark blue) of leaves infected by *Pst* DC3000 and *Pst* DC3000 *avrRpm1* (Fig. 8D). DAB polymerizes instantly and locally upon contact with H_2O_2 to form reddish brown polymers. *CaMBL1*-OX plants, which were either uninoculated or inoculated with *Pst* DC3000 or *Pst* DC3000 *avrRPM1*, exhibited DAB-stained spots, indicating H_2O_2 accumulation to high levels. *CaMBL1*-OX plants exhibited significantly increased dark blue zones (HR-like cell death) compared with wild-type plants after inoculation with *Pst* DC3000 and *Pst* DC3000 *avrRPM1*. Cell death associated with the HR was quantified using the electrolyte leakage assay (Fig. 8E). *CaMBL1*-OX leaves exhibited higher levels of electrolyte leakage compared with wild-type leaves during *Pst* DC3000 and *Pst* DC3000 *avrRpm1* infection. We next quantified H_2O_2 levels at different times after infection using the xylenol orange assay (Fig. 8F). H_2O_2 production was significantly promoted in *CaMBL1*-OX leaves during *Pst* DC3000 and *Pst* DC3000 *avrRPM1* infection, as observed by DAB staining. Together, these results suggest that *CaMBL1* overexpression in

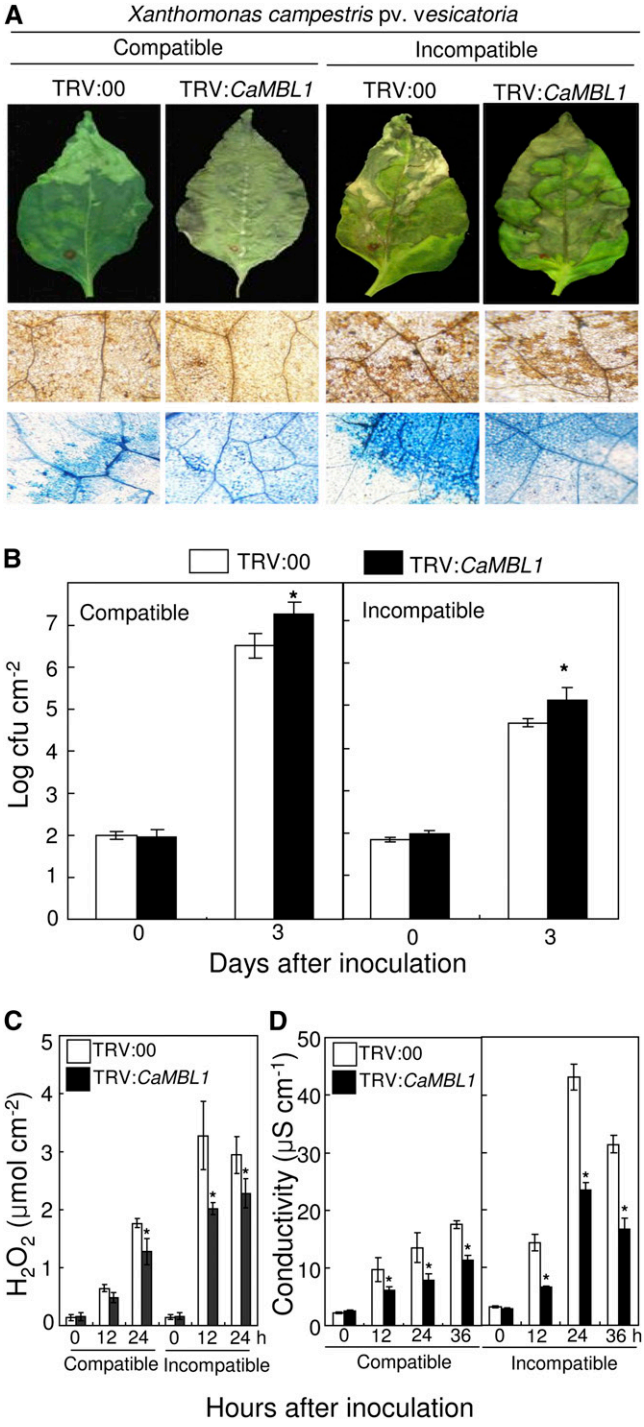

Figure 6. Enhanced susceptibility of *CaMBL1*-silenced pepper plants to *Xcv* infection with the virulent (compatible) strain Ds1 and the avirulent (incompatible) strain Bv5-4a. A, Disease symptoms developed on the empty vector control (TRV:00) and silenced (TRV:*CaMBL1*) leaves infected by *Xcv* strains. Infected leaves were stained with DAB and trypan blue. Bars = 0.2 mm. B, Bacterial growth in leaves of the empty vector control (TRV:00) and *CaMBL1*-silenced (TRV:*CaMBL1*) plants 0 and 3 d after inoculation with *Xcv* strains (5×10^4 cfu mL^{-1}). C, Quantification of H_2O_2 in leaves of empty vector control plants and *CaMBL1*-silenced plants after inoculation with *Xcv* strains using the xylenol orange assay. D, Measurement of electrolyte leakage from the leaf tissues at different time points after inoculation with *Xcv* strains. All experiments were repeated three times with similar results. Values are presented as means ± SD. Asterisks indicate significant differences between the means as determined by Student's *t* test ($P < 0.05$).

Arabidopsis may contribute to both basal defense and HR-mediated resistance to hemibiotrophic *Pst* infection.

Role of *CaMBL1* and the Arabidopsis Ortholog *At1g78830* in Resistance to *A. brassicicola* Infection

To investigate whether the expression of *CaMBL1* and the *Arabidopsis* ortholog *At1g78830* affects fungal infection, a spore suspension of *A. brassicicola* was drop inoculated on leaves of wild-type (ecotype Columbia [Col-0]), *CaMBL1*-OX, and *At1g78830*-defective Arabidopsis plants (Fig. 9). The predicted amino acid sequence of *At1g78830*, which contains an MBL domain, is 48% identical to that of *CaMBL1* (Supplemental Figs. S2 and S3). Two *At1g78830* T-DNA insertion mutants were investigated, *mbl1-1* (SALK_003821) and *mbl1-2* (SALK_121641; Fig. 9A). RT-PCR analysis did not detect *At1g78830* transcripts in *mbl1-1* or *mbl1-2* plants during *Pst* DC3000 *avrRpm1* infection (Fig. 9B), indicating that the *At1g78830* function is completely lost due to the T-DNA insertion. Typical spreading of susceptible lesions was observed in the leaves of *mbl1-1* and *mbl1-2* plants 5 d after inoculation with *A. brassicicola* (Fig. 9, C and E). However, wild-type and *CaMBL1*-OX leaves exhibited small necrotic lesions no larger than the initial inoculation droplet during the *A. brassicicola* infection. Trypan blue staining of infected leaf tissues showed restricted hyphal growth and cell death in wild-type and *CaMBL1*-OX leaves but extensive hyphal growth in *At1g78830* mutant leaves (Fig. 9D). These data indicate that the Arabidopsis ortholog *At1g78830* is required for resistance to *A. brassicicola* infection of Arabidopsis plants.

Distinct Responses of CaMBL1-OX and CaMBL1 Arabidopsis Ortholog, *mbl1*, Mutants to *Hyaloperonospora arabidopsidis* and *P. syringae* pv *tomato* Infection

To determine whether the Arabidopsis defense response to biotrophic oomycete infection is induced by *CaMBL1* and the *CaMBL1* Arabidopsis ortholog, we tested the responses of the wild type (Col-0), *CaMBL1*-OX Arabidopsis, and the *CaMBL1* Arabidopsis ortholog *At1g78830* mutant (*mbl1-1* and *mbl1-2*) plants to the biotrophic oomycete *H. arabidopsidis*. Over 100 wild-type and *CaMBL1*-OX plants were inoculated with an asexual spore suspension of *H. arabidopsidis* isolate Noco2 (5×10^4 conidiospores mL^{-1}), which is virulent to Arabidopsis Col-0. As shown in Supplemental Figure S4A, the infected cotyledons of wild-type plants exhibited high levels of mycelial growth, sporulation, and sporangiophores. *CaMBL1-OX* plants were also highly susceptible to *H. arabidopsidis* isolate

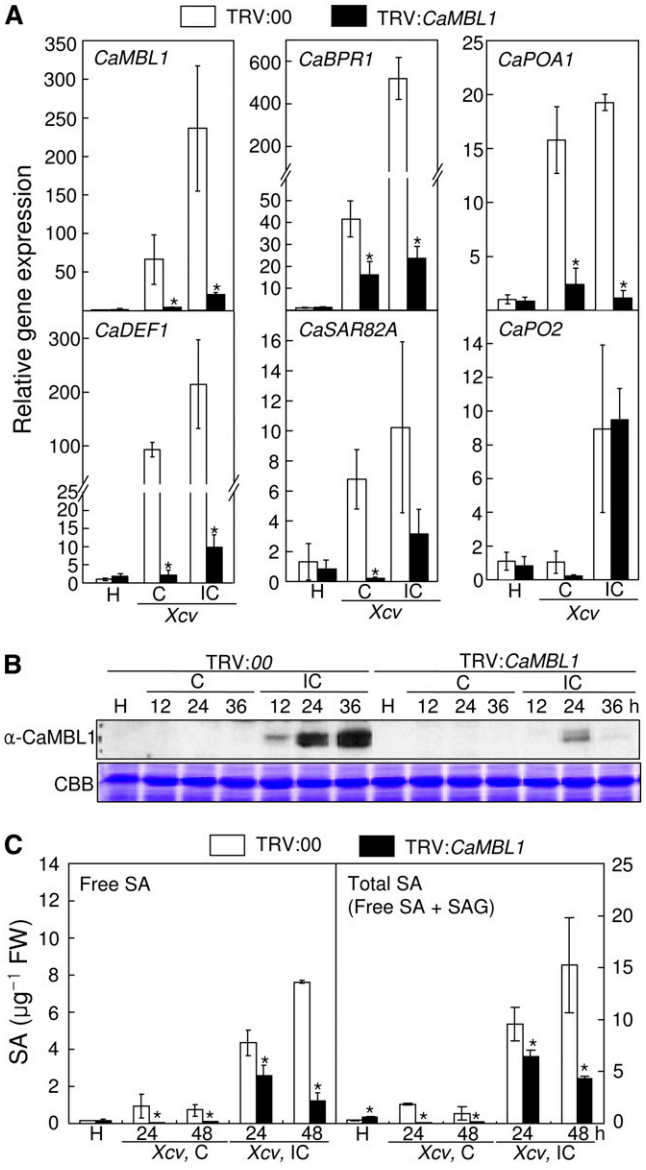

Figure 7. Effects of *CaMBL1* silencing on gene expression, protein expression, and SA accumulation in pepper leaves infected by the virulent (compatible) strain Ds1 and the avirulent (incompatible) strain Bv5-4a of *Xcv*. A, Real-time quantitative PCR analysis of the expression of *CaMBL1* and pepper defense-related genes in empty vector control leaves (TRV:00) and *CaMBL1*-silenced leaves (TRV:*CaMBL1*) at 18 h after inoculation with *Xcv*. The experiments were carried out three times. Transcript levels were normalized to the expression of pepper 18S ribosomal RNA measured in the same samples. H, Healthy leaves; C, compatible; IC, incompatible. B, Western-blot analysis of CaMBL1 proteins in empty vector control and *CaMBL1*-silenced leaves. Coomassie Brilliant Blue (CBB) staining is shown for the 60-kD regions of protein extracts. C, Levels of SA in empty vector control and *CaMBL1*-silenced leaves. Values are presented as means ± SD. Asterisks indicate significant differences with respect to empty vector control plants (Student's *t* test, $P < 0.05$). FW, Fresh weight. [See online article for color version of this figure.]

Noco2. No differences were observed between wild-type and *CaMBL1*-OX plants in mycelial growth, sporulation, and sporangiophores, as observed by trypan blue staining. The formation of conidiospores was scored for 7 d (Supplemental Fig. S4B). Extensive sporulation occurred in wild-type and *CaMBL1*-OX plants. Five days after inoculation, *H. arabidopsidis* infection was evaluated by measuring asexual sporangiophores on Arabidopsis cotyledons (Supplemental Fig. S4C). *CaMBL1*-OX plants exhibited the formation of sporangiophores similar to that on the cotyledons of wild-type plants. These results indicate that the *CaMBL1* gene does not confer enhanced defense response against the biotrophic oomycete *H. arabidopsidis* isolate Noco2. No significant defense responses to *H. arabidopsidis* isolate Noco2 and *Pst* DC3000 were observed in *CaMBL1* Arabidopsis ortholog *At1g78830* mutant plants (*mbl1-1* and *mbl1-2*), as assayed by measuring sporangiophores and bacterial growth (Supplemental Fig. S4, D and E).

DISCUSSION

MBLs (recently called the GNA-related lectins) are involved in many biological processes, including cell-to-cell and host-pathogen interactions, by specifically binding to carbohydrates (Peumans and Van Damme, 1995; Lis and Sharon, 1998; Van Damme et al., 2008). Notably, MBLs play a crucial role in the innate immune response through binding to carbohydrates on the surface of a wide range of microbial pathogens (Vijayan and Chandra, 1999; Barre et al., 2001). In this study, we isolated and functionally characterized the pepper *CaMBL1* gene, which was rapidly and strongly induced in pepper leaves during incompatible interactions with *Xcv*. The CaMBL1 protein contains the GNA-related lectin domain that is conserved in rice (Barre et al., 2001). Lectins known to be carbohydrate-binding proteins are ubiquitous in plants, and MBLs that contain the GNA-related lectin domain are representative of a new plant lectin family (Van Damme et al., 2008). Among the MBL genes, there are also *LecRLK* genes encoding receptor-like kinases with predicted extracellular bulb-type Man-specific B-lectin and intracellular Ser/Thr kinase domains (Riou et al., 2002). Some LecRLKs have been shown to be involved in *Rhizobium* symbiosis and in other cellular processes (Navarro-Gochicoa et al., 2003; Shiu et al., 2004). More recently, the rice *LecRLK* gene *Pi-d2* with these domains was suggested to confer resistance to the Chinese blast strain ZB15 of *Magnaporthe oryzae* (Chen et al., 2006). Intriguingly, a specific feature of the *CaMBL1* gene is that it contains only the GNA-related lectin domain, unlike these *LecRLK* genes, and phylogenetic analysis suggests that *CaMBL1* is more closely related to the group having only the GNA-related lectin domain. Notably, some pepper GNA-related lectins fall into similar clusters with CaMBL1 in the phylogenetic tree.

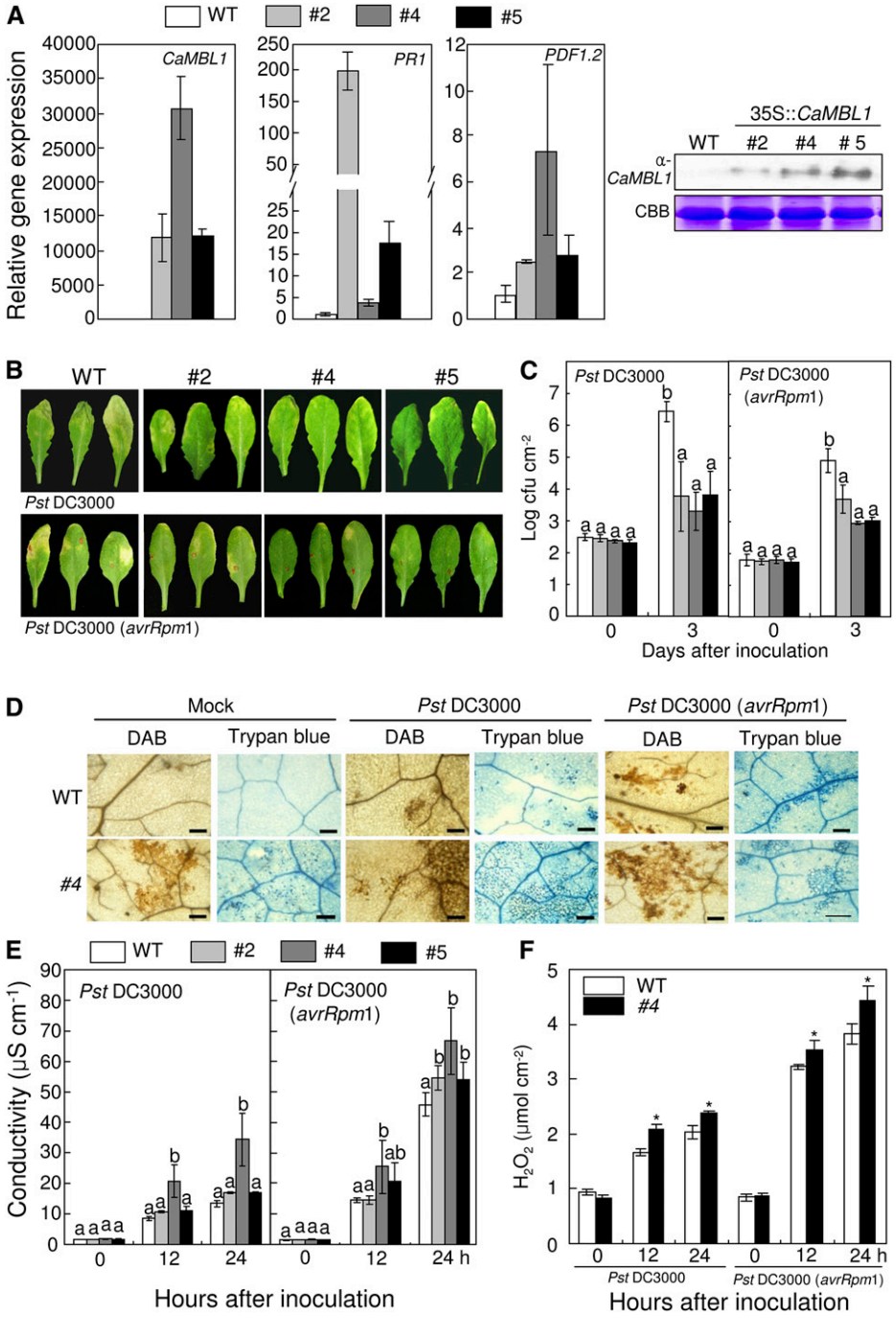

Figure 8. Enhanced resistance of *CaMBL1*-OX transgenic Arabidopsis plants to *P. syringae* pv *tomato* infection. A, Real-time quantitative PCR and western-blot analyses of the expression of *CaMBL1* and Arabidopsis defense-related genes in wild-type (WT) and transgenic plants (lines 2, 4, and 5). Transcript levels were normalized to the expression of pepper 18S ribosomal RNA measured in the same samples. B, Disease symptoms of wild-type and transgenic plants inoculated with *Pst* DC3000 strains. Leaves of 4-week-old Arabidopsis plants were infiltrated with a suspension (10^5 cfu mL^{-1}) of *Pst* DC3000 or *Pst* DC3000 *avrRpm1*. Disease symptoms were photographed 5 d after inoculation. C, Bacterial growth in leaves of wild-type and transgenic lines inoculated with *Pst* DC3000 and *Pst* DC3000 *avrRpm1*. D, DAB and trypan blue staining of leaf tissues of wild-type and transgenic plants 24 h after inoculation with *Pst* DC3000 or *Pst* DC3000 *avrRpm1*. Bars = 0.1 mm. E, Quantification of electrolyte leakage from leaf tissues inoculated with *Pst* DC3000 or *Pst* DC3000 *avrRpm1*. Samples were taken 0, 12, and 24 h after inoculation. F, Quantification of H_2O_2 in wild-type and transgenic plants after inoculation with *Pst* DC3000 or *Pst* DC3000 *avrRpm1* using the xylenol orange assay. Values are presented as means ± SD. Different letters indicate significant differences from three independent experiments based on the LSD test ($P < 0.05$). Asterisks indicate significant differences with respect to empty vector control plants (Student's *t* test, $P < 0.05$). [See online article for color version of this figure.]

It has been demonstrated that the consensus sequence motif (QxDxNxVxY) of GNA-related lectin domain is involved in α-D-Man recognition (Van Damme et al., 2008). However, CaMBL1 containing the GNA-related lectin domain lacks the putative Man-binding site sequence (QxDxNxVxY). Thus, we tested whether CaMBL1 has the Man-binding ability. Using D-Man-agarose (Sigma), we found that His-tagged CaMBL1 could bind to D-Man-agarose, suggesting that CaMBL1 may have Man-binding ability without the intact consensus sequence motif (QxDxNxVxY). The immunoblot analysis of purified CaMBL1 deletion mutants strongly supports the notion that its GNA-related lectin domain is responsible for binding D-Man, suggesting that pepper GNA-related lectin may recognize Man on the *Xcv* cell surface. However, we could not find any direct interaction (agglutination) between *Xcv* and purified CaMBP1 fusion protein (data not shown). Notably, the glycan array screening of CaMBL1 performed by the Consortium for Functional Glycomics also indicates that CaMBL1 has affinity toward Manα and/or

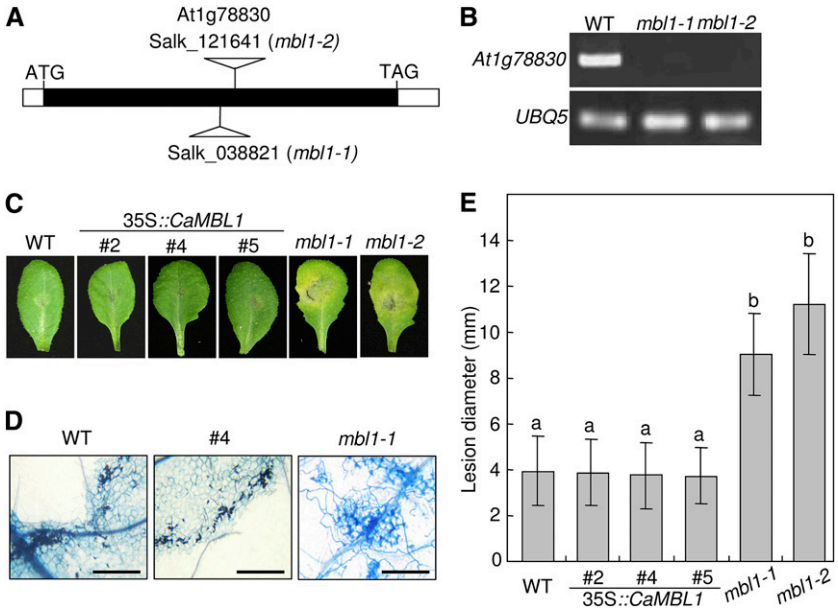

Figure 9. Disease development on the leaves of wild-type (WT), *CaMBL1*-OX, and Arabidopsis ortholog mutant (*mbl1-1* and *mbl1-2*) plants inoculated with *A. brassicicola*. Leaves of 4-week-old Arabidopsis plants were inoculated with 10-μL droplets of 5×10^4 spores mL^{-1}. A, Schematic representation of T-DNA insertion sites in *mbl1-1* (SALK_038821) and *mbl1-2* (SALK_121641) plants and the genomic structure of the *At1g78830* gene. The *At1g78830* exon is represented by the black box. B, RT-PCR analysis of expression of the *mbl1-1* and *mbl1-2* genes 15 h after inoculation with *Pst* DC3000 *avrRpm1*. C, Disease symptoms on leaves 5 d after inoculation. D, Microscopic images of fungal structures and damaged cells of infected leaves stained with trypan blue. Bars = 0.1 mm. E, Quantification of disease development based on the average diameter of lesions caused by *A. brassicicola*. The size of lesions is an average of 30 lesions per line. Values are presented as means ± SD. Different letters indicate significant differences from three independent experiments based on the LSD test ($P < 0.05$). [See online article for color version of this figure.]

Manβ and GalNAc residues. Tsutsui et al. (2006) revealed that only the first of the two consensus sequence motifs (QxDxNxVxY) is required for Man-binding activity of pufflectins identified in the skin mucus of the fugu (*Takifugu rubripes*). In that study, three mutants of the second motif (QxDxNxVxY) could bind to Man. These findings supports the possibility that the consensus sequence motif (QxDxNxVxY) of the GNA-related lectin domain is not necessarily essential for Man-binding activity.

Animal MBLs bind Man-based carbohydrates on bacterial plasma membranes (Fujita et al., 2004). The finding that *CaMBL1* encodes a GNA-related lectin domain is consistent with the observation that GFP-tagged CaMBL1 localizes to the plasma membrane of onion epidermal cells, suggesting that CaMBL1 does the same in its native pepper cells. We also tested the subcellular localization of CaMBL1 in pepper leaves. Indeed, CaMBL1 was mainly detected in the microsomal fraction including plasma membrane from pepper leaves during *Xcv* infection. In a subcellular localization experiment with a GNA domain:smGFP construct, we also confirmed that the GNA-related lectin domain is responsible for the localization of CaMBL1 to the plasma membrane. Together, these data suggest that the plasma membrane targeting of the CaMBL1 protein may be involved in the specific recognition of plant pathogens by GNA-related lectins. This proposal is well supported by the finding of Chen et al. (2006) that a B-lectin receptor kinase protein that triggers rice blast resistance is plasma membrane localized.

Most plant lectins may be involved in defense responses during pathogen infection by recognizing a wide range of pathogens (Peumans and Van Damme, 1995). Lectins are generally expressed at relatively low levels in a tissue- or organ-specific manner. In contrast, some lectins are induced to higher levels in plants following attack by other organisms or by stress. Plant lectins, including the GNA-related lectins, are believed to play a role in recognizing and binding high-Man glycans of foreign microorganisms or plant pathogens (Barre et al., 2001). GNA-related lectins are associated with specific resistance responses to herbivorous higher animals or phytophagous invertebrates (Peumans et al., 2002). In wheat (*Triticum aestivum*) resistant to the Hessian fly, induced GNA-related lectins recognize Man-rich glycans of microorganisms or plant predators, suggesting that this early recognition may play a significant role in defense responses (Subramanyam et al., 2008). However, with the exception of LecRLKs, the functions of plant lectins, such as those of GNA-related lectins in plant-pathogen interactions, are poorly understood. We found CaMBL1 expression to

be pathogen specific; however, the role of CaMBL1 as a defense protein in plants remains to be elucidated. In incompatible interactions with *Xcv*, *CaMBL1* was strongly and rapidly induced compared with its response in compatible interactions. These results suggest that *CaMBL1* is crucial in pepper plants for overall defense responses to pathogen invasion. Similarly, an MBL gene is expressed in maize (*Zea mays*) plants early during *Colletotrichum graminicola* infection (Sugui and Deising, 2002).

In plants, GNA-related lectins may be involved in pathogen recognition at an early stage of infection. The expression of *CaMBL1* was induced rapidly and strongly during incompatible interactions with *Xcv*. Thus, its expression may lead to the activation of defense-related genes. Silencing of *CaMBL1* resulted in enhanced disease susceptibility, increased bacterial growth, lowered accumulation of ROS, as well as reduced expression of *PR* genes in pepper leaves during virulent or avirulent *Xcv* infection. These findings support the notion that *CaMBL1* expression during *Xcv* infection activates basal resistance in pepper plants, which is accompanied by the induction of *PR* genes. Reduced SA levels in *CaMBL1*-silenced plants impaired their ability to mount responses such as defense-related gene expression and HR-like cell death. Taken together, these data suggest that the *CaMBL1* gene is a positive regulator of SA-dependent cell death and defense responses during *Xcv* infection.

Interestingly, CaMBL1 induced cell death responses in pepper leaves when it was transiently expressed by agroinfiltration, as evidenced by remarkably high levels of SA with concomitant *PR1* activation in *CaMBL1*-overexpressing pepper leaves. More importantly, domain analyses of *CaMBL1*-induced cell death in pepper leaves revealed that the GNA-related lectin domain is responsible for this phenomenon. Collectively, these results suggest that induction of *CaMBL1*, as a positive regulator of cell death and defense responses, causes SA to accumulate in pepper leaf tissues and trigger the expression of downstream defense-related genes, such as *CaBPR1* and *CaSAR82A*.

MBLs or GNA-related lectins may contribute to an innate immunity as a first line in plant defense (De Hoff et al., 2009). To determine whether overexpression of *CaMBL1* confers defense response to heterologous plants, we generated *CaMBL1*-OX transgenic lines in Arabidopsis, which were resistant to virulent and avirulent *P. syringae* pv *tomato* infection. Resistance responses in the transgenic plants included ROS accumulation, cellular membrane damage, and necrotic disease symptoms. These findings suggest that overexpression of the *CaMBL1* gene in Arabidopsis enhances basal or *R* gene-mediated resistance to bacterial infection. Inhibition of *P. syringae* pv *tomato* infection by overexpression of *CaMBL1*, which localizes to the plasma membrane, may be strongly supported by the activation of *PR* genes, induction of ROS accumulation, and cell death in Arabidopsis leaves following recognition of bacterial pathogens. However, there may be no correlation between CaMBL1 transcript and protein levels induced in the *CaMBL1-OX* Arabidopsis leaves by *Pst* DC 3000 infection. Furthermore, constitutively ectopic expression of the transgene *CaMBL1* distinctly primes the induction of other defense-related genes, such as *PR1*, which may be dependent on the transgenic Arabidopsis lines. The susceptible response of Arabidopsis ortholog *At1g78830* mutant plants to the fungal pathogen *A. brassicicola* also suggests that CaMBL1 is required for enhanced resistance to fungal pathogens in pepper plants.

GNA-related lectins constitute an extended superfamily of structurally and evolutionarily related proteins that play crucial roles for plant defenses. We have shown that *CaMBL1* is required for enhanced resistance to the bacterial pathogen *Xcv* as well as to the fungal pathogen *A. brassicicola*. These results support the notion that *CaMBL1* is effective for the recognition of bacterial and fungal pathogens on plant cell surfaces. Upon establishing contact with a host surface, pathogens release extracellular matrix-containing glycoproteins with Man residues (Sugui et al., 1998; Barre et al., 2001). Once GNA-related lectins recognize pathogens, their lectin domain could bind to Man or lipooligosaccharide residues on their surfaces. Therefore, expression of *CaMBL1* may provide cues for the plant to specifically recognize pathogens and to promote further defense responses.

Taking these results together, we propose a working model for *CaMBL1*-mediated defense responses in plants (Fig. 10). Plants recognize a diversity of attacking pathogens, including virulent and avirulent *Xcv*,

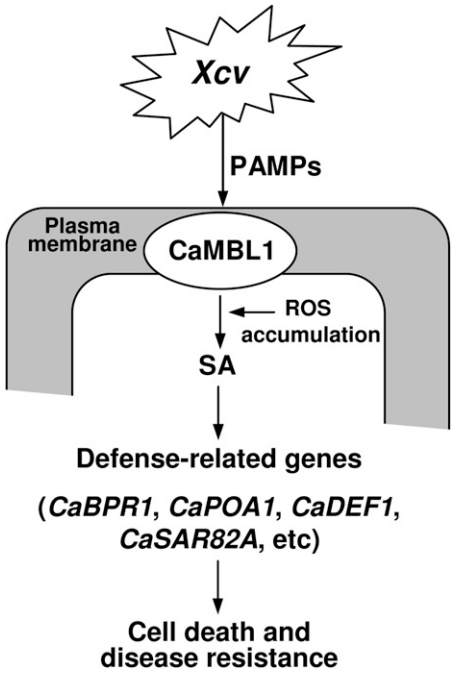

Figure 10. A working model for CaMBL1 function in pepper leaf cells during *Xcv* infection. PAMPs, Pathogen-associated molecular patterns.

most likely by interactions of GNA-related lectins with a subset of pathogen-associated molecular patterns in the host plasma membrane. In this study, we showed that pathogen recognition by *CaMBL1* triggers the accumulation of ROS and of the defense signal molecule SA in pepper plants, which in turn induces the expression of defense-related genes such as *CaBPR1*, *CaPOA1*, *CaDEF1*, and *CaSAR82A*. The *CaMBL1* transcript does not accumulate in the absence of pathogens, but as a result of rapid *CaMBL1* gene activation in response to their presence, pepper plants exhibit enhanced resistance to pathogens. In particular, plasma membrane-localized CaMBL1 is strongly expressed at an early stage of infection. However, it remains unclear how the recognition by CaMBL1 can lead to hypersensitive cell death, which is inevitably associated with ROS and SA accumulation, the expression of defense-related genes, and enhanced resistance. The presence of the GNA-related lectin domain supports a direct role for CaMBL1 in pathogen recognition, despite the absence of intracellular Ser/Thr kinase domains. Furthermore, experimental data using *CaMBL1*-silenced pepper and *CaMBL1*-OX Arabidopsis plants also support the notion that recognition by CaMBL1 may play a significant role in specific bacterial pathogen-plant interactions. Pathogen-induced *CaMBL1* gene expression may alert the plant to invading pathogens, ultimately leading to cell death and disease resistance responses. We propose that *CaMBL1* expression also triggers regulatory mechanisms to control defense responses in plants. Further studies of the GNA-related lectin protein CaMBL1 in pepper plants are required to reveal how this is achieved.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pepper plants (*Capsicum annuum* 'Nockwang') were used in this study. Pepper seeds were germinated at 27°C. Plants were raised in a plastic tray (55 × 35 × 15 cm) and were transplanted to pots at the two-leaf stage. All plants were cultivated in a soil mix (peat moss:vermiculite:perlite, 3:3:1, v/v/v) at 26°C ± 2°C under a 16-h-light/8-h-dark cycle.

Wild-type ecotype Col-0 and transgenic lines of Arabidopsis (*Arabidopsis thaliana*) were also studied. *CaMBL1* Arabidopsis ortholog *At1g78830* mutant seeds (*mbl1-1* and *mbl1-2*) were obtained from the Salk Institute T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress). Prior to sowing on soil (peat moss:vermiculite:perlite, 1:1:0.5, v/v/v), seeds were vernalized at 4°C under low light for 2 d. Arabidopsis plants were raised in a growth chamber at 24°C with a 14-h-light/10-h-dark cycle.

Pathogen Inoculation and Disease Rating

The virulent strain Ds1 and avirulent strain Bv5-4a of *Xanthomonas campestris* pv *vesicatoria* were used to inoculate pepper plants. To prepare bacterial inocula, *Xcv* was grown on yeast-nutrient broth (5 g of yeast extract, 8 g of nutrient broth, and 1 L of water) at 28°C for 18 h. Pepper plants at the six-leaf stage were inoculated by infiltrating with a suspension (5×10^8 cfu mL^{-1}) of the virulent strain Ds1 or the avirulent strain Bv5-4a or were mock infiltrated with 10 mM $MgCl_2$. The inoculated plants were incubated for 18 h in a moist chamber at 28°C and then transferred to a growth room. Infected leaves were sampled at various times after inoculation.

Pseudomonas syringae pv *tomato* DC3000 and DC3000 expressing *avrRpm1* were grown overnight at 28°C on yeast-nutrient broth containing rifampicin (50 μg mL^{-1}). Arabidopsis plants were infiltrated with a 10^5 cfu mL^{-1} bacterial suspension in 10 mM $MgCl_2$. To assess bacterial growth, infected leaves were collected 0 and 3 d after inoculation.

Hyaloperonospora arabidopsidis isolate Noco2 was maintained on Arabidopsis Col-0 by weekly subculturing. To produce a large quantity of inocula, 7- to 10-d-old seedlings were inoculated with *H. arabidopsidis*, and the spores produced on cotyledons were collected in water. The seedlings were spray inoculated with a suspension of asexual inoculum (5×10^4 conidiosporangia mL^{-1}), covered with a transparent dome to maintain high humidity (80%–100%), and incubated for 7 d at 17°C. Asexual sporulation of *H. arabidopsidis* was assessed by using a light stereomicroscope to count the number of sporangiophores on both sides of the cotyledons 7 d after inoculation. The extent of disease was assigned to five classes based on the number of sporangiophores per cotyledon: zero to five, six to 10, 11 to 15, 16 to 20, and over 20. For the spore-count assay, the infected cotyledons of each line were excised and shaken vigorously. Spores harvested from 20 cotyledons per replicate were counted using a hemacytometer.

Alternaria brassicicola was cultured on potato dextrose agar medium at 24°C for 10 d. Conidial concentration was determined using a hemacytometer and adjusted to 5×10^4 conidia mL^{-1}. *A. brassicicola* was inoculated by placing 10-μL droplets of suspension on leaves of 5-week-old plants. For mock treatment, 10-μL droplets of water were placed onto the leaves. Inoculated plants were kept at 100% relative humidity at 24°C. Four days after inoculation, lesion diameters were measured.

Treatment with SA and Methyl Jasmonate

The leaves of pepper plants at the six-leaf stage were treated with SA (5 mM) and methyl jasmonate (100 μM). SA and methyl jasmonate were sprayed onto pepper plants at the six-leaf stage. The pepper plants treated with methyl jasmonate were incubated in a vinyl bag. Control plants were sprayed with water. Pepper plants were sampled at various times after treatment, frozen in liquid nitrogen, and stored at −70°C for RNA isolation.

Isolation of the *CaMBL1* Gene from a Pepper cDNA Library

A pathogen-induced cDNA library was prepared from total RNA extracted from pepper leaves 18 h after inoculation with *Xcv* using a λZAPII-cDNA library synthesis kit (Jung and Hwang, 2000). Differential hybridization was performed to isolate pathogen-induced cDNAs from the library using cDNA probes from uninoculated pepper leaves or leaves inoculated with the *Xcv* avirulent strain Bv5-4a.

Gene-specific RT-PCR was performed to confirm the differential expression of genes identified on an expression array. Digoxigenin-labeled single-stranded cDNAs were synthesized using total RNA from healthy and bacteria-infected pepper leaves. Total RNA (20 μg) from pepper was mixed with 5× reaction buffer (5× avian myeloblastosis virus [AMV] RT buffer), AMV reverse transcriptase (Roche), and oligo$(dT)_{15}$ as a primer in diethyl pyrocarbonate water. To produce probes, RT-PCR was performed using RNA and primer mixes at 25°C for 10 min, 42°C for 60 min, and 99°C for 5 min. Equal amounts of the single-stranded cDNA probes from uninoculated control leaves and leaves inoculated with *Xcv* were hybridized to the two pepper cDNA expression arrays in a separate bottle for 18 h at 65°C in a mixture of 5× SSC, 0.1% sodium lauroylsarcosine, 0.02% SDS, and 10% blocking reagent. The hybridization membranes were washed twice in solution 1 (2× SSC and 0.1% SDS) for 10 min each at room temperature and then twice at 65°C for 5 min each in solution 2 (0.1× SSC and 0.1% SDS). The blotted membranes were exposed to x-ray film. The hybridization patterns produced by 96 samples each of uninoculated and inoculated samples were compared.

Several cDNA clones that were expressed in pepper leaves inoculated with the avirulent strain Bv5-4a of *Xcv* were isolated and sequenced with an ABI 310 DNA sequencer (PE Biosystems) using PRISM BigDYE Terminator sequencing-ready reaction kits. The DNA sequences were verified and analyzed using BLAST network services at the National Center for Biotechnology Information and the ExPASy Proteomics Server. A full-length cDNA clone encoding a pepper glycoprotein, designated *CaMBL1*, was isolated and analyzed.

Expression of the CaMBL1 Protein and Its Binding Assay with Man-Agarose

The *CaMBL1* gene was cloned into the vector pET28a to generate pET28a::*CaMBL1*, which created a translational fusion of CaMBL1 to a His tag at the

N terminus. The CaMBL1 protein construct was expressed in *Escherichia coli* and purified on a Man-agarose column. The *CaMBL1* deletion series was made by PCR with specific primers CaMBL1 forward (5′-GGATCCATGTCTCCTTCATGGACTACTAC-3′) and *CaMBL1* reverse (5′-AAGCTTTTAAATCCTGCTGTAAGTTACC-3′); *CaMBL1-1* forward (5′-GGATCCATGCAAGTTCCAGCTGAAAACA-3′) and *CaMBL1-1* reverse (5′-AAGCTTTTAAATCCTGCTGTAAGTTACC-3′); *CaMBL1-2* forward (5′-GGATCCATGATTGTCGAATACAGCGCTGA-3′) and *CaMBL1-2* reverse (5′-TTAAATCCTGCTGTAAGTTACC-3′); and *CaMBL1-3* forward (5′-GGATCCATGTATAGCTTGGTGGTGC-3′) and *CaMBL1-3* reverse (5′-AAGCTTTTAAATCCTGCTGTAAGTTAC-3′). To express the *CaMBL1* gene, pET28a::*CaMBL1* was transformed into *E. coli* BL21 (DE3). Recombinant strains were grown in 50 mL of Luria-Bertani medium at 37°C to an optical density at 600 nm (OD_{600}) = 0.4 to 0.5, induced with 1 mM isopropyl-D-thiogalactopyranoside for 3 h at 18°C, and harvested. Cells were collected by centrifugation, resuspended in 10 mL of Man-binding buffer (50 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM $CaCl_2$), and disrupted by sonication. Cell debris was removed by centrifugation, and the supernatant was applied to a D-Man-agarose column (Sigma). The column was incubated in the presence of the protein extract for 30 min. After unbound proteins were washed out with Man-binding buffer, bound proteins were eluted from the column using Man-binding buffer containing 100 mM D-Man. Purified CaMBL1 was subjected to 15% SDS-PAGE. Protein bands were electrotransferred from an unstained gel onto Hybond-P membranes (GE Healthcare Bioscience). For detection of proteins, an anti-His antibody (Sigma) was used at 1:5,000 dilution.

Subcellular Localization

Plasmids harboring *smGFP*, *CaMBL1*:*smGFP*, *GNA domain*:*smGFP*, and *CaMBL1*c:*smGFP* were transformed into onion (*Allium cepa*) epidermal cells by particle bombardment (Bio-Rad) using 5 μg of each plasmid DNA, which was precipitated onto gold particles. After bombardment, the onion epidermal cells on Murashige and Skoog agar plates were incubated for 18 h at 25°C in the dark. Subcellular localization of fused proteins was visualized in onion epidermal cells by confocal laser scanning fluorescence microscopy (Carl Zeiss LSM 5 Exciter).

Agrobacterium tumefaciens-Mediated Transient Expression

Constructs containing pBIN35S:*CaMBL1* or 35S:*CaMBL1*:8Myc were introduced into the *Agrobacterium* strain GV3101 by direct transformation. Recombinant *Agrobacterium* was grown overnight at 28°C, collected by centrifugation, and resuspended to a final concentration of OD_{600} = 1.0 in induction medium (10 mM ethanesulfonic acid, pH 5.7, and 10 mM $MgCl_2$). In experiments to test the efficacy of additives, acetosyringone was added to the liquid culture to a final concentration of 200 μM at pH 5.6. The cell suspensions were incubated at 28°C for 3 h before infiltration. *Agrobacterium* suspensions expressing *CaMBL1* were injected at different concentrations into pepper leaves.

VIGS

TRV-based vectors (pTRV1/2) were used for VIGS of the *CaMBL1* gene (Liu et al., 2002). An 861-bp fragment of the *CaMBL1* cDNA was amplified by PCR to specifically silence *CaMBL1*. The resulting PCR product was cloned into pTRV2 to generate pTRV2:*CaMBL1*. The TRV2 derivative *pTRV:CaMBL1* was transformed into *Agrobacterium* strain GV3101, and transformed cells were selected on yeast extract peptone medium with 50 $\mu g\ mL^{-1}$ kanamycin and 100 $\mu g\ mL^{-1}$ rifampicin. *Agrobacterium* GV3101 containing pTRV1 and pTRV2:*CaMBL1* VIGS vectors was coinfiltrated into fully expanded pepper cotyledons using a syringe (OD_{600} = 0.4). A positive control was infiltrated with a VIGS clone containing the *Phytoene Desaturase* gene. A negative control was infiltrated with an empty vector clone (TRV:00). The inoculated plants were transferred to a growth chamber maintained at 17°C for 2 d with 60% relative humidity and then placed in a growth room at 26°C ± 2°C with a 16-h-light/8-h-dark cycle. Unsilenced (TRV:00) and *CaMBL1*-silenced (TRV:*CaMBL1*) plants were inoculated with the virulent Ds1 and avirulent Bv5-4a strains of *Xcv* to examine gene expression (10^6 cfu mL^{-1}) and to assay bacterial growth (5×10^4 cfu mL^{-1}). Bacterial cells were counted 0 and 3 d after inoculation.

Generation of Arabidopsis Plants Overexpressing *CaMBL1*

To generate the *CaMBL1*-OE construct, the *CaMBL1* coding sequence obtained from a pBluescript SK2 (Stratagene) construct by *Xba*I and *Bam*HI restriction was subcloned into the vector pCR2.1-TOPO (Invitrogen). This fragment was ligated into the binary expression vector pBIN35S. The primers used in the PCR to generate *Xba*I and *Bam*HI sites were 5′-TCTAGAATGTCTCCTTCATGGACTACTAG-3′ (forward) and 5′-GGATCCTTAAATCCTGCTTAAGTTACC-3′ (reverse). The vector pBIN35S containing the cauliflower mosaic virus 35S promoter-*CaMBL1* construct was transformed into *Agrobacterium* strain EHA105 via electroporation.

To generate transgenic Arabidopsis, 4-week-old Arabidopsis plants were infected with *Agrobacterium* strain EHA105 by the floral dip method (Clough and Bent, 1998). Seeds were collected from transformed Arabidopsis, and plants were selected on Murashige and Skoog agar plates with 50 $\mu g\ mL^{-1}$ kanamycin to obtain independent transgenic lines. The presence of the transgene was confirmed by PCR.

Identification of T-DNA Insertion Mutant Lines

The T-DNA insertion lines (SALK_121641 and SALK_038821) of At1g78830 were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/) at Ohio State University. Homozygous mutant plants were identified by PCR using T-DNA and gene-specific primer sets as described on the T-DNA Express Web site (http://signal.salk.edu/tdnaprimers.html). Two sets of PCR were carried out using the three primers (LP, left gene-specific primer; RP, right gene-specific primer; LB, left border primer of the T-DNA insertion) for each Salk line: LP primer (5′-GAGCGGTATTCCAAGTGGAG-3′) and RP primer (5′-TACATCTGATTCGAACGGCTC-3′) for SALK_121641; LP primer (5′-ACGGTGGATCATTACAAGCAC-3′) and RP primer (5′-ATTCCAAATCTTACCCAACGG-3′) for SALK_038821; and LBa1 primer (5′-TGGTTCACGTAGTGGGCCATCG-3′).

Northern, RT-PCR, and Quantitative Real-Time PCR Analyses

Total RNA was isolated from pepper leaves, stems, roots, flowers, and fruits and from Arabidopsis plants using TRIzol (Invitrogen) according to the manufacturer's instructions. Leaf samples were harvested and immersed in liquid nitrogen at different times after inoculation with *Xcv*. Total RNA was extracted from the samples by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The total RNA concentration and purity were estimated by spectrophotometry and by ribosomal RNA staining with ethidium bromide, respectively. Total RNAs (10 μg) were fractionated on 1.2% agarose gels containing 7.4% formaldehyde and transferred onto nylon membranes (Hybond+; Amersham) for 18 h followed by UV cross-linking. A biotin probe (14-dCTP-biotin) was prepared from the corresponding pepper cDNA, and *CaMBL1* and *CaBPR1* were amplified with the following specific primer sets: *CaMBL1*, 5′-AAAATCAACAACGAGAAGTTCACATGTTAT-3′ (forward) and 5′-AACTAATTTATTGGACTAGCTGTGGACCCA-3′ (reverse); *CaBPR1*, 5′-ATGGGACACTCTAATATTGCC-3′ (forward) and 5′-GACATCAGTTGGAAGTTCCAA-3′ (reverse). After probe generation, membranes were prehybridized for 3 h at 65°C and then hybridized in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA at 65°C overnight. Membranes were washed twice with 2× SSC, 0.1% SDS for 10 min each at room temperature and twice in 0.1× SSC, 0.1% SDS for 15 min each at 65°C. Membranes were exposed to x-ray film.

For RT-PCR analysis, first-strand cDNA synthesis was carried out in a reaction volume of 20 μL with 1 μg of RNA, 10 units μL^{-1} AMV reverse transcriptase (BIO BASIC), 2 μL of oligo(dT), 2 μL of deoxyribonucleotide triphosphates, and 2× AMV-RT buffer at 42°C for 60 min. PCR was performed in a final volume of 50 μL per reaction containing 0.5 μg of cDNA, 250 nM forward and reverse primers, 10× Taq reaction buffer (Takara), 0.2 mM deoxyribonucleotide triphosphates (Takara), and 0.5 units of Taq polymerase (Takara). Thirty cycles of amplification were preceded by an initial denaturation at 95°C for 5 min, with each amplification cycle including a 30-s denaturation at 95°C, a 30-s annealing at 55°C to 58°C (primer pair specific), and a 1-min extension at 72°C. After the amplification cycles, the samples were subjected to a 10-min extension at 72°C.

Quantitative real-time PCR using the Bio-Rad iCycler System was performed to monitor levels of gene expression in plants. Each reaction (20 μL)

mix contained 10 μL of SYBR Green super mix (Bio-Rad) and 0.2 μM gene-specific primers. Thermal cycling conditions consisted of 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 55°C. Data acquisition and analysis were performed by using iCycler Real-Time PCR Detection System software (Bio-Rad). Transcript levels were normalized to the expression of pepper 18S ribosomal RNA measured in the same samples.

Protein Extraction and Immunoblotting

For immunoblotting with the anti-cMyc (Sigma) and anti-CaMBL1 antibodies, total protein was extracted from 0.5 g of leaf tissues homogenized in 1 mL of extraction buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, and 1× proteinase inhibitor cocktail [Roche]). Crude protein extracts were immunoprecipitated on an anti-c-Myc agarose affinity gel (Sigma) overnight at 4°C. Protein extracts were separated by 12% SDS-PAGE and blotted onto Hybond-P membranes (GE Healthcare Bioscience). Fusion proteins were detected using a mouse anti-c-Myc antibody (Sigma) at 1:2,000 dilution. An anti-CaMBL1 antibody was raised in a rabbit against a synthetic peptide corresponding to C-terminal residues 284 to 297 of CaMBL1 (AbFrontier). Leaf protein extracts were immunoblotted using the anti-CaMBL1 antibody at 1:5,000 dilution, which was able to detect only CaMBL1 from the leaf extracts.

Measurement of SA

SA and SA glycoside were extracted and quantified according to the method described by Verberne et al. (2002). Leaf tissue samples (0.5 g) were frozen in liquid nitrogen, ground to a fine power, and sequentially extracted with 90% and 100% methanol. As an internal standard for SA, 3-hydroxybenzoic acid (Sigma) was added at a mass ratio of 50 mg g^{-1} fresh weight. SA was determined by fluorescence (excitation, 305 nm; emission, 405 nm) after separation on a C_{18} reverse-phase HPLC column (Waters).

Measurement of Electrolyte Leakage

Pepper leaves were infiltrated with *Agrobacterium* strain GV3101 carrying the pBIN:*CaMBL1* construct to determine electrolyte leakage. Leaf discs (1 cm in diameter) were washed in 20 mL of double distilled water for 30 min and transferred into 20 mL of double distilled water. Ion leakage was measured 0, 12, 24, and 36 h after infiltration. Conductance was measured with a conductivity meter (model sensION7; Hach). Electrical conductivities of the medium were expressed in μS cm^{-1}.

Quantification of H_2O_2

H_2O_2 production in plants was measured by ferrous oxidation using the xylenol orange assay (Choi et al., 2007). One milliliter of freshly prepared assay reagent (25 mM $FeSO_4$ and 25 mM $[NH_4]_2SO_4$ dissolved in 2.5 M H_2SO_4) was added to 100 mL of 125 μM xylenol orange and 100 mM sorbitol. Leaf discs (0.5 cm^2) were floated for 10 min in 1 mL of distilled water, followed by centrifugation at 5,000*g* for 1 min. The supernatant (100 μL) was incubated for 30 min in 1 mL of xylenol orange reagent. H_2O_2 was determined at 560 nm using a standard H_2O_2 curve.

Staining with DAB and Trypan Blue

Infection and development of pathogens were assessed by staining inoculated plants with 1 mg mL^{-1} DAB (Sigma D8001) and lactophenol-trypan blue (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of trypan blue, dissolved in 10 mL of distilled water). After overnight treatment with DAB, the stained leaves were cleared by boiling for 10 min in absolute ethanol and then destained overnight in absolute ethanol. The inoculated leaves were also boiled in trypan blue staining solution for 5 min and destained overnight in chloral hydrate (2.5 g of chloral hydrate dissolved in 1 mL of distilled water). The destained plant tissues were mounted in 70% glycerol for observation with a microscope.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GQ265892 (*CaMBL1*), AF053343 (*CaBPR1*), AF442388 (*CaDEF1*), AF442387 (*CaPOA1*), AF313766 (*CaSAR82A*), DQ489711 (*CaPO2*), *PR1* (At2g14610), *PDF1.2* (At5g44420), NP_565191 (At1g78830), and *UBQ5* (At3g62250).

Supplemental Data

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

Supplemental Figure S1. Nucleotide and deduced amino acid sequences of pepper *CaMBL1* cDNA encoding a GNA-related lectin protein.

Supplemental Figure S2. Alignment of pepper *CaMBL1* with other GNA-related lectins.

Supplemental Figure S3. Phylogenetic tree analysis of *CaMBL1* with other GNA-related lectins.

Supplemental Figure S4. Disease responses of *CaMBL1*-OX and Arabidopsis ortholog mutants *mbl1-1* and *mbl1-2* to *H. arabidopsidis* isolate Noco2 and *Pst* DC3000 infection.

Supplemental Figure S5. Glycan-binding profile of CaMBL1 as analyzed by the Consortium for Functional Glycomics.

Supplemental Table S1. The glycan binding data of CaMBL1 as analyzed by the Consortium for Functional Glycomics.

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LITERATURE CITED

Barre A, Bourne Y, Van Damme EJM, Peumans WJ, Rougé P (2001) Mannose-binding plant lectins: different structural scaffolds for a common sugar-recognition process. Biochimie **83:** 645–651

Barre A, Herve C, Lescure B, Rougé P (2002) Lectin receptor kinases in plants. Crit Rev Plant Sci **21:** 379–399

Broekaert WF, van Parijs J, Leyns F, Joos H, Peumans WJ (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science **245:** 1100–1102

Chen X, Shang J, Chen D, Lei C, Zou Y, Zhai W, Liu G, Xu J, Ling Z, Cao G, et al (2006) A B-lectin receptor kinase gene conferring rice blast resistance. Plant J **46:** 794–804

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124:** 803–814

Choi HW, Kim YJ, Lee SC, Hong JK, Hwang BK (2007) Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO2 activates local and systemic cell death and defense response to bacterial pathogens. Plant Physiol **145:** 890–904

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156–159

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Cox KD, Layne DR, Scorza R, Schnabel G (2006) *Gastrodia* anti-fungal protein from the orchid *Gastrodia elata* confers disease resistance to root pathogens in transgenic tobacco. Planta **224:** 1373–1383

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826–833

Davies HA, Daniels MJ, Dow JM (1997) Induction of extracellular matrix glycoproteins in *Brassica* petioles by wounding and in response to *Xanthomonas campestris*. Mol Plant Microbe Interact **10:** 812–820

Deepak S, Shailasree S, Kini RK, Hause B, Shetty SH, Mithöfer A (2007) Role of hydroxyproline-rich glycoproteins in resistance of pearl millet against downy mildew pathogen *Sclerospora graminicola*. Planta **226:** 323–333

De Hoff PL, Brill LM, Hirsch AM (2009) Plant lectins: the ties that bind in root symbiosis and plant defense. Mol Genet Genomics **282:** 1–15

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell **7:** 1085–1097

Fujita T, Matsushita M, Endo Y (2004) The lectin-complement pathway: its role in innate immunity and evolution. Immunol Rev **198:** 185–202

Garcia-Brugger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B, Wendehenne D, Pugin A (2006) Early signaling events induced by elicitors of plant defenses. Mol Plant Microbe Interact **19:** 711–724

Hammond-Kosack KE, Parker JE (2003) Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. Curr Opin Biotechnol **14:** 177–193

Jones JDG, Dangl JL (2006) The plant immune system. Nature **444:** 323–329

Jung HW, Hwang BK (2000) Isolation, partial sequencing, and expression of pathogenesis-related cDNA genes from pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria*. Mol Plant Microbe Interact **13:** 136–142

Lis H, Sharon N (1998) Lectins: carbohydrate-specific proteins that mediate cellular recognition. Chem Rev **98:** 637–674

Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. Plant J **31:** 777–786

Mellersh DG, Heath MC (2001) Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell **13:** 413–424

Michiels K, Van Damme EJ, Smagghe G (2010) Plant-insect interactions: what can we learn from plant lectins? Arch Insect Biochem Physiol **73:** 193–212

Mur LA, Kenton P, Lloyd AJ, Ougham H, Prats E (2008) The hypersensitive response: the centenary is upon us but how much do we know? J Exp Bot **59:** 501–520

Naithani S, Chookajorn T, Ripoll DR, Nasrallah JB (2007) Structural modules for receptor dimerization in the S-locus receptor kinase extracellular domain. Proc Natl Acad Sci USA **104:** 12211–12216

Navarro-Gochicoa MT, Camut S, Timmers AC, Niebel A, Hervé C, Boutet E, Bono JJ, Imberty A, Cullimore JV (2003) Characterization of four lectin-like receptor kinases expressed in roots of *Medicago truncatula*: structure, location, regulation of expression, and potential role in the symbiosis with *Sinorhizobium meliloti*. Plant Physiol **133:** 1893–1910

Ngai PHK, Ng TB (2007) A lectin with antifungal and mitogenic activities from red cluster pepper (*Capsicum frutescens*) seeds. Appl Microbiol Biotechnol **74:** 366–371

Peumans WJ, Barre A, Bras J, Rougé P, Proost P, Van Damme EJM (2002) The liverwort contains a lectin that is structurally and evolutionary related to the monocot mannose-binding lectins. Plant Physiol **129:** 1054–1065

Peumans WJ, Van Damme EJM (1995) Lectins as plant defense proteins. Plant Physiol **109:** 347–352

Riou C, Herve C, Pacquit V, Dabos P, Lescure B (2002) Expression of an Arabidopsis lectin kinase receptor gene, *lecRK-a1*, is induced during senescence, wounding and in response to oligogalacturonic acid. Plant Physiol Biochem **40:** 431–438

Sequeira L, Graham TL (1977) Agglutination of avirulent strains of *Pseudomonas solanacearum* by potato lectin. Physiol Plant Pathol **11:** 43–54

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH (2004) Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. Plant Cell **16:** 1220–1234

Shridhar S, Chattopadhyay D, Yadav G (2009) PLecDom: a program for identification and analysis of plant lectin domains. Nucleic Acids Res **37:** W452–W458

Subramanyam S, Smith DF, Clemens JC, Webb MA, Sardesai N, Williams CE (2008) Functional characterization of HFR1, a high-mannose *N*-glycan-specific wheat lectin induced by Hessian fly larvae. Plant Physiol **147:** 1412–1426

Sudmoon R, Sattayasai N, Bunyatratchata W, Chaveerach A, Nuchadomrong S (2008) Thermostable mannose-binding lectin from *Dendrobium findleyanum* with activities dependent on sulfhydryl content. Acta Biochim Biophys Sin (Shanghai) **40:** 811–818

Sugui JA, Deising HB (2002) Isolation of infection-specific sequence tags expressed during early stages of maize anthracnose disease development. Mol Plant Pathol **3:** 197–203

Sugui JA, Leite B, Nicholson RL (1998) Partial characterization of the extracellular matrix released onto hydrophobic surfaces by conidia and conidial germlings of *Colletotrichum graminicola*. Physiol Mol Plant Pathol **52:** 411–425

Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8:** 397–403

Tsutsui S, Tasumi S, Suetake H, Kikuchi K, Suzuki Y (2006) Carbohydrate-binding site of a novel mannose-specific lectin from fugu (*Takifugu rubripes*) skin mucus. Comp Biochem Physiol B Biochem Mol Biol **143:** 514–519

Van Damme EJM, Allen AK, Peumans WJ (1987) Isolation and characterization of lectin with exclusive specificity towards mannose from snow drop (*Galanthus nivalis*) bulbs. FEBS Lett **215:** 140–144

Van Damme EJM, Barre A, Rougé P, Peumans WJ (2004) Cytoplasmic/nuclear plant lectins: a new story. Trends Plant Sci **9:** 484–489

Van Damme EJM, Culerrier R, Barre A, Alvarez R, Rougé P, Peumans WJ (2007a) A novel family of lectins evolutionarily related to class V chitinases: an example of neofunctionalization in legumes. Plant Physiol **144:** 662–672

Van Damme EJM, Lannoo N, Peumans WJ (2008) Plant lectins. Adv Bot Res **48:** 108–209

Van Damme EJM, Nakamura-Tsuruta S, Smith DF, Ongenaert M, Winter HC, Rougé P, Goldstein IJ, Mo H, Kominami J, Culerrier R, et al (2007b) Phylogenetic and specificity studies of two-domain GNA-related lectins: generation of multispecificity through domain duplication and divergent evolution. Biochem J **404:** 51–61

Van Damme EJM, Peumans WJ, Barre A, Rougé P (1998) Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Sci **17:** 575–692

Verberne MC, Brouwer N, Delbianco F, Linthorst HJ, Bol JF, Verpoorte R (2002) Method for the extraction of the volatile compound salicylic acid from tobacco leaf material. Phytochem Anal **13:** 45–50

Vijayan M, Chandra N (1999) Lectins. Curr Opin Struct Biol **9:** 707–714

Wang XC, Bauw G, Van Damme EJM, Peumans WJ, Chen ZL, Van Montagu M, Angenon G, Dillen W (2001) Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. Plant J **25:** 651–661