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The pepper GNA-related lectin and PAN domain protein gene, *CaGLP1*, is required for plant cell death and defense signaling during bacterial infection

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ABSTRACT

Carbohydrate-binding proteins, commonly referred to as lectins or agglutinins, function in defense responses to microbial pathogens. Pepper (*Capsicum annuum*) GNA-related lectin and PAN-domain protein gene *CaGLP1* was isolated and functionally characterized from pepper leaves infected with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). *CaGLP1* contained an amine-terminus prokaryotic membrane lipoprotein lipid attachment site, a *Galanthus nivalis* agglutinin (GNA)-related lectin domain responsible for the recognition of high-mannose N-glycans, and a carboxyl-terminus PAN/apple domain. RNA gel blot and immunoblot analyses determined that *CaGLP1* was strongly induced in pepper by compatible and incompatible *Xcv* infection. CaGLP1 protein localized primarily to the plasma membrane and exhibited mannose-binding specificity. *CaGLP1*-silenced pepper plants were more susceptible to compatible or incompatible *Xcv* infection compared with that of non-silenced control plants. *CaGLP1* silencing in pepper leaves did not accumulate H_2O_2 and induce cell death during incompatible *Xcv* infection. Defense-related *CaDEF1* (defensin) gene expression was significantly reduced in *CaGLP1*-silenced pepper plants. *CaGLP1*-overexpression in *Arabidopsis thaliana* enhanced resistance to *Pseudomonas syringae* pv. *tomato*. Defense-related *AtPDF1.2* expression was elevated in *CaGLP1*-overexpression lines. Together, these results suggest that *CaGLP1* is required for plant cell death and defense responses through the reactive oxygen species burst and downstream defense-related gene expression in response to bacterial pathogen challenge.

1. Introduction

Plants have evolved a multilayered innate immune system to overcome the challenges presented by a wide range of pathogens such as bacteria, fungi, and viruses [1]. An active plant immune system specifically recognizes pathogen and altered-self molecules generated during pathogen infection [2]. Pathogen recognition is mediated by pattern-recognition receptors that bind to conserved pathogen-associated molecular patterns (PAMPs) that are shared by broad classes of microorganisms [3]. Defense response pathways initiated by pattern-recognition receptors generate antimicrobial phytoalexins, reactive oxygen species (ROS) and pathogenesis-related (PR) proteins that inhibit pathogen proliferation [4].

Lectins are proteins that reversibly and non-enzymatically bind specific carbohydrates [5]. Lectins were first identified as plant proteins that agglutinate human red blood cells [6]. Lectins recognize and bind specific monosaccharides or oligosaccharides. Plant lectins comprise an elaborate collection of proteins capable of recognizing and interactin with specific structures, either originating from the invading microorganisms or from damaged plant cell wall structures [7]. Plant lectins bind with high affinity to

Abbreviations: DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; GNA, *Galanthus nivalis* agglutinin; HR, hypersensitive response; *Hpa*, *Hyaloperonospora arabidopsidis*; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; MBL, mannose-binding lectin; MES, 2-(N-morpholino) ethanesulfonic acid; MS, Murashige and Skoog; NO, nitric oxide; OX, overexpression; PAMPs, pathogen-associated molecular patterns; PCR, polymerase chain reaction; PR, pathogenesis-related; *Pst*, *Pseudomonas syringae* pv. *tomato*; ROS, reactive oxygen species; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing; *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*.

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glycan moieties of glycoproteins, glycolipids, and polysaccharides, and recognize the glycoconjugates present on the surfaces of bacteria and fungi [8–10]. Some lectins have been demonstrated to be involved in specific protein-carbohydrate interactions within cytoplasmic and/or nuclear compartments [10,11]. Plant lectins are induced by various biotic and abiotic stresses, and they have antibacterial, antifungal and anti-insect activities [11–13]. It is well established that lectin-carbohydrate interactions play a major role in the immune system in plants [14] The soybean (*Glycine max*) lectin, β-glucan binding protein, shows high-affinity binding activity to β-glucan, a potent PAMP of the oomycetal pathogen *Phytophthora sojae* [15]. The potato (*Solanum tuberosum*) lectin causes agglutination of avirulent strains of *Ralstonia solanacearum*, which immobilizes them on the plant cell wall [16]. The *Arabidopsis thaliana* lectin gene *RESTRICTED TEV MOVEMENT1* (*RTM1*) has a crucial role to inhibit systemic spread of tobacco-etch virus [17]. Among many roles proposed for plant lectins, the most likely function for the lectins is in plant defense [12,13]. Recently, pepper (*Capsicum annuum*) mannose-binding lectin gene *CaMBL1* has been demonstrated to regulate plant cell death and defense responses to microbial pathogens [18]. The *CaMBL1* gene contains a predicted *Galanthus nivalis* agglutinin (GNA)-related lectin domain responsible for the recognition of high-mannose N-glycans.

Cell death plays a crucial role in plant defense responses to a variety of stimuli including developmental cues, abiotic stress, and biotic stress [19]. During pathogen infection, cell death is associated with disease susceptibility or resistance depending on the pathogen lifestyle [20,21]. The best characterized plant cell-death response associated with disease resistance is the hypersensitive response (HR), which involves localized cell death at the site of infection and rapid activation of defense responses that limit the spread of pathogens [20]. Oxidative burst [22], ion fluxes [22–24], nitric oxide [25], and the interaction between some of these signals [25] appear to govern HR-like cell death. By contrast, cell-death responses associated with disease susceptibility occur relatively late during infection with compatible pathogens. Pepper *CaABR1* (abscisic acid-responsive 1), *CaRBP1* (RNA-binding protein), *CaADC1* (arginine decarboxylase), *CaDC1* (cysteine/histidine-rich DC1-domain protein), and *CaSGT1* (suppressor of the G2 allele of *skp1*) are required for plant cell death and defense responses [26–30].

Here, we report the isolation and identification of the pepper GNA-related lectin and PAN-domain protein gene *CaGLP1* from leaves infected with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). *CaGLP1* contains an N-terminus prokaryotic membrane lipoprotein lipid attachment site, a GNA-related lectin domain responsible for recognition of high-mannose N-glycans, and a C-terminus PAN/apple domain. *CaGLP1* transcript and protein levels were rapidly and strongly elevated in pepper leaves during compatible and incompatible *Xcv* infection. CaGLP1 localized primarily to the plasma membrane and displayed mannose-binding activity; however, the signal-peptide region and the GNA-related lectin domain were not required for CaGLP1 binding to D-mannose. *CaGLP1* silencing attenuated resistance to *Xcv* infection in pepper, which was accompanied by reduced H_2O_2 accumulation and cell-death induction. *CaGLP1* overexpression in *Arabidopsis* enhanced resistance to *Pseudomonas syringae* pv. *tomato*. Taken together, these results suggest that *CaGLP1* has an important role in regulating plant cell death and defense responses.

2. Materials and methods

2.1. Plant materials and pathogen inoculation

Pepper (*C. annuum* L., cv. Nockwang) and *Nicotiana benthamiana* plants were grown in soil mix (perlite:vermiculite:loam, 1:1:3, v/v/v) at 25 °C with 16 h day length and light intensity of 65 μmol photons m^{-2} s^{-1}. The virulent (compatible) strain Ds1 and the avirulent (incompatible) strain Bv5-4a of *X. campestris* pv. *vesicatoria* (*Xcv*) were cultured in yeast nutrient (YN) broth (5 g L^{-1} yeast extract and 8 g L^{-1} nutrient broth). Bacterial suspensions were syringe-infiltrated into the abaxial side of fully expanded pepper leaves of plants at six- to eight-leaf stages. Infected leaves were harvested at various time points after inoculation for analysis of bacterial proliferation and RNA gel blot [31].

A. thaliana ecotype Columbia (Col-0) seeds were planted on MS growth media [1× Murashige and Skoog, 1% (w/v) sucrose] [32] after surface sterilization and stratification at 4 °C for 3 days to break dormancy. Seedlings at the two- or three-leaf stage were transplanted to pots containing vermiculite:perlite:loam (1:1:2, v/v/v) and were grown in a climate chamber under controlled conditions (24 °C, 12 h light/12 h dark cycle, 130 μmol photons m^{-2} s^{-1}, and 60% humidity). *P. syringae* pv. *tomato* (*Pst*) DC3000 or DC3000 (*avrRpm1*) was grown overnight in King's B medium (10 g L^{-1} peptone, 1.5 g L^{-1} K_2HPO_4, 15 g L^{-1} glycerol, 5 mM $MgSO_4$, pH 7.0) containing 50 mg mL^{-1} rifampicin or 50 mg mL^{-1} kanamycin. To assess bacterial proliferation, *Arabidopsis* leaves were infiltrated with *Pst* suspensions using a needleless syringe. Infected leaves were harvested at different time intervals after inoculation. One-week-old *Arabidopsis* seedlings were spray-inoculated with spore suspensions (5×10^4 conidiospores mL^{-1}) of *Hyaloperonospora arabidopsidis* isolate Noco2. The infected plants were incubated at 17 °C in a controlled-environment chamber. Sporangiophores produced on the cotyledons were counted 6 days after inoculation.

2.2. Isolation and sequence analysis of CaGLP1

We constructed cDNA libraries from pepper leaves inoculated with avirulent *Xcv* strain Bv5-4a. The 1395 bp full-length *CaGLP1* (accession no. KJ605498) cDNA was isolated from the cDNA libraries using the differential hybridization technique [33]. The *CaGLP1* cDNA clone hybridized markedly with the cDNA probe from leaves inoculated with the *Xcv* avirulent strain Bv5-4a. Sequence analysis was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [34]. Expasy Proteomics Server (http://www.expasy.org) was used to identify homologous proteins and protein domains.

2.3. Protein extraction and immunoblotting

Total proteins were extracted from pepper leaves inoculated with *Xcv*. Leaf tissues were homogenized in extraction buffer [20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 0.2% Triton X-100, and plant protease inhibitor cocktail (Roche)]. The supernatant obtained after centrifugation at 13,000 × *g* for 20 min was used for immunoblot analysis. Proteins were separated on 10% SDS-PAGE gels, transferred to membranes, and immunoblotted with anti-*CaGLP1* antibody. The anti-*CaGLP1* antibody was raised in rabbits using synthetic peptides derived from the C-terminal sequence (NNEKDRTRYDSVAF) of *CaGLP1* (Abfrontier, Seoul, Korea). Immunoblotting was performed with the WERT-ZOL plus protein gel blotting substrate (Intron, Seoul, Korea) according to the manufacturer's instructions.

2.4. Subcellular localization of CaGLP1 protein

CaGLP1 or *CaGLP1*ΔSP-smGFP was inserted into the binary vector pBIN35S under control of the CaMV 35S promoter for transient expression in *N. benthamiana* leaves. *Agrobacterium tumefaciens* strain GV3101 harboring the constructs was grown in liquid Luria–Bertani (LB) medium, harvested, and resuspended in induction media [10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.6, 10 mM $MgCl_2$, and 200 μM acetosyringone] to OD_{600} = 1.0.

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