

The Pepper Extracellular Xyloglucan-Specific Endo- β -1,4-Glucanase Inhibitor Protein Gene, *CaXEGIP1*, Is Required for Plant Cell Death and Defense Responses^{1[C][W][OA]}

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Plants produce various proteinaceous inhibitors to protect themselves against microbial pathogen attack. A *xyloglucan-specific endo- β -1,4-glucanase inhibitor1* gene, *CaXEGIP1*, was isolated and functionally characterized in pepper (*Capsicum annuum*) plants. *CaXEGIP1* was rapidly and strongly induced in pepper leaves infected with avirulent *Xanthomonas campestris* pv *vesicatoria*, and purified *CaXEGIP1* protein significantly inhibited the hydrolytic activity of the glycoside hydrolase74 family xyloglucan-specific endo- β -1,4-glucanase from *Clostridium thermocellum*. Soluble-modified green fluorescent protein-tagged *CaXEGIP1* proteins were mainly localized to the apoplast of onion (*Allium cepa*) epidermal cells. *Agrobacterium tumefaciens*-mediated overexpression of *CaXEGIP1* triggered pathogen-independent, spontaneous cell death in pepper and *Nicotiana benthamiana* leaves. *CaXEGIP1* silencing in pepper conferred enhanced susceptibility to virulent and avirulent *X. campestris* pv *vesicatoria*, accompanied by a compromised hypersensitive response and lowered expression of defense-related genes. Overexpression of dexamethasone: *CaXEGIP1* in *Arabidopsis* (*Arabidopsis thaliana*) enhanced resistance to *Hyaloperonospora arabidopsidis* infection. Comparative histochemical and proteomic analyses revealed that *CaXEGIP1* overexpression induced a spontaneous cell death response and also increased the expression of some defense-related proteins in transgenic *Arabidopsis* leaves. This response was also accompanied by cell wall thickening and darkening. Together, these results suggest that pathogen-inducible *CaXEGIP1* positively regulates cell death-mediated defense responses in plants.

Plant cell walls provide a physical barrier that separates challenging pathogens from the internal contents of plant cells. Additionally, the cell walls regulate cell expansion and differentiation (York et al., 2004; Flors et al., 2007; Cantu et al., 2008). Polysaccharides, such as cellulose, hemicellulose, and pectic polysaccharides, are the main components of primary cell walls. Xyloglucan (XG), the most abundant hemicellulose in the primary cell wall, plays a structural role by forming strong hydrogen bonds with cellulose microfibrils (Carpita and Gibeau, 1993). The primary

structure of XG contains a common β -(1 \rightarrow 4)-D-glucan backbone, which is repeatedly substituted with α -(1 \rightarrow 6)-D-xylopyranosyl residues. Depolymerization of XG is proposed to play an important role during both cell wall expansion and pathogen invasion (Bourquin et al., 2002; Qin et al., 2003; Baumann et al., 2007). During cell wall expansion, plant XG endo-transglycosylases cut and rejoin XG chains to allow the cellulose microfibrils to move apart. From a pathogen point of view, the carbon-rich complex represents a useful energy source for pathogen growth. To facilitate penetration into the plant tissues and to catabolize carbon sources, many plant pathogens secrete a mixture of cell wall-degrading enzymes, such as polygalacturonases, pectin methyl esterases, pectin/pectate lyases, xylanases, and endoglucanases (Valette-Collet et al., 2003; DeBoy et al., 2008). Some microbial glycoside hydrolase (GH) family proteins, including GH5, GH12, and GH74, reportedly hydrolyze plant-derived XG (Martinez-Fleites et al., 2006; Gloster et al., 2007).

To inhibit pathogen-derived cell wall-degrading enzymes, plants secrete a mixture of inhibitor proteins into the cell wall (Qin et al., 2003; An et al., 2008; Xie et al., 2008). Some of the best characterized inhibitor proteins are polygalacturonase-inhibiting proteins (PGIPs; Albersheim and Anderson, 1971; De Lorenzo and Ferrari, 2002; Federici et al., 2006). In bean (*Phaseolus*

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vulgaris), two pairs of PGIPs, PvPGIP1/PvPGIP2 and PvPGIP3/PvPGIP4, are present in the genome. These genes may have originated from independent gene duplication events (D'Ovidio et al., 2004a). PvPGIP2 strongly inhibits polygalacturonases from *Fusarium phyllophilum* and *Aspergillus niger* via three conserved Asp residues (Spinelli et al., 2009). PGIPs reduce the hydrolytic activity of polygalacturonases to favor the accumulation of long-chain oligogalacturonides, known as elicitors of a variety of defense responses (Côté and Hahn, 1994; D'Ovidio et al., 2004b). Furthermore, transgenic expression of pear (*Pyrus communis*) PGIP in transgenic tomato (*Solanum lycopersicum*) plants limited fungal colonization, suggesting a role of PGIPs in plant defense (Powell et al., 2000).

The proteinaceous inhibitor of the cell wall-degrading enzyme xyloglucan-specific endo- β -1,4-glucanase (XEG) was identified from suspension-cultured tomatoes (Qin et al., 2003). The purified xyloglucan-specific endo- β -1,4-glucanase inhibitor protein (XEGIP) strongly inhibited XEG activity through the formation of a 1:1 protein:protein complex with XEG of *Aspergillus aculeatus*. More recently, two putative XEGIPs were isolated from *Nicotiana benthamiana* based on conserved regions found in plant XEGIP genes, and these genes were functionally characterized using virus-induced gene silencing (VIGS; Xie et al., 2008). VIGS of *NbXEGIP1* strongly enhanced the wilting symptoms exhibited following infection by virulent *Pseudomonas syringae* pv *tabaci*. This finding supports the notion that *NbXEGIP1* may act as an inhibitor of bacterial cell wall-degrading enzymes in *N. benthamiana* plants.

Programmed cell death (PCD) has been extensively characterized in plants (Lam, 2004). The hypersensitive response (HR), a well-known form of plant PCD, is one of the most efficient and immediate resistance reactions of plants. The HR is characterized by the rapid death of cells in the local region surrounding an infection site. As a result, the growth and spread of the pathogen is restricted or confined. During HR cell death development, cell wall strengthening occurs. Histochemical analyses of cells involved in melon (*Cucumis melo*)-powdery mildew (*Podosphaera fusca*) interactions demonstrate the reinforcement of the cell wall compartment as part of HR cell death-mediated resistance (Romero et al., 2008). Treatment of suspension-cultured tobacco (*Nicotiana tabacum*) cells with cryptogein, a 10-kD protein secreted by the oomycete *Phytophthora cryptogea*, induces a HR on tobacco leaves, accompanied by induced strengthening of the cell wall (Kieffer et al., 2000). However, the role of cell wall strengthening in HR cell death is poorly understood. A second type of PCD is thought to be associated with the differentiation of procambium into tracheary elements in the xylem of vascular plants (Fukuda, 2000; Lam, 2004). During the early formation of mature tracheary elements, vacuoles accumulate degradation enzymes and the cell wall is remodeled into a highly reticulated form. A similar phenomenon occurs during some plant developmental processes,

including senescence and aerenchyma formation in roots (Jones, 2001).

In this study, we have isolated and functionally characterized a pepper (*Capsicum annuum*) xyloglucan-specific endo- β -1,4-glucanase inhibitor-protein1 gene (*CaXEGIP1*). Expression of *CaXEGIP1* was strongly induced in pepper leaves infected with avirulent *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) strain Bv5-4a. The purified *CaXEGIP1* protein inhibited the hydrolytic activity of GH74 family XEG from the thermophilic bacterium *Clostridium thermocellum*. The soluble-modified GFP (smGFP)-fused *CaXEGIP1* protein was localized in the external and intercellular regions of onion epidermal cells. Importantly, *Agrobacterium tumefaciens*-mediated transient expression of *CaXEGIP1* induced the hypersensitive cell death response in pepper and *N. benthamiana* leaves. VIGS of *CaXEGIP1* significantly enhanced the growth of virulent and avirulent *Xcv* in pepper leaves, accompanied by compromised HR cell death and lowered expression of *CaPR1* (pathogenesis-related protein1 [PR1]) and *CaDEF1* (defensin [DEF1]). We also investigated the role of *CaXEGIP1* in plant cell death and defense responses using transgenic Arabidopsis (*Arabidopsis thaliana*) plants harboring the dexamethasone (DEX)-inducible *CaXEGIP1* transgene. Overexpression of *CaXEGIP1* triggered spontaneous cell death and modification of the cell wall compartment in Arabidopsis plants. Together, these results suggest that the pathogen-responsive *CaXEGIP1* is involved in plant cell death-mediated defense signaling.

RESULTS

CaXEGIP1 Encodes a Putative Extracellular XEGIP

To isolate pepper genes induced during the HR, we performed macro complementary DNA (cDNA) array analysis using a cDNA library constructed from pepper leaves infected with avirulent *Xcv* strain Bv5-4a (Jung and Hwang, 2000; Hwang and Hwang, 2010, 2011; Hwang et al., 2011). Among the defense-related genes selected, we isolated the putative pepper *CaXEGIP1* gene. This gene was strongly induced during the HR. The *CaXEGIP1* cDNA (accession no. JQ673414) consists of 1,415 bp, including a 30-bp poly(A) tail, and encodes a protein of 430 amino acids with a predicted molecular mass of 47.2 kD and a pI of 9.15. The translated *CaXEGIP1* amino acid sequence shared high amino acid sequence identity with various plant XEGIPs (Supplemental Fig. S1). The putative amino acid sequence encoded by the *CaXEGIP1* cDNA was 45% identical to a putative XEGIP from grape (*Vitis vinifera*; accession no. XP_002272235) and 42% identical to a XEGIP from tomato (accession no. AAN87262; Qin et al., 2003).

CaXEGIP1 Is Strongly Induced in Leaves by Avirulent *Xcv* Infection

RNA gel-blot analysis was performed to investigate the expression of *CaXEGIP1* in pepper leaves during

compatible and incompatible interactions with *Xcv* (Fig. 1A). *CaXEGIP1* was strongly induced in leaves inoculated with the *Xcv* avirulent (incompatible) strain Bv5-4a harboring *avrBsT*, which induces HR cell death (Kim et al., 2010; Kim and Hwang, 2011). *CaXEGIP1* induction was detected as early as 5 h after *Xcv* avirulent Bv5-4a infection and was notably strong prior to the appearance of the HR. In contrast, *CaXEGIP1* expression was low in leaves 20 and 25 h after inoculation with the virulent (compatible) strain Ds1. *CaXEGIP1* transcripts were only faintly detected in mock (10 mM MgCl₂)-inoculated leaves.

Immunoblot analysis using an antiserum raised against a CaXEGIP1 peptide demonstrated that infection by avirulent *Xcv* strain Bv5-4a strongly induced CaXEGIP1 accumulation in pepper leaves as compared with virulent Ds1 infection (Fig. 1B). As observed with the RNA gel-blot analyses, CaXEGIP1 expression was induced 5 h after avirulent Bv5-4a infection, which occurred prior to the appearance of the HR cell death. However, the induction of CaXEGIP1 was not detected in mock-inoculated leaves and was only faintly detected in leaves inoculated with virulent *Xcv*. This pronounced CaXEGIP1 expression during the HR cell death indicates a significant role of *CaXEGIP1* in the HR-like resistance response of pepper plants to the bacterial spot pathogen *Xcv*.

Inhibition of XEG Activity by CaXEGIP1 Protein

Recombinant CaXEGIP1 protein was purified from the extracts of isopropyl- β -D-thiogalactoside (IPTG)-induced *Escherichia coli* BL21 cells using Ni²⁺-nitrilotriacetic acid affinity chromatography (Fig. 2A). The

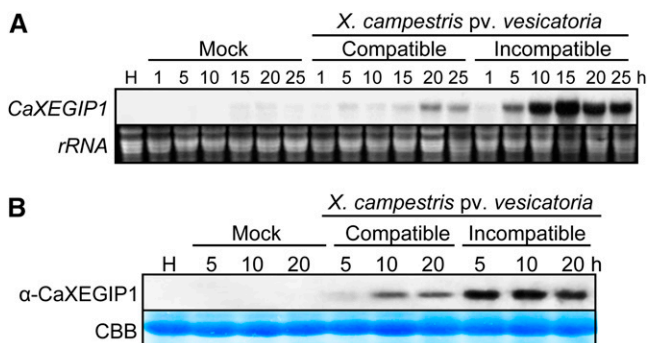


Figure 1. Induction of *CaXEGIP1* in pepper leaves by *Xcv* infection. Shown are RNA gel-blot (A) and immunoblot (B) analyses of *CaXEGIP1* expression in pepper leaves at various time points after inoculation with the *Xcv* virulent strain Ds1 (compatible) and avirulent strain Bv5-4a (incompatible) at the six-leaf stage. H, Healthy leaves; Mock, leaves treated with 10 mM MgCl₂. Equal loading of total RNA (20 μ g per lane) was verified by visualizing ribosomal RNA (rRNA) on a gel stained with ethidium bromide. Protein loading was verified by Coomassie Brilliant Blue (CBB) staining. [See online article for color version of this figure.]

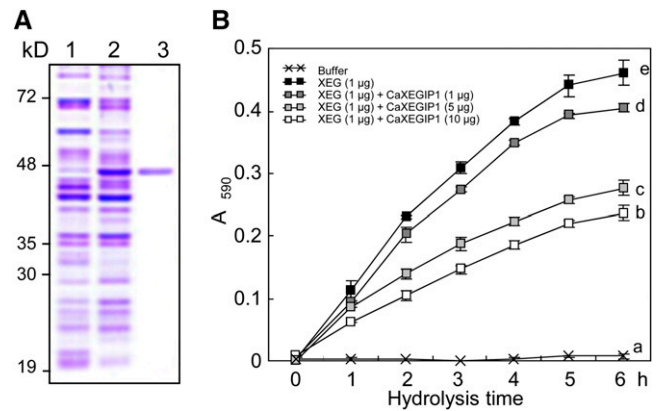


Figure 2. Inhibitory effect of the recombinant CaXEGIP1 protein on XEG activity. A, Expression and purification of recombinant CaXEGIP1 in *E. coli* BL21 (DE3) cells. Protein loading was verified by Coomassie Brilliant Blue staining. Lane 1, uninduced *E. coli* cell extracts; lane 2, crude protein extracts of *E. coli* cells expressing the His-tagged CaXEGIP1, as induced by 1 mM IPTG; lane 3, purified His-tagged CaXEGIP1. B, Inhibition of XEG activity by CaXEGIP1. The data represent means \pm SD ($n = 4$) from three independent experiments. Different letters indicate significant differences, as determined by Fisher's LSD test ($P < 0.05$, $n = 4$). [See online article for color version of this figure.]

homogeneity of the purified protein was confirmed by a single band on a Coomassie Brilliant Blue-stained SDS-PAGE gel (Fig. 2A, lane 3). To investigate whether recombinant CaXEGIP1 inhibits the hydrolytic activity of XEG, various amounts of the purified CaXEGIP1 were mixed with 1 μ g of GH74 family XEG enzyme from *C. thermocellum* F7/YS (Martinez-Fleites et al., 2006). The resulting mixtures were incubated at 60°C for 1 h to permit complex formation and then added to AZO-XG solutions (Megazyme). Aliquots of each reaction mixture were taken at various time points, and the amount of reducing sugar was examined spectrophotometrically. As shown in Figure 2B, the addition of increasing amounts of purified CaXEGIP1 protein strongly inhibited XEG-catalyzed depolymerization of XG.

Subcellular Localization of CaXEGIP1

Computational analysis of the predicted protein sequence indicated that CaXEGIP1 is a secreted protein (PSORT, 82%; TargetP, 99%) and contains a signal peptide cleavage site between Ala-24 and Glu-25 (Supplemental Fig. S1). To analyze the subcellular localization of the CaXEGIP1 protein, we performed a biolistic transformation experiment. The *smGFP* gene was fused to the C-terminal region of *CaXEGIP1*. As shown in Figure 3, the CaXEGIP1:smGFP fusion protein was mainly localized in the external and intercellular regions of the onion cell, while the control smGFP protein was uniformly distributed throughout the cell. Different levels of expression of the CaXEGIP1:smGFP

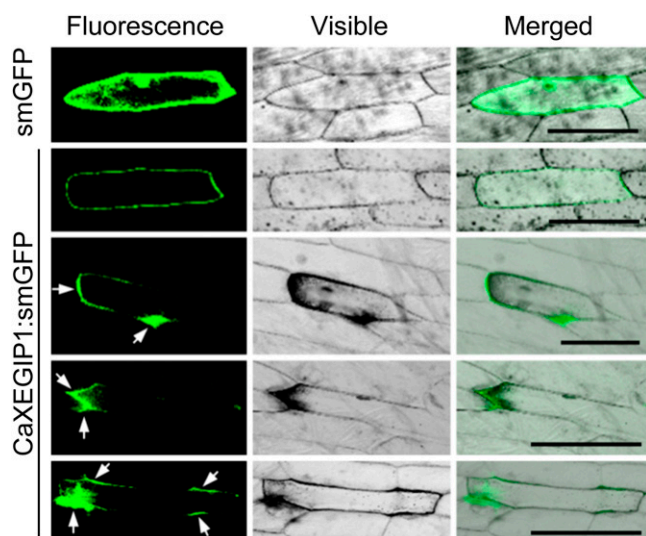


Figure 3. Subcellular localization of CaXEGIP1 protein by transient expression of the *CaXEGIP1:smGFP* construct in onion epidermal cells. The *smGFP* gene was fused to the *CaXEGIP1* 3' region. Transient expression of *smGFP* or *CaXEGIP1:smGFP* was detected by confocal laser scanning microscopy 24 h after biolistic transformation. White arrows indicate the localization of CaXEGIP1 protein in the external and intercellular regions of the onion epidermal cells. Bars = 100 μ m.

fusion protein were visualized based on the GFP fluorescence signaling intensity. Interestingly, the focal and strong accumulation of CaXEGIP1 proteins darkened the peripheral and apoplastic regions where the proteins localized. However, in the cells expressing lower levels of GFP-tagged CaXEGIP1, darkening of the cell peripheral region was not observed. These observations support the notion that the darkened microscopic images of onion epidermal cells resulted from the localized thickening and darkening of the cell wall by the accumulation of CaXEGIP1.

Transient Expression of CaXEGIP1 Triggers Cell Death in Pepper and *N. benthamiana* Leaves

To examine the *in vivo* function of *CaXEGIP1* in defense responses, we used *A. tumefaciens*-mediated transient expression in pepper and *N. benthamiana* plants, as described previously (Choi et al., 2009, 2011; Hwang and Hwang, 2010; Choi and Hwang, 2011; Kim and Hwang, 2011). Reverse transcription (RT)-PCR and immunoblot analyses revealed the expression of *CaXEGIP1* in *A. tumefaciens*-infiltrated pepper leaves at both the mRNA and protein levels (Fig. 4, A and B). Transient expression of *CaXEGIP1* in pepper leaves triggered a strong cell death response 48 h after agroinfiltration (Fig. 4C). This cell death induction by *CaXEGIP1* transient expression was supported by the strong and differential staining of the cell death response with trypan blue and also high electrolyte leakage from the agroinfiltrated leaves (Fig. 4, D and E).

We next investigated whether transient expression of CaXEGIP1 triggers hypersensitive cell death in *N. benthamiana* leaves (Fig. 5). Successful transient expression of CaXEGIP1 in *N. benthamiana* leaves was confirmed by RT-PCR and immunoblot analyses (Fig. 5, A and B). The tomato R gene *Pto* and the corresponding *Pseudomonas syringae* pv *tomato* (*Pst*) effector gene *avrPto* were included as positive controls. As shown in Figure 5C, transient expression of *avrPto* alone triggered the chlorotic phenotype, whereas empty vector or *Pto* expression did not impair *N. benthamiana* leaves. However, transient expression of *CaXEGIP1* or *Pto/avrPto* induced the typical hypersensitive cell death response in *N. benthamiana* leaves. In support of these visual cell death phenotypes, expression of *CaXEGIP1* significantly enhanced electrolyte leakage from the agroinfiltrated leaves as compared with empty vector-, *Pto*-, or *avrPto*-expressing leaves (Fig. 5D). Trypan blue staining of *N. benthamiana* leaves also revealed *CaXEGIP1*-induced cell death and darkened cell wall compartments (Supplemental Fig. S2). Collectively, these results indicate that CaXEGIP1 transient expression triggers pathogen-independent hypersensitive cell death in plant tissues.

Silencing of *CaXEGIP1* Confers Enhanced Susceptibility to *Xcv* Infection

VIGS was used to investigate *CaXEGIP1* loss of function in the defense responses of pepper plants (Choi et al., 2007; Hwang and Hwang, 2011; Hwang et al., 2011). Interestingly, the silencing of *CaXEGIP1* enhanced the growth of the pepper plants (Fig. 6A). The efficiency of VIGS was evaluated using RT-PCR in empty vector control (TRV:00) and *CaXEGIP1*-silenced (TRV:*CaXEGIP1*) pepper leaves 12 h after inoculation with *Xcv* virulent and avirulent strains (Fig. 6B). *Xcv* infection did not induce *CaXEGIP1* expression in the silenced leaves, indicating that *CaXEGIP1* was fully silenced in the pepper leaves. To determine whether *CaXEGIP1* silencing affects the defense response pathway in pepper, the expression of some defense-related genes was further analyzed by RT-PCR (Fig. 6B). Silencing of *CaXEGIP1* greatly reduced the induction of *CaPR1* in pepper leaves during virulent *Xcv* infection. Significantly, *CaDEF1* expression was not induced in the *CaXEGIP1*-silenced pepper leaves inoculated with both virulent and avirulent *Xcv*, although *CaDEF1* was strongly induced in the infected, unsilenced leaves. In the *CaXEGIP1*-silenced plants, *Xcv* growth was approximately 10-fold greater than that in the empty vector control plants (Fig. 6C), indicating that *CaXEGIP1* silencing enhances *Xcv* growth in pepper. Trypan blue staining of avirulent *Xcv*-infected leaves revealed a compromised HR cell death response in *CaXEGIP1*-silenced pepper leaves (Fig. 6D). Furthermore, *CaXEGIP1* silencing significantly reduced electrolyte leakage from avirulent *Xcv*-infected leaf discs (Fig. 6E). However, VIGS of empty vector

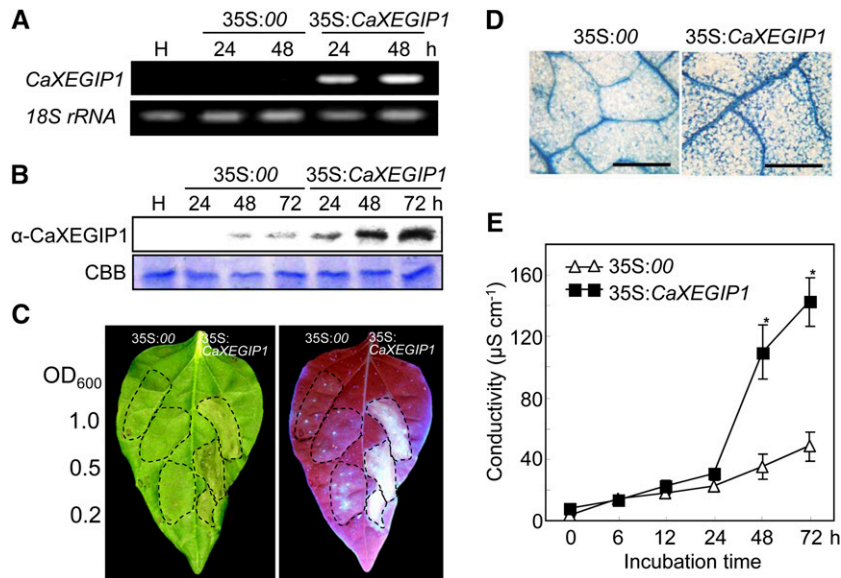


Figure 4. Induction of the cell death response in pepper leaves by *A. tumefaciens*-mediated transient expression of *CaXEGIP1*. A and B, RT-PCR (A) and immunoblot (B) analyses of *CaXEGIP1* expression in pepper leaves. Expression of pepper *18S rRNA* served as a loading control. Protein loading was verified by Coomassie Brilliant Blue (CBB) staining. C, Induction of cell death in pepper leaves. The leaf areas between the lateral veins were infiltrated with *A. tumefaciens* at the indicated OD_{600} concentrations. Photographs were taken 48 h after agroinfiltration: left, visible symptoms; right, UV exposure to visualize the deposition of fluorescent pigments. D and E, Trypan blue staining (D) and electrolyte leakage measurements (E) from the leaves infiltrated by *A. tumefaciens* ($\text{OD}_{600} = 0.5$) strains carrying the indicated constructs. The data represent means \pm SD from three independent experiments. Asterisks indicate significant differences in electrolyte leakage, as determined by the two-tailed Student's *t* test ($P < 0.05$). Bars = 200 μm .

(TRV:00) in pepper leaves did not affect *Xcv* growth and electrolyte leakage in pepper leaves compared with those in wild-type pepper leaves (Supplemental Fig. S3, A and B). Together, these results indicate that *CaXEGIP1* expression is required for hypersensitive cell death signaling in pepper leaves during avirulent *Xcv* infection.

Generation and Analysis of the DEX:CaXEGIP1 Arabidopsis Transgenic Plants

We failed to successfully generate Arabidopsis transgenic lines constitutively expressing *CaXEGIP1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Thus, the DEX-inducible promoter system was used to generate DEX:CaXEGIP1 transgenic Arabidopsis plants. DEX:CaXEGIP1 line 3 strongly expressed the *CaXEGIP1* gene following induction with DEX, and this line was selected for further experiments. We did not observe any apparent phenotypic differences between wild-type and DEX:CaXEGIP1 transgenic plants (data not shown). The accumulation of CaXEGIP1 protein in the transgenic Arabidopsis leaves was detected using rabbit polyclonal antibodies raised against a CaXEGIP1 peptide (Fig. 7A). CaXEGIP1 protein greatly accumulated in the transgenic Arabidopsis leaves 24 and 48 h after DEX treatment. However, mock treatments did not

induce *CaXEGIP1* transcripts and protein in the DEX:CaXEGIP1 Arabidopsis leaves. The conditional induction of *CaXEGIP1* by DEX treatment triggered the cell death response in transgenic Arabidopsis leaves (Fig. 7B). Visible cell death phenotypes were primarily observed in the marginal region of the DEX:CaXEGIP1 Arabidopsis leaves 2 d after DEX treatment but not in mock-treated leaves. Microscopic observations of DEX-treated leaves showed the trypan blue-stained cell death response and cell wall compartments in the DEX:CaXEGIP1 transgenic leaf tissues (Fig. 7C). However, mock and DEX treatments did not trigger any cell death response in leaves of wild-type Arabidopsis plants, as observed by visible plant growth phenotypes and trypan blue staining (Supplemental Fig. S4, A and B).

Seven-day-old transgenic Arabidopsis seedlings were transplanted to Murashige and Skoog (MS) medium containing 3 μM DEX. Interestingly, the transgenic seedlings grown on this medium exhibited unidirectional root bending (Fig. 7D, bottom panel). However, no phenotypic differences were observed in the transgenic seedlings grown in the absence of DEX (Fig. 7D, top panel). To visualize the root bending at the cellular level, transverse sections of the root tissue were stained with 0.1% Calcofluor and observed with a microscope equipped with a UV lamp (Fig. 7E). Compared with the mock-treated wild-type and transgenic seedling roots, the DEX-treated transgenic

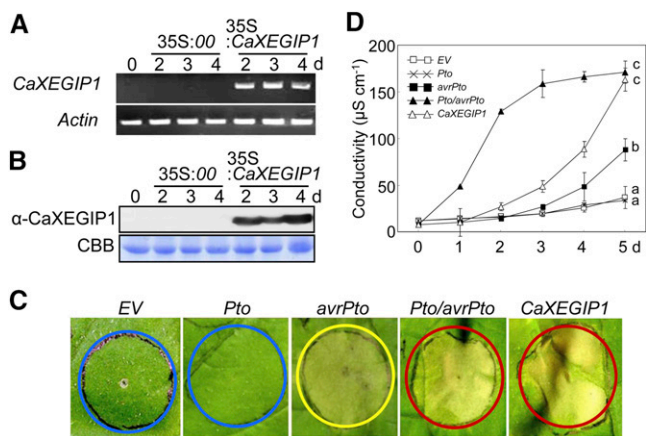


Figure 5. Effect of *A. tumefaciens*-mediated transient expression of *Pto*, *avrPto*, *Pto/avrPto*, and *CaXEGIP1* on the cell death response of *N. benthamiana* leaves. A and B, RT-PCR (A) and immunoblot (B) analyses of *CaXEGIP1* expression in *N. benthamiana* leaves. Expression of the *N. benthamiana Actin* gene served as a control. Protein loading was verified by Coomassie Brilliant Blue (CBB) staining. C, Induction of cell death in *N. benthamiana* leaves. D, Measurements of electrolyte leakage from *N. benthamiana* leaves infiltrated with *A. tumefaciens* (OD₆₀₀ = 0.5) carrying the indicated constructs. The blue, yellow, and red circles indicate no cell death, intermediate cell death, and full cell death, respectively. The data represent means ± SD from three independent experiments. Different letters indicate statistically significant differences, as determined by Fisher's LSD test ($P < 0.05$).

root tissues showed shrinkage of the epidermal layers. Similar abnormal phenotypes were also observed in the epidermal layers of the hypocotyls of DEX-treated transgenic seedlings. Collectively, these results indicate that DEX-induced overexpression of *CaXEGIP1* triggered the cell death response in the leaves, roots, and hypocotyls of transgenic Arabidopsis plants.

CaXEGIP1 Overexpression Confers Enhanced Cell Death But Does Not Suppress *Pst* Growth in Arabidopsis

We next investigated *CaXEGIP1* gain of function in DEX:*CaXEGIP1* transgenic Arabidopsis plants during *Pst* infection. Seven days after inoculation with virulent and avirulent *Pst* DC3000, more severe cell death developed on the leaves of DEX-treated transgenic plants compared with the mock-treated plants (Fig. 8, A and B). Interestingly, infection of the halves of leaves with avirulent *Pst* DC3000 (*avrRpm1*) induced the spreading cell death in the DEX-treated Arabidopsis plants but not in the mock-treated plants. As shown in Figure 8B, the cell death in the mock-treated plants was confined to the pathogen infection region; however, cell death spread over the infection region in the DEX-treated plants that overexpressed *CaXEGIP1*. In contrast to this cell death response, no significant differences in bacterial growth were detected between mock- and DEX-treated transgenic plants as well as between mock- and DEX-treated wild-type plants during both virulent and avirulent *Pst* DC3000 infection (Fig. 8C; Supplemental Fig. S4C). These results indicate that *CaXEGIP1* overexpression enhances the cell death response but does not induce the resistance of Arabidopsis plants to *Pst* DC3000 infection.

CaXEGIP1 Overexpression Confers Reduced Susceptibility to *Hyaloperonospora arabidopsidis*

To determine whether *CaXEGIP1* overexpression enhances disease resistance to a biotrophic oomycete pathogen, we inoculated DEX-treated cotyledons of 7-d-old DEX:*CaXEGIP1* transgenic plants with *H. arabidopsidis* (*Hpa*) isolate Noco2. DEX-induced overexpression of *CaXEGIP1* induced cell death and also

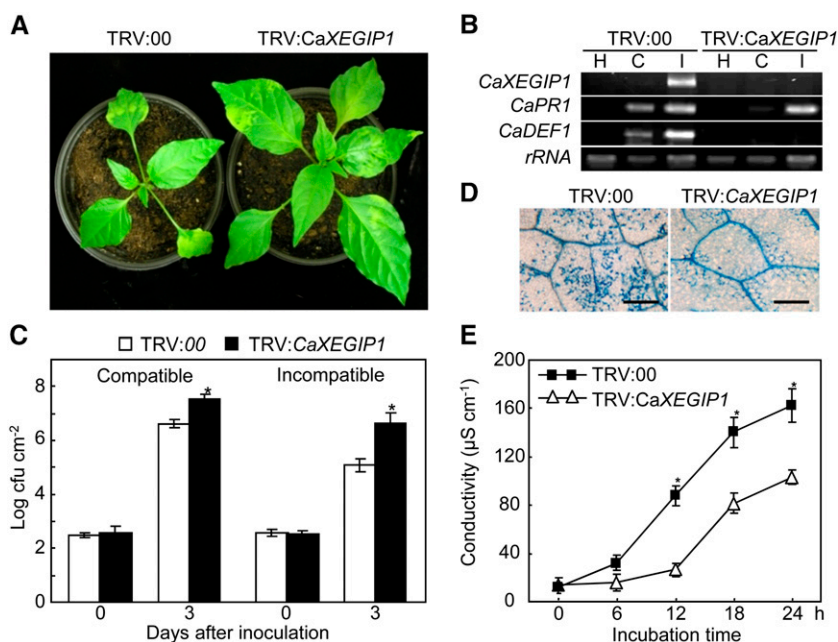


Figure 6. Enhanced susceptibility of *CaXEGIP1*-silenced pepper plants to infection with the *Xcv* virulent strain Ds1 and the avirulent strain Bv5-4a. A, Enhanced growth of *CaXEGIP1*-silenced pepper plants. B, RT-PCR analysis of the expression of *CaXEGIP1* and defense-related genes in empty vector control (TRV:00) and gene-silenced (TRV:*CaXEGIP1*) pepper plants 12 h after inoculation with *Xcv*. Expression of the pepper *18S rRNA* gene served as a control. H, Uninoculated healthy leaves; C, compatible; I, incompatible. C, Bacterial growth in leaves. Asterisks indicate significant differences, as determined by the two-tailed Student's *t* test ($P < 0.05$). D and E, Trypan blue staining (D) and electrolyte leakage measurements (E) from leaves inoculated with avirulent *Xcv*. The data represent means ± SD from three independent experiments. Asterisks indicate significant differences, as determined by the two-tailed *t* test ($P < 0.05$). Bars = 200 μm. [See online article for color version of this figure.]

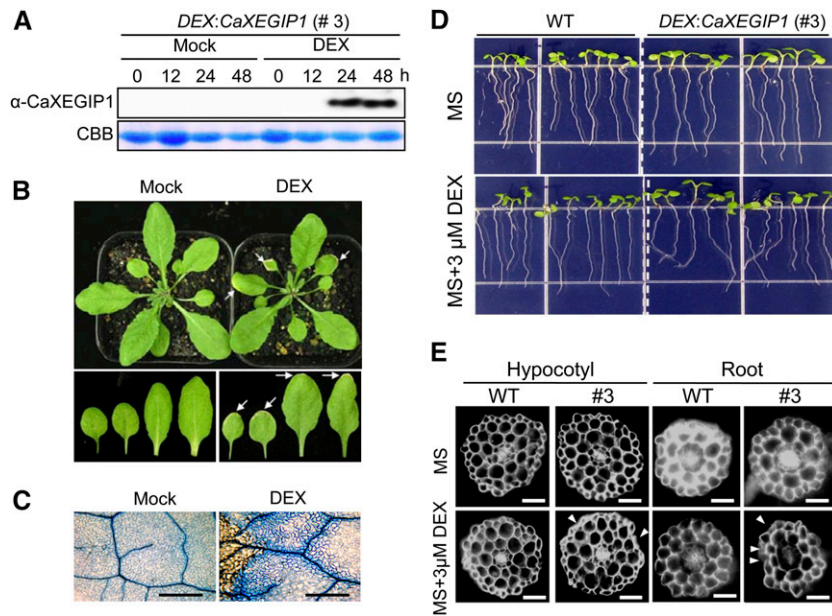


Figure 7. *CaXEGIP1*-induced cell death and abnormal growth of DEX:*CaXEGIP1* transgenic Arabidopsis plants after DEX treatment. A, Immunoblot analysis of *CaXEGIP1* expression in leaves of transgenic plants at different time intervals after mock or 30 μM DEX treatment. Protein loading was verified by Coomassie Brilliant Blue (CBB) staining. B, *CaXEGIP1*-induced cell death phenotype in the leaves of DEX:*CaXEGIP1* transgenic Arabidopsis plants. The visible cell death phenotype is mainly seen in the marginal region of DEX-treated leaves but not in that of mock-treated leaves, as indicated by white arrows. Photographs were taken 2 d after treatment. C, Trypan blue staining of leaves 24 h after mock or DEX treatment. Bars = 500 μm . D, Root-waving phenotype of transgenic Arabidopsis plants grown on MS medium with or without 3 μM DEX. E, Transverse sections from the roots and hypocotyls of wild-type (WT) and transgenic seedling plants (#3) grown on MS medium with or without DEX. Thin cross-sections of the roots and hypocotyls were stained with 0.1% Calcofluor (Fluorescent Brightener 28; Sigma) in water. Samples were observed with a microscope equipped with a UV lamp. Arrows indicate abnormal growth of root and hypocotyl cells of DEX:*CaXEGIP1* seedling plants. [See online article for color version of this figure.]

significantly reduced the oomycete growth in Arabidopsis cotyledons (Fig. 9A). Three days after inoculation, the mock- or DEX-pretreated cotyledons of transgenic plants were stained with trypan blue to visualize *Hpa* growth. As shown in Figure 9B, DEX-treated plants showed significantly reduced disease severity compared with mock-treated plants. Trypan blue staining of infected leaves revealed a lower level of colonization with less hyphal growth and sporulation in DEX-treated transgenic plants compared with mock-treated plants (Fig. 9C). Five days after inoculation, the number of sporangiophores on over 50 cotyledons was counted with a stereomicroscope (Fig. 9D). The number of sporangiophores was significantly reduced in the DEX:*CaXEGIP1* transgenic lines. These results indicate that *CaXEGIP1* overexpression in Arabidopsis plants confers enhanced resistance to the obligate biotrophic oomycete pathogen *Hpa*.

Proteomic Analysis of DEX:*CaXEGIP1* Transgenic Arabidopsis Leaves

Comparative two-dimensional electrophoresis (2-DE) was used to isolate novel defense response proteins from the proteome in DEX:*CaXEGIP1* transgenic plants. The entire 2-DE gel images of total proteins from mock- or DEX-treated transgenic Arabidopsis

leaves are shown in Supplemental Figure S5. Among the various protein spots, four proteins newly induced in *CaXEGIP1*-overexpressing leaves (Supplemental Fig. S5C) were excised from the two-dimensional gels and analyzed by matrix-assisted laser-desorption/ionization time of flight (Supplemental Table S1). Interestingly, two of these proteins were identified as PR proteins, PR5 (N1, thaumatin; AT1G75040) and PR2 (N2, β -1,3-glucanase2 [BGL2]; AT3G57260). The other protein spots were identified as Gln biosynthesis-related proteins, GSR2 (N3, copper ion binding/Gln-ammonia ligase; AT1G66200) and GSR1 (N4, Gln synthetase; AT5G37600). To determine whether *CaXEGIP1* overexpression induces the genes encoding these four proteins, quantitative RT-PCR was performed (Fig. 10). In DEX:*CaXEGIP1* transgenic leaves, none of the tested genes were significantly induced 24 and 48 h after mock treatment. In contrast, PR5, PR2, GSR1, and GSR2 were all significantly induced in DEX:*CaXEGIP1* transgenic plants 24 h after DEX treatment. These results indicate that *CaXEGIP1* overexpression triggers some defense response genes.

DISCUSSION

The HR cell death-mediated defense signaling genes of pepper were isolated from a pepper cDNA library

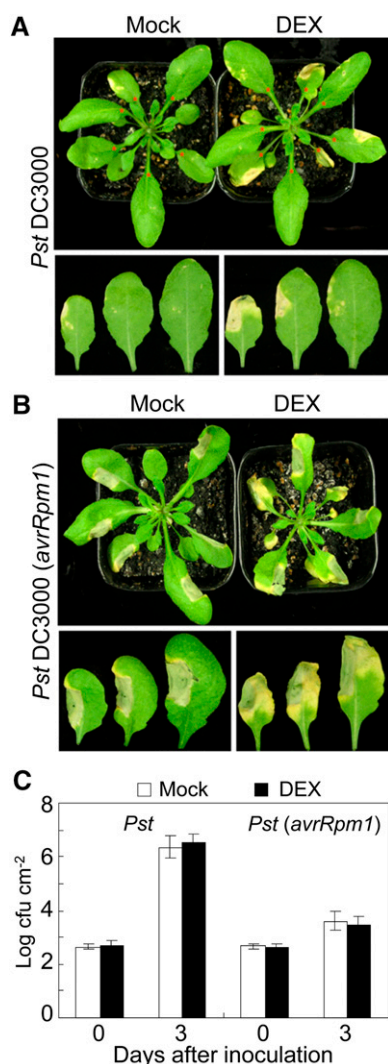


Figure 8. Cell death phenotype and bacterial growth in DEX:CaXEGIP1 transgenic Arabidopsis plants infiltrated with *Pst* DC3000. A and B, Disease symptoms developed on the leaves of DEX:CaXEGIP1 transgenic Arabidopsis 8 d after inoculation with virulent *Pst* DC3000 (A) and avirulent *Pst* DC3000 (*avrRpm1*) (B; 10^7 cfu mL⁻¹). Arabidopsis plants were inoculated with *Pst* DC3000 24 h after mock treatment or 30 μ M DEX treatment. C, Bacterial growth in DEX:CaXEGIP1 transgenic Arabidopsis leaves 0 and 3 d after inoculation with virulent *Pst* DC3000 and avirulent *Pst* DC3000 (*avrRpm1*) strains (10^5 cfu mL⁻¹). The data represent means \pm SD from three independent experiments. [See online article for color version of this figure.]

using differential hybridization and RNA gel-blot analyses (Choi et al., 2007, 2008, 2009). One of the isolated defense response genes was identified as *CaXEGIP1* based on sequence homology with tomato XEGIP1 (Qin et al., 2003). *CaXEGIP1* expression is rapidly and strongly induced in pepper leaves challenged with the avirulent *Xcv* strain Bv5-4a. The avirulent *Xcv* strain Bv5-4a was recently reported to harbor the type III secretion effector protein AvrBsT, which triggers the hypersensitive cell death response and the strong induction of PR genes in pepper plants

(Kim et al., 2010). In contrast, infection with virulent *Xcv* strain Ds1 causes susceptible symptoms accompanied by lower PR gene expression (Lee and Hwang, 2005; Choi et al., 2007). Thus, the rapid and strong induction of *CaXEGIP1* in pepper leaves by infection with avirulent *Xcv* strain Bv5-4a strongly raises the possibility that *CaXEGIP1* may be involved in the HR cell death-mediated resistance of pepper plants.

XEGIPs from tomato, tobacco, and carrot (*Daucus carota*) have been demonstrated to have in vitro inhibitory activity against the fungus *A. niger* XEG enzyme, a GH12 family XEG (Pauly et al., 1999; Qin et al., 2003; Naqvi et al., 2005; Yoshizawa et al., 2012). At the beginning of our study, we expected that the pathogen-induced CaXEGIP1 would inhibit the pathogen-derived XEG enzyme activity to reduce the pathogen virulence. However, the fungus *A. niger*-derived or other XEG enzymes that belong to the GH12 family were not available for us to test the inhibitory activity of CaXEGIP1. Thus, we first tested commercial XEG enzyme from the thermophilic bacterium *C. thermocellum* F7/YS, which belongs to a GH74 family XEG (PROZOMIX). Fortunately, we were able to detect the inhibitory effect of CaXEGIP1 on this GH74 family XEG from *C. thermocellum*. These results suggest that CaXEGIP1 is a functional xyloglucanase inhibitor protein. In the genome sequence of *Xcv* strain 85-10, the closest relative of *Xcv* strain Bv5-4a, 92 genes were predicted to encode putative GH family proteins (www.cazy.org). Further studies of the inhibitory effect of CaXEGIP1 on *Xcv*-derived GH family enzymes may determine whether CaXEGIP1 can target cell wall-degrading enzymes of invading pathogens.

Recently, structural studies using XEGIP protein from carrot and GH12 family XEG protein from *A. aculeatus* revealed that the two critical Arg residues from XEGIP proteins located in the inhibition loop 1 (IL-1; Arg-322) and inhibition loop 2 (IL-2; Arg-403) are conserved in XEGIP homologs from tomato and tobacco (Yoshizawa et al., 2012). All of the XEGIP homologs from carrot, tomato, and tobacco showed specific inhibitory activity on GH12 family XEG enzyme activity (Qin et al., 2003; Naqvi et al., 2005; Yoshizawa et al., 2012). However, the GH12 family XEG enzymes are structurally and mechanistically distinct from GH74 family XEG enzymes (Master et al., 2008). The inhibitory effects of plant XEGIPs on the GH74 family XEG enzymes are not yet reported. In addition, CaXEGIP1 does not have the two conserved Arg residues from IL-1 and IL-2 (Supplemental Fig. S1). Recently, Yoshizawa et al. (2011) reported the crystal structure of soybean (*Glycine max*) XEGIP and found that it lacks inhibitory activity on GH12 and GH11 family XEG enzymes, due to the lack of key amino acid residues, Arg-322 and Arg-403, on their IL-1 and IL-2. More importantly, the role of XEGIPs on plant cell death regulation is not fully understood. Here, we showed that overexpression of *CaXEGIP1* induces pathogen-independent cell death in pepper, *N. benthamiana*, and Arabidopsis plants. These findings

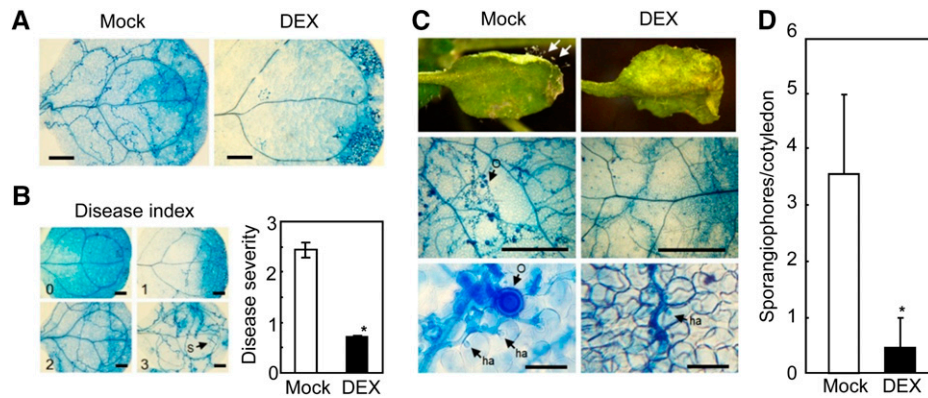


Figure 9. Enhanced resistance of DEX:CaXEGIP1 transgenic Arabidopsis plants to infection with *Hpa* isolate Noco2. A, Trypan blue staining of *Hpa*-infected cotyledons of untreated and DEX-treated transgenic plants 3 d after inoculation. Arabidopsis seedlings were inoculated with *Hpa* 24 h after mock treatment or 30 μM DEX treatment. Bars = 200 μm . B, Reduced growth of *Hpa* in DEX-treated transgenic Arabidopsis plants. The disease severity was rated on a 0 to 3 scale after trypan blue staining to observe fungal structure development (0, no hyphal growth; 1, minor hyphal growth; 2, severe hyphal growth but no sporangiophore formation; 3, severe hyphal growth and sporangiophore formation). S, Sporangiophore. C, Disease symptoms on the cotyledons of untreated and DEX-treated transgenic plants 5 d after spray inoculation with *Hpa* (5×10^4 spores mL^{-1}). Arrows indicate sporangiophores. Trypan blue-stained fungal structures are shown at higher magnification (bottom panels). ha, Haustorium; O, oospore. Bars = 500 μm (middle row) and 50 μm (bottom row). D, The number of sporangiophores per cotyledon of untreated or DEX-treated transgenic plants 7 d after inoculation with *Hpa*. Asterisks indicate significant differences in disease severity, as determined by the two-tailed Student's *t* test ($P < 0.05$).

support the notion that the inhibitory mechanisms of CaXEGIP1 on GH74 family XEG may be different from those of other reported XEGIPs on GH12 family XEG enzymes. Alternatively, CaXEGIP1 may target intrinsic XG-modifying enzymes to initiate cell death signaling. However, molecular mechanisms underlying the effects of CaXEGIP1 on GH74 family XEG or the intrinsic target of CaXEGIP1 should be further elucidated.

Our topology analysis using smGFP revealed that CaXEGIP1 localizes to the cell wall and apoplast, locations where certain pathogenic microorganisms initially colonize and multiply (Fritig et al., 1998; Lee et al., 2008). Significantly, purified CaXEGIP1 protein inhibited the hydrolytic activity of GH74 family XEG from *C. thermocellum* F7/Y5. Another important finding is that CaXEGIP1 accumulation darkens and thickens the cell wall in plant tissues, as stained by trypan blue. Interestingly, CaXEGIP1 is also ubiquitously distributed around the thickened cell walls of onion epidermal cells. Similarly, *A. tumefaciens*-mediated or DEX-induced overexpression of CaXEGIP1 in *N. benthamiana* or Arabidopsis leaves, respectively, triggered dense, pronounced staining of the cell wall compartment with trypan blue. In contrast, VIGS of CaXEGIP1 in pepper plants significantly compromised avirulent *Xcv*-induced HR cell death. Together, the topology and gain- or loss-of-function data support a potential functional relationship between CaXEGIP1 expression and cell wall strengthening during the HR cell death-mediated resistance response. In some plant-pathogen interactions, the resistance responses accompany rapid cell death and cell wall thickening at the site of attempted infection (Politis and Goodman, 1978; Devarenne and Martin, 2007; Jacobs, 2008). For example, infection of resistant potato (*Solanum*

tuberosum) plants with *Phytophthora infestans* triggers the formation of papilla at the penetration site and thickening of the entire cell wall prior to HR-like cell death (Schmelzer, 2002). Localized cell wall apposition was also observed in tobacco leaves infected with avirulent *Pseudomonas pisi* (Politis and Goodman,

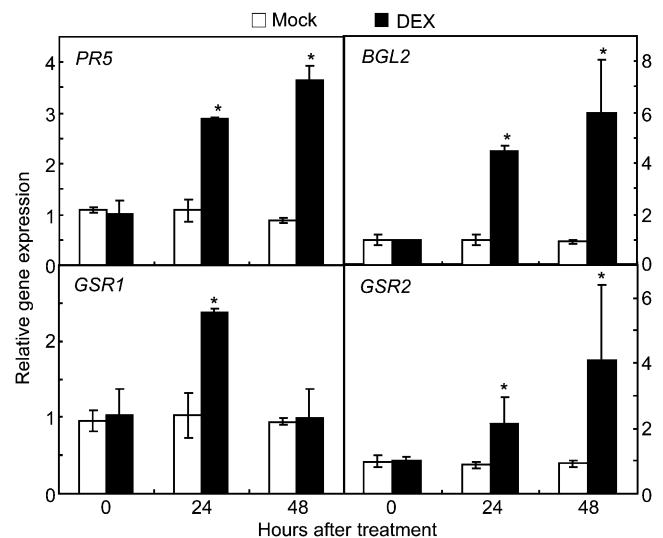


Figure 10. Real-time RT-PCR analyses of the expression of Arabidopsis genes that are newly induced in DEX:CaXEGIP1 transgenic plants. RNA was extracted from the leaves of 4-week-old transgenic Arabidopsis plants 24 and 48 h after mock or DEX treatment. The relative amount of the gene transcript was normalized against the expression level of the *UBQ* gene. *PR5*, Pathogenesis-related gene5 (AT1G75040); *BGL2*, β -1,3-glucanase2 (AT3G57260); *GSR1*, Gln synthetase (AT5G37600); *GSR2*, copper-ion binding/Glu-ammonia ligase (AT1G66200). Asterisks indicate significant differences, as determined by the two-tailed Student's *t* test ($P < 0.05$).

1978). In contrast, virulent *P. syringae* infection suppresses cell wall appositions in Arabidopsis leaves by secreting the type III effector proteins (Hauck et al., 2003). Our findings, along with earlier studies, suggest that *CaXEGIP1* expression may strengthen the cell wall barrier, thus activating HR cell death-mediated defense responses.

Overexpression of *CaXEGIP1* in pepper, *N. benthamiana*, and Arabidopsis leaves induced pathogen-independent, spontaneous cell death and, concomitantly, enhanced accumulation of CaXEGIP1 protein. Notably, VIGS of *CaXEGIP1* in pepper plants compromised HR cell death but prompted plant growth under normal environmental conditions, as compared with empty vector control plants. These findings support the notion that pathogen-induced CaXEGIP1 up-regulates plant cell death via modification of cell wall structure. Increasing experimental evidence points to a key role for the XG family polysaccharides during various plant developmental processes, including seed germination, fruit ripening, and rapid cell wall expansion (Bourquin et al., 2002; Baumann et al., 2007; Miedes and Lorences, 2009; Anderson et al., 2010). XG is known to coat and cross-link cellulose microfibrils. Thus, the breakage of XG from the XG-cellulose network into oligosaccharides leads to cell wall loosening and cell expansion. However, the significance of xyloglucanase-mediated modification of cell wall structure during cell death signaling remains to be understood.

Silencing of *CaXEGIP1* resulted in the enhanced growth of both virulent and avirulent *Xcv* in pepper leaves. These data suggest a potential role for *CaXEGIP1* in the plant defense response to bacterial pathogens. Importantly, the high level of bacterial growth in *CaXEGIP1*-silenced plants was accompanied by the reduced expression of defense-related genes, such as *CaPR1* (Kim and Hwang, 2000) and *CaDEF1* (Do et al., 2004). These defense-related genes are strongly induced by avirulent *Xcv* infection. Notably, ectopic expression of *CaPR1* confers tobacco (cv Xanthi) plants with a significantly enhanced resistance to a diverse range of plant pathogens, including the oomycete pathogen *Phytophthora nicotianae* and the bacterial pathogens *Ralstonia solanacearum* and *P. syringae* pv *tabaci* (Sarowar et al., 2005). Together, these findings along with other available information suggest that the *CaXEGIP1* gene plays a pivotal role in the disease resistance and downstream defense signaling of pepper plants.

Unexpectedly, *CaXEGIP1* overexpression did not significantly suppress the proliferation of *Pst* DC3000 in transgenic Arabidopsis leaves. However, the virulent and avirulent *Pst* DC3000 infection distinctly induced susceptible and hypersensitive cell death phenotypes in *CaXEGIP1*-overexpressing transgenic plants, respectively. These results suggest that *CaXEGIP1* overexpression does not directly impair *Pst* DC3000 itself but instead positively regulates plant cell death signaling. In contrast, *CaXEGIP1* overexpression did significantly suppress infection by the biotrophic

oomycete pathogen *Hpa* by enhancing the cell death response. Comparative proteomics and quantitative PCR analyses of transgenic Arabidopsis plants identified four genes (*PR2*, *PR5*, *GSR1*, and *GSR2*) newly induced by *CaXEGIP1* overexpression. Arabidopsis *PR2* and *PR5*, which encode β -1,3-glucanase and thaumatin-like protein, respectively, have been widely used as defense molecular markers. Notably, thaumatin-like *PR5* families are involved in resistance against oomycetes (van Loon et al., 2006). Reduced susceptibility of the *CaXEGIP1*-overexpressing transgenic Arabidopsis plants against *Hpa* infection may be partially due to enhanced expression of *PR2* and *PR5*.

In summary, we have identified *CaXEGIP1* as essential for the HR cell death-mediated defense response of pepper plants. Silencing of *CaXEGIP1* confers pepper plants with significantly enhanced susceptibility to *Xcv* infection. This is accompanied by compromised HR cell death and decreased expression of *CaPR1* and *CaDEF1*. In contrast, overexpression of *CaXEGIP1* induces a pathogen-independent, spontaneous cell death response and enhances resistance to the obligate biotrophic downy mildew pathogen in Arabidopsis via cell wall modification. Together, these data suggest that extracellular and cell wall-bound CaXEGIP1 may enhance disease resistance by strengthening the host cell wall.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pepper plants (*Capsicum annuum* 'Nockwang') and *Nicotiana benthamiana* were raised in a growth room at 28°C with a 16-h photoperiod at a light intensity of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Arabidopsis (*Arabidopsis thaliana* ecotype Columbia [Col-0]) and transgenic plants were grown at 24°C under long-day conditions (16 h of light/8 h of dark) with a photosynthetic flux of 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Isolation and Sequence Analysis of *CaXEGIP1* cDNA

A cDNA library was constructed using poly(A⁺) mRNA extracted from pepper leaves inoculated with the avirulent *Xanthomonas campestris* pv *vesicatoria* strain Bv5-4a (Jung and Hwang, 2000; Choi et al., 2007). Differential hybridization screening was performed using the cDNA library, and selected clones were sequenced on the ABI 310 DNA sequencer (Applied Biosystem).

Purification and Activity Assay of CaXEGIP1

The *CaXEGIP1* coding region was cloned in the pET30a vector (Novagen). *Escherichia coli* BL21 (DE3) (Invitrogen) was grown at 37°C until the optical density at 600 nm (OD_{600}) reached 0.5. Gene expression was induced by adding IPTG to a final concentration of 500 μM . After 18 h of incubation at 18°C with shaking, bacterial cells were harvested by centrifugation at 3,000g for 30 min. The cells were resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 0.3 M NaCl, and 10 mM imidazole) and lysed by sonication. Cell debris was removed by centrifugation at 15,000g for 1 h at 4°C. The soluble His-tagged CaXEGIP1 protein was purified from the supernatant by affinity chromatography using Ni²⁺-nitrilotriacetic acid agarose resin (Qiagen).

Recombinant XEG from *Clostridium thermocellum* F7/YS was purchased from PROZOMIX. The inhibitory effect of CaXEGIP1 on the enzymatic activity of XEG was assayed using AZO-XG (Megazyme) as a substrate according to the manufacturer's instructions. Varying amounts of CaXEGIP1 were preincubated with 1 μg of XEG at 60°C for 1 h, then mixed and

incubated with 0.5 mL of 10 mg mL⁻¹ AZO-XG in 100 mM sodium acetate buffer (pH 4.5) at 60°C for 10 min. The reaction was terminated, and the high-*M_r* substrates were precipitated by the addition of 2 mL of 100% methanol. After centrifugation at 1,000g for 10 min, the amount of reducing sugars was measured by spectrophotometric examination of the absorbance of the supernatant solutions containing depolymerized Remazol Brilliant Blue-dyed sugars at 590 nm.

GFP Localization by Confocal Microscopy

Biolistic transformation of onion (*Allium cepa*) epidermal cells was performed as described previously (Choi et al., 2007). Briefly, the *CaXEGIP1* coding region was cloned between the CaMV 35S promoter and the *smGFP* region of the binary vector p326GFP to generate a C-terminal fusion of *smGFP* to *CaXEGIP1*. The resulting plasmids were purified using the Qiagen plasmid maxi kits according to the manufacturer's instructions. Onion epidermis was bombarded with gold particles coated with plasmids using a Bio-Rad PDS-1000/He particle-delivery system. Bombarded specimens were incubated for 24 h on 1× MS agar medium at 24°C and observed using a MRC-1024 confocal laser scanning microscope (Bio-Rad).

Pathogen Inoculation

The *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) virulent strain Ds1 and the avirulent strain Bv5-4a were prepared as described previously (Choi et al., 2007). Briefly, bacteria were cultured overnight in YN broth (5 g of yeast extract and 8 g of nutrient broth L⁻¹) at 28°C. Prior to inoculation, bacterial cells were collected by centrifugation and resuspended in 10 mM MgCl₂ (10⁸ colony-forming units [cfu] mL⁻¹). Pepper plants at the six-leaf stage were inoculated by infiltrating the bacterial suspension into the abaxial side of fully expanded leaves using a syringe without a needle. The infected plants were incubated in a controlled chamber at 28°C with 100% relative humidity for 16 h.

Pseudomonas syringae pv *tomato* DC3000 and DC3000 (*avrRpm1*) were used to infect Arabidopsis. The leaves of wild-type (Col-0) and DEX:*CaXEGIP1* plants were infiltrated with *Pst* at 10⁷ cfu mL⁻¹ (for disease symptom or HR observation) or 10⁵ cfu mL⁻¹ (for the bacterial growth assay). Bacterial growth in leaves was monitored on King's B agar medium containing 50 μg mL⁻¹ rifampicin and 50 μg mL⁻¹ kanamycin.

Hyaloperonospora arabidopsidis isolate Noco2 was maintained on the susceptible Arabidopsis ecotype Col-0 (Reignault et al., 1996). One-week-old seedlings of wild-type and DEX:*CaXEGIP1* plants were inoculated with a suspension of 5 × 10⁴ conidiosporangia mL⁻¹ in distilled tap water containing 0.05% Tween 20. Seedlings were covered with a transparent dome to maintain high humidity and were grown at 17°C with a 14-h photoperiod. Asexual sporulation was assessed by counting the sporangiophores per cotyledon 5 d after inoculation.

RNA Gel-Blot and RT-PCR Analyses

Total cellular RNA was extracted from pepper and Arabidopsis leaves using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. For RNA gel blotting, equal quantities of RNA were separated on 1.2% formaldehyde-agarose gels in the presence of ethidium bromide and transferred to nylon membranes (Hybond N⁺; Amersham). To generate a gene-specific probe, the N-terminal region of *CaXEGIP1* was amplified using the following primers: forward (5'-ATGGTGACATTCAAACTCCCT-3') and reverse (5'-CTTCTGGTGAATCTAGGGTC-3'). A PCR-amplified fragment of 588 bp was ³²P labeled using a random priming kit (Boehringer Mannheim). Agarose gel electrophoresis, RNA transfers, and hybridization with the *CaXEGIP1* fragment were performed using standard procedures.

Pepper and Arabidopsis cDNA was synthesized from total RNA (2 μg) using avian myeloblastosis virus reverse transcriptase (Roche) with oligo(dT)₁₅ as a primer (Roche) according to the manufacturer's instructions. RT-PCR was performed using ExTaq polymerase (Takara Biomedicals). The reaction mixture contained aliquots of 1 μL of RT reaction products, 0.5 μM each of the forward and reverse primers, 1× ExTaq buffer, 250 nM deoxyribonucleotide triphosphates, and 0.5 units of ExTaq polymerase in a 50-μL solution. RT-PCR conditions were 95°C for 10 min and 30 cycles of 95°C for 30 s each, 52°C for 30 s, and 72°C for 30 s. Single bands of PCR products were confirmed on an agarose gel. The following gene-specific primers were used for RT-PCR

analyses: 5'-GTGGCATCTGAAGAGTACTATATC-3' (forward) and 5'-CTAGCCTAGAGTGGTATTATCG-3' (reverse) for *CaXEGIP1*; 5'-CAGGATGCAACTCTGGTGG-3' (forward) and 5'-ATCAAAGGCCGGTTGGTC-3' (reverse) for *CaPR1* (accession no. AF053343); and 5'-CAGGATGCAACTCTGGTGG-3' (forward) and 5'-ATCAAAGGCCGGTTGGTC-3' (reverse) for *CaDEF1* (accession no. AF442388).

Agrobacterium tumefaciens-Mediated Transient Expression Assay

A. tumefaciens-mediated transient expression experiments were performed as described previously (Choi et al., 2011, 2012; Kim and Hwang, 2011). Briefly, the coding region of *CaXEGIP1* was cloned between the CaMV 35S promoter and the *nos* terminator region of the binary vector pBIN35S. The resulting plasmid was transformed into *A. tumefaciens* GV3101. *A. tumefaciens* containing the empty vector (pBIN:00) or the pBIN35S:*CaXEGIP1* construct was grown overnight at 28°C in YEP medium (10 g of yeast extract, 10 g of peptone, and 5 g of sodium chloride [Difco] L⁻¹; pH 7.5 adjusted with NaOH) containing 50 μg mL⁻¹ rifampicin and 50 μg mL⁻¹ kanamycin. The bacterial cells were suspended in infiltration buffer (10 mM MgCl₂, 10 mM MES [pH 5.7], and 200 μM acetosyringone) to a final OD₆₀₀ of 1.0, followed by serial dilutions. Bacterial cells were infiltrated into pepper or *N. benthamiana* leaves. For comparison, *Pto*, *avrPto*, and *Pto/avrPto* were transiently expressed in *N. benthamiana* leaves.

VIGS

The tobacco rattle virus (TRV)-based VIGS system was used to silence *CaXEGIP1* in pepper plants (Liu et al., 2002; Choi and Hwang, 2011; Lee et al., 2011). The C-terminal region of *CaXEGIP1* was amplified by PCR with the following primers: 5'-GTGGCATCTGAAGAGTACTATATC-3' (forward) and 5'-CTAGCCTAGAGTGGTATTATCG-3' (reverse). PCR-amplified fragments containing the 558-bp 3' region of *CaXEGIP1* were cloned into the pTRV2 vector to yield pTRV2:*CaXEGIP1*. The resulting plasmid was transformed into *A. tumefaciens* strain GV3101. Transformants carrying pTRV1 or pTRV2:*CaXEGIP1* were coinfiltrated into the fully expanded cotyledons of pepper plants (OD₆₀₀ = 0.2). Plants were placed in a growth room at 25°C with a 16-h-light/8-h-dark photoperiod to allow viral growth and spread. Pepper plants silenced for 4 to 5 weeks were used for pathogen inoculation.

Arabidopsis Transformation

The full-length *CaXEGIP1* cDNA sequence was excised from pBluescript SK- by digestion with *XhoI* and *XbaI* and subsequently cloned into pTA7002. The binary plasmids were introduced into *A. tumefaciens* strain GV3101 via electroporation. Transgenic Arabidopsis plants harboring the DEX-inducible *CaXEGIP1* gene were generated using the floral dipping method (Clough and Bent, 1998). Transgenic plants were selected on MS agar plates containing 25 μg mL⁻¹ hygromycin. For DEX treatment, 30 μM DEX was sprayed onto Arabidopsis leaves.

Immunoblot Analyses

After SDS-PAGE, proteins were electrotransferred to polyvinylidene difluoride membranes (GE Healthcare). Membranes were incubated with a 1:10,000 dilution of polyclonal rabbit antibodies raised against the *CaXEGIP1*-specific peptide NIYNPKKIDISKD (AbFrontier). Antigen-antibody complexes were detected using peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich).

Histochemistry

Trypan blue staining was performed as described previously (Choi et al., 2007). To visualize cell death and fungal structure, leaves were stained by boiling in lactophenol-trypan blue solution (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) followed by destaining with chloral hydrate (2.5 g mL⁻¹). To monitor the modification of the cell wall structure in DEX:*CaXEGIP1* transgenic Arabidopsis seedlings, transverse hand sections were obtained from agarose-embedded seedlings and stained with 0.1% Fluorescent Brightener 28 (Sigma; Lin and Schiefelbein, 2001).

Electrolyte Leakage Measurement

Six leaf discs of defined area (0.6 cm in diameter) were removed and washed with distilled water following infiltration. These discs were incubated in 3 mL of distilled water at room temperature. The conductivity of the incubation medium was recorded at various time points with a conductivity meter (model sensION7; Hach; <http://www.hach.com>).

2-DE and Protein Staining

2-DE was performed as described previously (Choi and Hwang 2011) with minor modifications. Total soluble proteins were extracted from the leaves of mock-treated or 30 μ M DEX-treated transgenic Arabidopsis plants using extraction buffer (10% [w/v] trichloroacetic acid and 0.07% [w/v] dithiothreitol [DTT] in cold acetone [-20°C]). Protein extracts (800 μ g) were suspended in rehydration buffer (9 M urea, 100 mM DTT, 4% [w/v] CHAPS, 0.5% [v/v] Bio-lyte 3/10 carrier ampholytes, and 0.002% bromophenol blue) and applied to an immobilized pH gradient (IPG) strip for in-gel rehydration. Strips were rehydrated at 50 V for 24 h. Isoelectric focusing was performed using 24-cm IPG strips (Bio-Rad) with a nonlinear pH 4 to 7 gradient. Isoelectric focusing was run using a PROTEAN IEF Cell (Bio-Rad) at gradient steps of 250 V for 1 h, 500 V for 1 h, 1,000 V for 2 h, and 10,000 V for 4 h, then a final step of 10,000 V toward a total of 90 kVh. IPG strips were incubated in the equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% [v/v] glycerol, and 2% [w/v] SDS) containing 1% (w/v) DTT for 15 min for the first equilibration step. This was followed by incubation in 4% (w/v) iodoacetamide for the second equilibration step. For 2-DE, IPG strips were sealed on the top of the 12.5% two-dimensional gel using 1% low-melting agarose in SDS-electrophoresis buffer (25 mM Tris, 0.2 M Gly, and 0.1% SDS). SDS-PAGE was performed in an Ettan DALTsix electrophoresis unit (Amersham Biosciences) at 5 W per gel for 1 h, followed by 15 W per gel for 6 h until the bromophenol blue front reached the end of the gel.

For Coomassie Brilliant Blue staining, gels were fixed in a 40% (v/v) methanol and 7% acetic acid solution for 2 h and then stained in a Coomassie Brilliant Blue solution (0.1% [w/v] Coomassie Brilliant Blue G 250, 34% [v/v] methanol, 3% [v/v] phosphoric acid, and 17% [w/v] ammonium sulfate). Coomassie blue-stained gels were scanned with the UMAX PowerLook 1100XL scanner, and the images were analyzed using ImageMaster 2D Platinum 6.0 (Amersham Biosciences). The two-dimensional gels were compared and matched, and a quantitative determination of spot volumes was performed.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JQ673414 (*CaXEGIPI*), AF053343 (*CaPRI*), AF442388 (*CaDEF1*), AT1G75040 (*PR5*), AT3G57260 (*BGL2*), AT5G37600 (*GSR1*), AT1G66200 (*GSR2*), and AT3G62250 (*UBQ5*).

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of pepper CaX-EGIP1 with putative XEGIPs from grape (accession no. XP_002272235), petunia (*Petunia \times hybrida*; accession no. ACM68432), carrot (accession no. BAA03413), tomato (accession no. AAN87262), potato (accession no. AAP84703), and Arabidopsis (accession no. AAM61574).

Supplemental Figure S2. Trypan blue staining of CaXEGIPI-induced cell death in *N. benthamiana* leaves.

Supplemental Figure S3. VIGS of empty vector (TRV:00) in pepper leaves does not affect *Xcv* growth and electrolyte leakages in pepper leaves compared with those in wild-type pepper leaves.

Supplemental Figure S4. No cell death phenotypes and no differences in bacterial growth are seen in mock- and DEX-treated wild-type Arabidopsis (Col-0) plants.

Supplemental Figure S5. Two-dimensional gel images of the proteome in mock- or DEX-treated DEX:CaXEGIPI transgenic Arabidopsis leaves.

Supplemental Table S1. Proteins newly induced by DEX:CaXEGIPI expression in Arabidopsis, as identified by matrix-assisted laser-desorption ionization time of flight mass spectrometry.

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