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

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ABSTRACT

Plants produce various proteinaceous inhibitors to protect themselves against microbial pathogen attack. A xyloglucan-specific endo-\(\beta\)-1,4-glucanase inhibitor 1 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 (Xcv), and purified CaXEGIP1 protein significantly inhibited the hydrolytic activity of glycoside hydrolase 74 family xyloglucan-specific endo-\(\beta\)-1,4-glucanase (XEG) from Clostridium thermocellum. Soluble-modified green fluorescent protein (smGFP)-tagged CaXEGIP1 proteins were mainly localized to the apoplast of onion epidermal cells. Agrobacterium-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 Xcv, accompanied by compromised HR and the lowered expression of defense-related genes. Overexpression of dexamethasone (DEX):CaXEGIP1 in Arabidopsis 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.
INTRODUCTION

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 Gibeaut, 1993). The primary structure of XG contains a common β-(1→4)-D-glucan backbone, which is repeatedly substituted with α(1→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 xyloglucan endotransglycosylases 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 *Phaseolus 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 (PGs) from *Fusarium phyllophilum* and *Aspergillus niger* via three conserved aspartic acid residues (Spinelli et al., 2009). PGIPs reduce the hydrolytic activity of PGs to favor the accumulation of long-chain oligogalacturonides (OGs), known as elicitors of a variety of defense responses (Côté and Hahn, 1994; D'Ovidio et al., 2004b). Furthermore, transgenic expression of pear PGIP in transgenic tomato 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–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 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 L.) 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 xyloglucan-specific endo-β-1,4-glucanase (XEG) from the thermophilic bacterium Clostridium thermocellum. The soluble-modified green fluorescent protein (smGFP)-fused CaXEGIP1 protein was localized in the external and intercellular regions of onion epidermal cells. Importantly, Agrobacterium-mediated transient expression of CaXEGIP1 induced the hypersensitive cell death response in pepper and Nicotiana benthamiana leaves. Virus-induced gene silencing (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 (PR1) and CaDEF1 (defensin). We also investigated the role of CaXEGIP1 in plant cell death and defense responses using transgenic Arabidopsis plants harboring the dexamethasone-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 Xyloglucan-Specific Endo-β-1,4-Glucanase Inhibitor Protein

To isolate pepper genes induced during the hypersensitive response (HR), we performed macro cDNA array analysis using a cDNA library constructed from pepper leaves infected with avirulent X. campestris pv. vesicatoria (Xcv) strain Bv5-4a (Jung and Hwang, 2000; Hwang and Hwang, 2010 and 2011; Hwang et al., 2011). Among the defense-related genes selected, we isolated the putative pepper xyloglucan-specific endo-β-1,4-glucanase inhibitor protein 1 (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 kDa 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 (Solanum lycopersicum, accession no. AAN87262) (Qin et al., 2003).

CaXEGIP1 Is Strongly Induced in Leaves by Avirulent Xanthomonas campestris pv. vesicatoria 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 to 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 Xyloglucan-Specific Endo-β-1,4-Glucanase (XEG) Activity by CaXEGIP1 Protein

Recombinant CaXEGIP1 protein was purified from the extracts of isopropyl-β-D-thio-galactoside (IPTG)-induced E. coli BL21 cells using Ni-NTA affinity chromatography (Fig. 2A). The homogeneity of the purified protein was
confirmed by a single band on a Coomassie Brilliant Blue (CBB)-stained SDS-PAGE gel (Fig. 2A, Lane 3). To investigate whether recombinant CaXEGIP1 inhibits the hydrolytic activity of xyloglucan-specific endo-β-1,4-glucanase (XEG), various amounts of the purified CaXEGIP1 were mixed with 1 μg of GH74 family XEG enzyme from *Clostridium 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-xyloglucan solutions (Megazyme, Co. Wicklow, IE). 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 xyloglucan.

**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 24-Ala (A) and 25-Glu (E) (Supplemental Fig. S1). To analyze the subcellular localization of the CaXEGIP1 protein, we performed a biolistic transformation experiment. The soluble-modified green fluorescent protein (*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 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 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 *Agrobacterium*-mediated transient expression in pepper and *Nicotiana benthamiana* plants, as described previously (Choi et al., 2009 and 2011; Hwang and Hwang, 2010; Choi and Hwang, 2011; Kim and Hwang, 2011). Reverse-transcription polymerase chain reaction (RT-PCR) and immunoblot analyses revealed expression of CaXEGIP1 in *Agrobacterium*-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 *Nicotiana 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* 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 EV-, *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 X. campestris pv. vesicatoria 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 (pathogenesis-related protein 1) 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, virus-induced gene silencing of empty vector (TRV:00) in pepper leaves did not affect Xcv growth and electrolyte leakages in pepper leaves compared to those in wild-type
pepper leaves (Supplemental Figure 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 CaMV 35S promoter. Thus, the dexamethasone (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 dexamethasone, and this line was selected for further experiments. We did not observe any apparent phenotypic differences between wild-type (WT) 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 days 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 \textit{DEX:CaXEGIP1} transgenic leaf tissues (Fig. 7C). However, mock- and DEX treatments did not trigger any cell death response in leaves of wild-type \textit{Arabidopsis} plants, as observed by visible plant growth phenotypes and trypan blue staining (Supplemental Fig. S4, A and B).

Seven-day-old transgenic \textit{Arabidopsis} seedlings were transplanted to MS media containing 3 μM DEX. Interestingly, the transgenic seedlings grown on this media exhibited undirectional root bending (Fig. 7D, lower panel). However, no phenotypic differences were observed in the transgenic seedlings grown in the absence of DEX (Fig. 7D, upper panel). To visualize the root bending at the cellular level, transverse sections of the root tissue were stained with 0.1% Calcofluor and observed under a microscope equipped with a UV lamp (Fig. 7E). Compared to the mock-treated WT and transgenic seedling roots, the DEX-treated transgenic 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 \textit{CaXEGIP1} triggered the cell death response in the leaves, roots and hypocotyls of transgenic \textit{Arabidopsis} plants.

\textbf{\textit{CaXEGIP1} Overexpression Confers Enhanced Cell Death but Does Not Suppress \textit{Pseudomonas syringae pv. tomato} growth in \textit{Arabidopsis}}

We next investigated CaXEGIP1 gain-of-function in DEX:CaXEGIP1 transgenic Arabidopsis plants during Pseudomonas syringae pv. tomato (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 to 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 did 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 which 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 and 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 Hp. arabidopsidis
(Hpa) isolate Noco2. DEX-induced overexpression of CaXEGIP1 induced cell death and also 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 to 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 (see Supplemental Figure S5C) were excised from the 2-D gels and analyzed by MALDI-TOF (see Supplemental Table S1).
Interestingly, two of these proteins were identified as pathogenesis-related (PR) proteins, PR5 (N1, thaumatin, AT1G75040) and PR2 [N2, \( \beta \)-1,3-glucanase 2 (BGL2), AT3G57260]. The other protein spots were identified as glutamine (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 (*Capsicum annuum*) were isolated from a pepper cDNA library using differential hybridization and RNA gel blot analyses (Choi et al., 2007, 2008 and 2009). One of the isolated defense response genes was identified as a xyloglucan-specific endoglucanase inhibitor 1 protein (CaXEGIP1) gene 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 have been demonstrated to have in vitro inhibitory activity against the fungus Aspergillus 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 inhibits the pathogen-derived XEG enzyme activity to reduce the pathogen virulence. However, the fungus Aspergillus-derived or other XEG enzymes that belong to GH12 family were not available for us to test inhibitory activity of CaXEGIP1. Thus, we first tested commercial XEG enzyme from a thermophilic bacterium Clostridium thermocellum F7/YS, which belongs to a GH74 family XEG (PROZOMIX, Northumberland, UK). 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, ninety two 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 the CaXEGIP1 can target cell wall-degrading enzymes of invading pathogens.

Recently, structural studies using XEGIP protein from carrot and GH12 family XEG protein from *Aspergillus aculeatus* revealed that the two critical Arginine (Arg) residues from XEGIP proteins located in the inhibition loop 1 (IL-1) (Arg322) and IL-2 (Arg403) 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 Figure 1). Recently, Yoshizawa et al., (2011) reported the crystal structure of soybean XEGIP and found that it lacks inhibitory activity on GH12 and GH11 family XEG enzymes, due to the lack of key amino acid residues, Arg322 and Arg403 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 support the notion that the inhibitory mechanisms of CaXEGIP1 on GH74 family XEG may be different from those of other reported XEGIP on GH12 family XEG enzymes. Alternatively, CaXEGIP1 may target intrinsic xyloglucan-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 soluble-modified green fluorescent protein (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 xyloglucan endo-β-1, 4-glucanase (XEG) from Clostridium thermocellum F7/YS. 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, Agrobacterium-mediated or dexamethasone (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 plants with
Phytophthora infestans triggers 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, 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 to empty vector control plants. These findings support the notion that pathogen-induced CaXEGIP1 upregulates plant cell death via modification of cell wall structure. Increasing experimental evidence points to a key role for the xyloglucan 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). Xyloglucan is known to coat and cross-link cellulose microfibrils. Thus, the breakage of xyloglucan from the xyloglucan-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 (pathogenesis-related protein1) (Kim and Hwang, 2000) and CaDEF1 (defensin) (Do et al., 2004). These defense-related genes are strongly induced by avirulent Xcv infection. Notably, ectopic expression of CaPR1 confers tobacco (Nicotiana tabacum 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 Pseudomonas 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 Pseudomonas syringae (Pst) DC3000 in transgenic Arabidopsis leaves. However, the virulent and avirulent Pst DC3000 infection distinctly induced susceptible and hypersensitive cell death phenotypes in CaXEGIP1-OX 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 *Hyaloperonospora arabidopsidis* (*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*-OX transgenic *Arabidopsis* plants against *Hpa* infection may be partially due to enhanced expression of *PR2* and *PR5*.

In summary, we have identified the *xyloglucan-specific endoglucanase inhibitor 1* gene *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 L. cv. Nockwang) and Nicotiana benthamiana were raised in a growth room at 28 °C with a 16 h photoperiod at a light intensity of 100 μmol photons m−² sec⁻¹. Arabidopsis (Arabidopsis thaliana ecotype Columbia, Col-0) and transgenic plants were grown at 24 °C under long-day conditions (16 h light/8 h dark) with a photosynthetic flux of 130 μmol photons m⁻² sec⁻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 (Xcv) 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, Foster City, CA).

Purification and Activity Assay of CaXEGIP1

The CaXEGIP1 coding region was cloned in the pET30a vector (Novagen, Madison, WI). Escherichia coli BL21 (DE3) (Invitrogen, Carlsbad, USA) was grown at 37 °C until the OD₆₀₀ reached 0.5. Gene expression was induced by adding isopropyl-β-D-thio-galactoside (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 3000 g for 30 min. The cells were resuspended in lysis buffer
[50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 10 mM imidazole] and lysed by sonication. Cell debris was removed by centrifugation at 15,000 g for 1 h at 4 °C. The soluble His-tagged CaXEGIP1 protein was purified from the supernatant by affinity chromatography using Ni²⁺-nitrilotriacetic acid (NTA) agarose resin (Qiagen, Hilden, Germany).

Recombinant xyloglucan endo-β-1,4-glucanase (XEG) from Clostridium thermocellum F7/YS was purchased from PROZOMIX (Haltwhistle, Northumberland, UK). The inhibitory effect of CaXEGIP1 on the enzymatic activity of XEG was assayed using AZO-XYLOGLUCAN (Megazyme, Co. Wicklow, IE) as a substrate according to the manufacturer’s instructions. Varying amounts of CaXEGIP1 were pre-incubated with 1 μg of XEG at 60 °C for 1 h, and mixed and incubated with 0.5 mL of 10 mg mL⁻¹ of AZO-XYLOGLUCAN in 100 mM sodium acetate buffer (pH 4.5) at 60 °C for 10 min. The reaction was terminated and the high-molecular weight substrates were precipitated by the addition of 2 mL of 100% methanol. After centrifugation at 1000 g 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 epidermal cells was performed as described previously (Choi et al., 2007). Briefly, the CaXEGIP1 coding region was cloned between the CaMV35S 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 (Qiagen, Valencia, CA). Onion (Allium cepa) epidermis was bombarded with gold particles coated with plasmids using a Bio-Rad (Hercules, CA) PDS-1000/He particle delivery system. Bombarded specimens were incubated for 24 h on 1X Murashige and Skoog (MS) agar media at 24 °C and observed using a MRC-1024 confocal laser scanning microscope (Bio-Rad, Hercules, CA).

Pathogen Inoculation

The X. 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 yeast extract, 8 g nutrient broth L⁻¹) at 28°C. Prior to inoculation, bacterial cells were collected by centrifugation and resuspended in 10 mM MgCl₂ (10⁸ 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 (Pst) DC3000 and DC3000 (avrRpm1) were used to infect Arabidopsis. The leaves of WT (Col-0) and DEX:CaXEGIP1 plants were infiltrated with Pst at 10⁷ cfu mL⁻¹ (for disease symptom or hypersensitive response observation) or 10⁵ cfu mL⁻¹ (for the bacterial growth
Bacterial growth in leaves was monitored on King’s B agar medium containing 50 μg mL⁻¹ rifampicin (Rf) and 50 μg mL⁻¹ kanamycin (Km).

Hyaloperonospora arabidopsidis isolate Noco2 was maintained on the susceptible Arabidopsis ecotype Col-0 (Reignault et al., 1996). One-week-old seedlings of WT 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 days after inoculation.

RNA Gel Blot and RT-PCR Analyses

Total cellular RNA was extracted from pepper and Arabidopsis leaves using TRIzol Reagent (Invitrogen, Carlsbad) 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’-ATGGTGACATTCAAACTTCCCT-3’) and reverse (5’-CTTCCTGGTGAATCTAGGGTC-3’). A PCR-amplified fragment of 588 bp was ³²P-labeled using a random priming kit (Boehringer Mannheim, Mannheim, Germany). 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 AMV reverse transcriptase (Roche, Mannheim, Germany) with oligo (dT)15 as a primer (Roche, Mannheim, Germany) according to the manufacturer’s instructions. RT-PCR was performed using ExTaq polymerase (Takara Biomedicals, Otsu, Japan). The reaction mixture contained aliquots of 1 μl RT-reaction products, 0.5 μM each of the forward and reverse primers, 1× ExTaq buffer, 250 nM dNTPs, 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′-CAGGATGCAACACTCTGGTGG-3′ (forward) and 5′-ATCAAAGGCCGGTTGGTC-3′ (reverse) for *CaPR1* (accession no. AF053343); and 5′-CAGGATGCAACACTCTGGTGG-3′ (forward) and 5′-ATCAAAGGCCGGTTGGTC-3′ (reverse) for *CaDEF1* (accession no. AF442388).

**Agrobacterium-Mediated Transient Expression Assay**

*Agrobacterium*-mediated transient expression experiments were performed as described previously (Choi et al., 2011; Kim and Hwang, 2011; Choi et al., 2012). Briefly, the coding region of *CaXEGIP1* was cloned between the *CaMV35S* promoter and the *nos* terminator region of the binary vector pBIN35S. The resulting plasmid was transformed into *A. tumefaciens* GV3101.
Agrobacterium containing the empty vector (pBIN:00) or the pBIN35S:CaXEGIP1 construct was grown overnight at 28°C in YEP media [10 g of yeast extract, 10 g of peptone, and 5 g of sodium chloride (Difco, Detroit) per liter; pH 7.5 adjusted with NaOH] containing 50 μg mL⁻¹ Rf and 50 μg mL⁻¹ Km. The bacterial cells were suspended in infiltration buffer [10 mM MgCl₂, 10 mM 2-(N-morpholino) ethane sulfonic acid (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.

Virus-Induced Gene Silencing

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 558-bp 3’ region of CaXEGIP1 were cloned into the pTRV2 vector to yield pTRV2:CaXEGIP1. The resulting plasmid was transformed into Agrobacterium tumefaciens strain GV3101. Transformants carrying pTRV1 or pTRV2:CaXEGIP1 were co-infiltrated 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 4–5 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 dexamethasone (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 electro-transferred 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, Seoul, Korea). 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-trypa blue solution (10 mL lactic acid, 10 mL glycerol, 10
g phenol, and 10 mg trypan blue dissolved in 10 mL distilled water), followed by
destaining with chloral hydrate (2.5 g mL\(^{-1}\)). To monitor the modification of the
cell wall structure in \(\text{DEX:CaXEGIP1}\) transgenic \textit{Arabidopsis} seedlings,
transverse hand sections were obtained from agarose-embedded seedlings and
stained with 0.1% Fluorescent Brightener 28 (Sigma, MO) (Lin and Schiefelbein,
2001).

**Electrolyte Leakage Measurement**

Six-leaf disks of defined area (0.6 cm in diameter) were removed and washed
with distilled water following infiltration. These disks 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;

**2D Electrophoresis and Protein Staining**

Two dimensional electrophoresis (2-DE) was performed as described
previously (Choi and Hwang 2011) with minor modification. Total soluble
proteins were extracted from the leaves of mock- or 30 \(\mu\text{M}\) DEX-treated
transgenic \textit{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 \(\mu\text{g}\)) 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 (IEF) was performed using 24 cm IPG strips (Bio-Rad) with a nonlinear pH 4–7 gradient. IEF was run using a PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA) 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, and 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% 2-dimensional gel using 1% low-melting agarose in SDS-electrophoresis buffer (25 mM TRIS, 0.2 M glycine, 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 CBB staining, gels were fixed in a 40% (v/v) methanol and 7% acetic acid solution for 2 h and then stained in a CBB 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-stained gels were scanned with the UMAX PowerLook 1100XL scanner and the images were analyzed using ImageMaster 2D Platinum 6.0 (Amersham Biosciences). The 2D 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 (CaXEGIP1), AF053343 (CaPR1), 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 xyloglucan-specific endoglucanase inhibitor 1 (CaXEGIP1) with other putative XEGIPs.

Supplemental Figure S2. Trypan blue staining of CaXEGIP1-induced cell death in Nicotiana benthamiana leaves.

Supplemental Figure S3. 2D gel images of the proteome in mock- or DEX-treated DEX:CaXEGIP1 transgenic Arabidopsis leaves.

Supplemental Table S1. Proteins newly induced by DEX: CaXEGIP1 expression in Arabidopsis, as identified by MALDI-TOF MS

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**FIGURE LEGENDS**

**Figure 1.** Induction of *CaXEGIP1* in pepper leaves by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) infection. (A) RNA gel blot and (B) immunoblot 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 MgCl$_2$. Equal loading of total RNA (20 μg per lane) was verified by visualizing rRNA on a gel stained with ethidium bromide. Protein loading was verified by CBB staining.

**Figure 2.** Inhibitory effect of the recombinant CaXEGIP1 protein on xyloglucan-specific β-1,4-endoglucanase (XEG) activity. (A) Expression and purification of recombinant CaXEGIP1 in *E. coli* BL21 (DE3) cells. Protein loading was verified by CBB 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 the means ± standard deviations (n=4) from three independent experiments. Different letters indicate significant differences, as determined by Fisher’s Least Significant Difference (LSD) test (P < 0.05, n = 4).

**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. Scale bars = 100 µm.

**Figure 4.** Induction of the cell death response in pepper leaves by *Agrobacterium*-mediated transient expression of *CaXEGIP1*. (A) RT-PCR and (B) immunoblot analyses of *CaXEGIP1* expression in pepper leaves. Expression of pepper 18S rRNA served as a loading control. Protein loading was verified by CBB staining. (C) Induction of cell death in pepper leaves. The leaf areas between the lateral veins were infiltrated with *Agrobacterium* at the indicated OD<sub>600</sub> concentrations. Photographs were taken 48 h after agroinfiltration. Visible: Visible symptoms. UV: UV-exposure to visualize the deposition of fluorescent pigments. (D) Trypan blue staining and (E) electrolyte leakage measurements from the leaves infiltrated by *Agrobacterium* (OD<sub>600</sub> = 0.5) strains carrying the indicated constructs. The data represent the means ± standard deviations from three independent experiments. Asterisks indicate significant differences in electrolyte leakage, as determined by the two-tailed t test (P < 0.05). Scale bars = 200 µm.

**Figure 5.** Effect of *Agrobacterium*-mediated transient expression of *Pto*, *avrPto*, *Pto/avrPto* and *CaXEGIP1* on the cell death response of *Nicotiana benthamiana* leaves. (A) RT-PCR and (B) immunoblot analyses of *CaXEGIP1* expression in *N. benthamiana* leaves. Expression of the *N. benthamiana Actin* gene served as a control. Protein loading was verified by CBB staining. (C) Induction of cell death in *N. benthamiana* leaves. (D) Measurements of electrolyte leakage from
*N. benthamiana* leaves infiltrated with *Agrobacterium* (OD$_{600}=0.5$) 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 the means ± standard deviations from three independent experiments. Different letters indicate statistically significant differences, as determined by Fisher's LSD test ($P < 0.05$).

**Figure 6.** Enhanced susceptibility of *CaXEGIP1*-silenced pepper plants to infection with the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) virulent strain Ds1 (C, compatible) and the avirulent strain Bv5-4a (I, incompatible). (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, *CaPR1*: pepper PR1, *CaDEF1*: pepper defensin. (C) Bacterial growth in leaves. Asterisks indicate significant differences, as determined by the two-tailed *t* test ($P < 0.05$). (D) Trypan blue staining and (E) electrolyte leakage measurements from leaves inoculated with avirulent *Xcv*. The data represent the means ± standard deviations from three independent experiments. Asterisks indicate significant differences, as determined by the two-tailed *t* test ($P < 0.05$). Scale bars = 200 µm.
Figure 7. CaXEGIP1-induced cell death and abnormal growth of DEX:CaXEGIP1 transgenic Arabidopsis plants after dexamethasone (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 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. Pictures were taken 2 days 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 media 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 media 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.

Figure 8. Cell death phenotype and bacterial growth in DEX:CaXEGIP1 transgenic Arabidopsis plants infiltrated with Pseudomonas syringae pv. tomato (Pst) DC3000. (A and B) Disease symptoms developed on the leaves of DEX:CaXEGIP1 transgenic Arabidopsis 8 days after inoculation with (A) virulent Pst DC3000 and (B) avirulent Pst DC3000 (avrRpm1) (10^7 cfu ml^-1).
Arabidopsis plants were inoculated with Pst DC3000 24 h after mock or 30 μM DEX treatment. (C) Bacterial growth in DEX:CaXEGIP1 transgenic Arabidopsis leaves 0 and 3 days after inoculation with virulent Pst DC3000 and avirulent Pst DC3000 (avrRpm1) strains (10⁵ cfu ml⁻¹). The data represent the means ± standard deviations from three independent experiments.

Figure 9. Enhanced resistance of DEX:CaXEGIP1 transgenic Arabidopsis plants to infection with Hyaloperonospora arabidopsidis (Hpa) isolate Noco2. (A) Trypan blue staining of Hpa-infected cotyledons of untreated and DEX-treated transgenic plants 3 days after inoculation. Arabidopsis seedling plants were inoculated with Hpa 24 h after mock or 30 μM DEX treatment. Scale bars = 200 μm. (B) Reduced growth of Hpa in DEX-treated transgenic Arabidopsis plants. The disease severity was rated on a 0–3 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 days after spray inoculation with Hpa (5×10⁴ spores mL⁻¹). Arrows indicate sporangiophores. Trypan blue-stained fungal structures are shown at higher magnification (lower panel). O, oospore; Ha, haustorium. Scale bars = 500 μm (middle); 50 μm (lower). (D) The number of sporangiophores per cotyledon of untreated or DEX-treated transgenic plants 7 days after inoculation with Hpa. Asterisks indicate significant differences in disease severity, as determined by
the two-tailed t test (P<0.05).

**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 gene 5* (AT1G75040); *BGL2*, β-1,3-glucanase 2 (AT3G57260); *GSR1*, glutamine synthetase (AT5G37600); *GSR2*, copper ion binding/glutamate-ammonia ligase (AT1G66200). Asterisks indicate significant differences, as determined by the two-tailed t test (P<0.05).

**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure S1.** Amino acid sequence alignment of pepper xyloglucan-specific endoglucanase inhibitor 1 (CaXEGIP1) with putative XEGIPs from grape (*Vitis vinifera*, accession no. XP_002272235), petunia (*Petunia x hybrida*, accession no. ACM68432), carrot (*Daucus carota*, accession no. BAA03413), tomato (*Solanum lycopersicum*, accession no. AAN87262), potato (*Solanum tuberosum*, accession no. AAP84703), and *Arabidopsis* (*Arabidopsis thaliana*, accession no. AAM61574). Identical amino acid residues are shaded in black. Dashes indicate the addition of spaces in the amino acid sequences for proper alignment. The putative signal peptide (SP)
sequence and inhibitory loops 1 and 2 (IL-1 and IL-2) are indicated by dashes and thick overlines, respectively (Yoshizawa et al., 2012). Conserved two arginine residues required for inhibitory activity of plant XEGIPs against glycosyl hydrolase 12 (GH12) family xyloglucan-specific endo-β-1,4-glucanase (XEG) enzymes are marked by red dashed boxes (Yoshizawa et al., 2012). CaXEGIP1 lacks the two arginine residues on the predicted IL-1 and IL-2.

**Supplemental Figure S2.** Trypan blue staining of CaXEGIP1-induced cell death in *Nicotiana benthamiana* leaves. *Agrobacterium* (OD$_{600}$=0.5) strains carrying the indicated constructs were infiltrated into *N. benthamiana* leaves. Trypan blue staining was performed 3 days after agroinfiltration. CaXEGIP1 overexpression induced cell death and darkened the cell wall compartment. Infiltrated regions are marked with red dashed lines. Scale bars = 500 µm.

**Supplemental Figure S3.** Virus-induced gene silencing of empty vector (TRV:00) in pepper leaves does not affect *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) growth and electrolyte leakages in pepper leaves compared to those in wild-type pepper leaves. (A) Multiplication of the virulent strain Ds1 (compatible) and avirulent strain Bv5-4a (incompatible) of *Xcv* in wild-type (WT) or empty vector control (TRV:00) pepper leaves. (B) Electrolyte leakage measurements from leaves inoculated with of the virulent strain Ds1 (compatible) and avirulent strain Bv5-4a (incompatible) of *Xcv*. The data represent the means ± standard deviations from three independent experiments.
Supplemental Figure S4. No cell death phenotypes and no differences in bacterial growth in mock- and dexamethasone (DEX)-treated wild-type Arabidopsis (Col-0) plants. (A) No visible cell death phenotypes in mock- or DEX-treated leaves. Pictures were taken 2 days after 30 μM DEX treatment. (B) Trypan blue staining of leaves 24 h after mock or 30 μM DEX treatment. No cell death responses were observed in mock- or DEX-treated leaves. (C) Bacterial growth in mock- or DEX-treated leaves 0 and 3 days after inoculation with virulent Pseudomonas syringae pv. tomato (Pst) DC3000 and avirulent Pst DC3000 (avrRpm1) strains (10^5 cfu ml^-1). The data represent the means ± standard deviations from three independent experiments.

Supplemental Figure S5. 2D gel images of the proteome in mock- or DEX-treated DEX:CaXEGIP1 transgenic Arabidopsis leaves. (A and B) Proteins in mock- or DEX-treated DEX:CaXEGIP1 transgenic Arabidopsis leaves. Proteins extracted 24 h after mock- or DEX treatment were separated in the first dimension on IPG strips (24 cm, pI 4-7 gradient) and in the second dimension on a 12.5% polyacrylamide SDS gel. Newly induced proteins are numbered as N1, N2, N3 and N4. The spot numbers were assigned arbitrarily and are shown in Supplemental Table S1. The pI range of the pI gradient strips is shown above the gel images, and the positions of the protein molecular weight markers, MW (kD), are indicated on the left. (C) Enlarged images of the newly expressed protein spots from the 2D gel images (A and B). Spot densities of the protein spots are shown at the right. Spot density is expressed as the percentage
volume, as calculated by ImageMaster 2D Platinum 6.0 (Amersham Biosciences).

**Supplemental Table S1.** Proteins newly induced by dexamethasone (*DEX*): *CaXEGIP1* expression in *Arabidopsis*, as identified by MALDI-TOF MS.
Figure 1. Choi et al.
Figure 2. Choi et al.
Figure 3. Choi et al.
Figure 4. Choi et al.
Figure 5. Choi et al.
Figure 6. Choi et al.
Figure 7. Choi et al.
Figure 8. Choi et al.
Figure 9. Choi et al.
Figure 10. Choi et al.