Molecular Characterization of a Pathogen-Inducible Bidirectional Promoter from Hot Pepper (*Capsicum annuum*)

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In hot pepper, the sesquiterpene phytoalexin capsidiol is catalyzed by the two final-step enzymes, a sesquiterpene cyclase (EAS) and a hydroxylase (EAH), which are genetically linked and present as head-to-head orientation in the genome. Transcriptomic analysis revealed that a subset of *EAS* and *EAH* is highly induced following pathogen infection, suggesting the coregulation of *EAS* and *EAH* by a potential bidirectional activity of the promoter (pCaD). A series of the nested deletions of pCaD in both directions verified the bidirectional promoter activity of the pCaD. Promoter deletion analysis revealed that the 226 bp of the adjacent promoter region of *EAS* and GCC-box in *EAH* orientation were determined as critical regulatory elements for the induction of each gene. Based on promoter analyses, we generated a set of synthetic promoters to maximize reporter gene expression within the minimal length of the promoter in both directions. We found that the reporter gene expression was remarkably induced upon infection with *Phytophthora capsici*, *Phytophthora infestans*, and bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 but not with necrotrophic fungi *Botrytis cinerea*. Our results confirmed the bidirectional activity of the pCaD located between the head-to-head oriented phytoalexin biosynthetic genes in hot pepper. Furthermore, the synthetic promoter modified in pCaD could be a potential tool for pathogen-inducible expression of target genes for developing disease-resistant crops.

Keywords: disease resistance, hot pepper, pathogen-inducible promoter, phytoalexin, transient expression

Crop loss caused by biotic stress is one of the most serious problems of sustainable crop production (Carvalho 2017;

Chakraborty and Newton 2011; Ficke et al. 2018; Nalley et al. 2016). Application of fungicides and pesticides to prevent pathogen infection causes unexpected outcomes, such as a deleterious effect on soil microorganisms and agricultural poisoning (Baćmaga et al. 2016; Wyszowska and Kucharski 2004). To minimize the loss by pathogens for the maximal crop yield, various approaches were executed to identify plant defense-related genes and to introduce those genes for ectopic expression in the crops (Alexander et al. 1993; Brogue et al. 1991; Truong et al. 2012; Zhao et al. 2005; Zhu et al. 2012). Among these trials, a series of inducible or constitutive promoters have been explored to express target genes to obtain disease-resistant crops (Boni et al. 2018; Cao et al. 1998; Chen and Chen 2002; Li et al. 2004; Yamamizo et al. 2006). However, despite experimental usefulness, expression of the genes by constitutive promoters resulted in undesirable features, including morphological changes by the excessive expression of the introduced gene (Chen and Chen 2002; Fitzgerald et al. 2004; Li et al. 2004; Yi and Richards 2007). To overcome the defects of constitutive promoters, inducible defense systems have been paid considerable attention to identify appropriate promoters for inducible gene expression in response to pathogen infection (Keller et al. 1999; Xu et al. 2017).

Plants have evolved an array of defense components against pathogen attack, encompassing physical barriers, antimicrobial compounds, and induced immune responses (Ham et al. 2007; Heath 2000; Lee et al. 2017; Nürnberger and Lipka 2005). Recognition of pathogen-associated molecular patterns or secreted effector proteins from pathogens results in defense responses such as activation of a mitogen-activated protein kinase cascade, callose deposition for reinforcement of physical barrier of host cells, the release of reactive oxygen species, induction of defense-related phytoalexins and hormone biosynthesis, and often resulted in hypersensitive response (Boller and Felix 2009; Kamoun et al. 1999; Robert-Seilantantz et al. 2007; Vleeshouwers et al. 2000).

Phytoalexins are low-molecular weight antimicrobial compounds that de novo synthesized upon pathogen recognition or biotic stress (Choi et al. 1992, 1994; Kuc 1995; Li et al. 2015; Yang et al. 2017). Phytoalexins are categorized into several subclasses of secondary metabolites, such as phenolics, alkaloids, or terpenes, and are synthesized in plant species-specific manners (Bednarek et al. 2011; Gust et al. 2007; Piasecka et al. 2015; Stoessl et al. 1972).

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Capsidiol, a bicyclic sesquiterpene, possesses antifungal and antimicrobial activity against fungal and oomycete pathogens (Giannakopoulou et al. 2014; Stoessl et al. 1972). It is a typical phytoalexin of *Nicotiana* and *Capsicum* genera and catabolized by two key enzymes, EAS (5-*epi*-aristolochene synthase) and EAH (5-*epi*-aristolochene hydroxylase) in response to elicitor treatments and pathogen infections (Bailey et al. 1976; Li et al. 2015; Ralston et al. 2001; Starks et al. 1997; Stoessl et al. 1972; Vögeli and Chappell 1988). EAS is the sesquiterpene cyclase that converts farnesyl diphosphate to 5-*epi*-aristolochene and EAH hydroxylates 5-*epi*-aristolochene to capsidiol (Ralston et al. 2001; Whitehead et al. 1989).

Recently, capsidiol has been reported to play a critical role in defense against nonadapted pathogen *P. infestans* in hot pepper (Lee et al. 2017). The expression of EAS and EAH was highly induced in *Capsicum annuum* cv. CM334 following infection with an adapted pathogen, *P. capsici*, as well as a nonadapted pathogen, *P. infestans* (Lee et al. 2017). Furthermore, reduction of the capsidiol by EAS gene silencing allows the colonization of *P. infestans* in its nonadapted host pepper, indicating that capsidiol may play a pivotal role in the nonhost resistance of pepper plants against *P. infestans* (Lee et al. 2017).

According to genomic sequence analysis, 29 EAS and 28 EAH orthologs are found in the *C. annuum* cv. CM334 genome and subsets of EAS and EAH are located in a head-to-head manner in chromosomes, suggesting the transcriptional coregulation by putative bidirectional promoters.

In this study, we further analyzed the bidirectional inducible property of capsidiol biosynthetic genes. The intergenic region of the EAS/EAH gene pair located in chromosome 2 was selected as a putative promoter region named pCaD. Based on *in silico* prediction of *cis*-acting elements, we attended to ethylene-responsive elements (GCC-box) and W-box elements that are the binding sites of WRKY transcription factors, because ethylene-responsive factors (ERFs) and WRKYs are well-characterized plant defense-related transcription factors (Lu et al. 2013; Seo et al. 2015; Ülker and Somssich, 2004). Based on one GCC-box and four W-box sequences, we generated a series of deletion promoters with two reporter genes to identify the regions that required for the bidirectional inducible property of pCaD. In addition, a minimal synthetic promoter, named pMin, from pCaD was constructed to confirm enhanced reporter gene expression following pathogen inoculation. Since there are only a few cases of the characterization of bidirectional promoters in plants and their application for enhancing crop resistance (Arnaiz et al. 2019; Shin et al. 2003), our study could provide more efficient pathogen-inducible promoters in response to biotic stresses that are of benefit for developing disease-resistant crops expressing a candidate gene (or genes) of interest driven by the versatile promoters.

RESULTS

Inoculation of *P. capsici* or *P. infestans* significantly enhanced the expression of reporter genes fused to pCaD bidirectionally.

Compared with tomato, the genomic regions between the genes that have a syntenic relationship specifically have been expanded in hot pepper and one of the capsidiol biosynthetic gene pairs, CA02g09520 (EAS) and CA02g09530 (EAH), is highly induced following infection of *P. capsici* and *P. infestans* in *C. annuum* 'CM334' (Lee et al. 2017). Intriguingly, EAS and EAH are arrayed in a head-to-head orientation in the genome (Fig. 1A), suggesting that the intergenic promoter region (1,156 bp) between EAS and EAH (named as pCaD) has a potential to be a bidirectional promoter induced by infection of *P. capsici* and *P. infestans*. To examine a dual promoter activity

of pCaD, pCaD was fused to two reporter genes, super humanized *Renilla luciferase* (*shRLUC*) (to the direction of EAH transcription, pCaDrev::shRLUC [Woo et al. 2008]) and enhanced green fluorescent protein (EGFP) (to the direction of EAS transcription, pCaD::EGFP) (Fig. 1B). The dual reporter gene cassette (shRLUC::pCaD::EGFP) was transiently expressed in *Nicotiana benthamiana* leaves by agroinfiltration (Sparkes et al. 2006) and inoculation of *P. capsici* and *P. infestans* was conducted after 24 h after agroinfiltration. Significantly enhanced expression of EGFP and shRLUC was observed in pCaD driven construct compare with that of pNos driven construct at 12 and 24 h postinoculation (hpi) (Fig. 1C and D). This result suggested that pCaD possesses bidirectional promoter activity with inducible properties against pathogen infection.

Three W-box elements and a GCC-box element in pCaD differentially regulate the bidirectional promoter activity.

In silico prediction showed that the pCaD has putative *cis*-acting elements including a GCC-box that ERFs bind to and W-box elements that were known to be the binding site of WRKY transcription factor (Eulgem et al. 2000; Fujimoto et al. 2000; Ohme-Takagi and Shinshi 1995) (Fig. 1B). In earlier studies, it has been reported that ERFs and WRKYs mediate defense responses in various plant species (Adachi et al. 2015; Jin et al. 2016; Sarris et al. 2015; Song et al. 2019).

To identify the crucial region of pCaD promoter activity, serial deletion of the pCaD promoter was performed, based on GCC- and W-box sequences in both directions (Supplementary Fig. S1). pCaD and its deleted promoters were fused to EGFP and shRLUC reporter genes and were expressed in *N. benthamiana* leaves using agroinfiltration (Figs. 2 and 3). Two days after agroinfiltration, EGFP expression regulated by pCaD and its deletion promoters showed different activities of each *cis* element to the pCaD promoter activity (Fig. 2). A complete loss of EGFP fluorescence was observed when the 264-bp promoter region including the fourth W-box (W4) was deleted (pGW₁₂₃). A substantial recovery of EGFP expression by a 226-bp fragment of the pCaD (GW_{null}) indicates that 226 bp of pCaD is critical for transcript expression toward the direction of EAS. Interestingly, the deletion of the GCC-box and the second W-box (W2) resulted in an increase of EGFP expression, suggesting that both *cis* elements might negatively regulate pCaD activity in EAS transcription.

For the direction of EAH, shRLUC was fused to pCaDrev and its deletion series (Fig. 3). Remarkably, the GCC-box is sufficient to recover the full strength of the pCaDrev promoter activity (pG_{only}). Apparently, the presence of W1 has an additive effect on shRLUC expression, while W3 has a negative effect to pCaDrev promoter activity (Fig. 3). Taken together, these results indicate that a 199-bp fragment containing the GCC-box for EAH and a downstream region of 226 bp for EAS are minimal promoters for the pathogen-inducible expression of the head-to-head arrayed phytoalexin biosynthetic genes.

A minimal synthetic promoter drives enhanced reporter gene expression following inoculation of *P. capsici*, *P. infestans*, and *Pseudomonas syringae* pv. *tomato* DC3000 but not of the necrotrophic pathogen *Botrytis cinerea*.

To identify the minimal region of the promoter that maintains the inducibility by infections with other pathogens and sustains the strength of the promoter activity, five synthetic promoters were constructed in combination with the *cis* elements (Fig. 4A). EGFP and shRLUC expression driven by synthetic promoters was measured 2 days after agroinfiltration. shRLUC expression driven by synthetic promoters significantly differs depending on the existence of the W-box elements, while the fluorescence intensities of EGFP were elevated significantly by the deletion of the W-boxes (Fig. 4A).

Among the series of deleted promoters, we chose the shortest synthetic promoter, pMin, to test whether pMin responds to various pathogen infections. At 24 h after agroinfiltration of dual reporter cassettes driven by pCaD and pMin in *N. benthamiana* leaves, inoculation of *P. capsici*, *P. infestans*, *Pseudomonas syringae* pv. *tomato* DC3000, and *B. cinerea* were conducted as described below.

EGFP expression not only was driven by pCaD but was also driven by pMin and significantly increased after infection of *P. capsici*, *P. infestans*, and *Pseudomonas syringae* pv. *tomato* DC3000 at 12 hpi (Fig. 4B, C, and D). On the other hand, increased shRLUC activity was also detected after pathogen infection except in the case of *B. cinerea* inoculation (Fig. 4B, C, and D). *P. capsici* and *P. infestans* infection cause enhanced

shRLUC expression at 24 hpi (Fig. 4B and C). In the case of *Pseudomonas syringae* pv. *tomato* DC3000, shRLUC activity was significantly elevated at 12 hpi (Fig. 4D), while *B. cinerea* inoculation resulted in significantly lower shRLUC expression in the early infection stage (6 hpi) (Fig. 4E). These results confirmed that not only pCaD but also pMin have pathogen-inducible properties against *P. capsici*, *P. infestans*, and *Pseudomonas syringae* pv. *tomato* DC3000 infections.

Pvr4-CC expression driven by pCaD and pMin elicits stronger cell death than that driven by the 35S promoter.

Generating transgenic plants using pathogen-inducible promoters and resistance-related genes can enhance resistance

Fig. 1. Intergenic region of *CA02g09530* and *CA02g09520* (pCaD) bidirectionally regulates reporter gene expression. **A**, Location of *CA02g09530* and *CA02g09520* in *Capsicum annuum* 'CM334' (yellow box). **B**, Scheme of dual reporter gene cassette. *shRLUC* and *enhanced green fluorescent protein* (EGFP) were used to measure the transcriptional activity of pCaD. **C**, EGFP expression and **D**, shRLUC activity was detected after *Phytophthora capsici* and *P. infestans* inoculation. Reporter gene expression driven by the Nos promoter (pNos) was used as a negative control. EGFP expression induced by *P. capsici* was measured at 12 h after inoculation (hpi) ($n = 64$, error bar, standard error of the mean [SEM]) and shRLUC activity was measured at 24 hpi ($n = 40$, error bar, SEM). EGFP and shRLUC expression induced by *P. infestans* was observed at 12 and 24 hpi ($n = 64$, error bar, $\pm$ SEM and $n = 32$, error bar, $\pm$ SEM, respectively). Relative expressions indicate fold change of EGFP and shRLUC expression compared with mock-treated samples. Significant differences were detected using the Mann-Whitney test. Asterisks (****) indicate $P < 0.05$.

against pathogens by cell death to restrict pathogen spread (Boni et al. 2018; Keller et al. 1999; Yamamizo et al. 2006). Recently, it has been reported that the coiled-coil domain of *Pvr4* (Pvr4-CC), including an untranslated region (UTR), can induce cell death under the control of the 35S promoter (Kim et al. 2018). To make constructs that induce cell death after pathogen recognition, Pvr4-CC, except for its 5' UTR, was fused to either end of pCaD and pMin for bidirectional transcription (Fig. 5A; Supplementary Fig. S1B). Pvr4-CC, except for its 5' UTR, fused to the 35S promoter was used as a positive control, while 10-1-CC, which cannot induce cell death but is

included in same subclade with *Pvr4*, was used as a negative control for cell death upon expression (Lee et al. 2020). *Agrobacterium* containing each construct was infiltrated into *N. benthamiana* leaves and cell death was observed at 2 days after agroinfiltration.

Enhanced cell death driven by pCaD and pMin was observed, compared with that driven by the 35S promoter in a transient expression assay (Fig. 5B). Cell death intensity was measured by comparing photosynthetic efficiency in each area (Fig. 5C and D). Relative *Fv/Fm* ratio was decreased in pCaD::Pvr4-CC and pMin::Pvr4-CC expressed

Fig. 2. Relative expression of enhanced green fluorescent protein (EGFP) according to the nested deletion of pCaD. *EGFP* driven by deletion promoters was transiently expressed in *Nicotiana benthamiana* via agroinfiltration. Fluorescence intensity was normalized by the expression of pNos_ *EGFP*. $n = 72$, error bar, $\pm$ standard error of the mean. One-way analysis of variance and Dunn's multiple comparisons test was performed to compare expression driven by series of promoters. Asterisks (****) indicate $P < 0.0001$.

Fig. 3. Relative expression of shRLUC following removal of *cis*-acting elements in pCaD. Luminescence driven by a series of deletion promoters was measured after coelenterazine treatment. Final coelenterazine concentration was adjusted to 2 μ M in each well. shRLUC activity was normalized by the value of pNos_ *shRLUC*. Error bar, $\pm$ standard error of the mean, $n = 72$. One-way analysis of variance and Dunn's multiple comparisons test was performed to compare expression driven by deletion promoters. Three asterisks (***) indicate $P < 0.001$, two (**) $P < 0.01$.

Fig. 4. Minimal synthetic promoter (pMin) drive enhanced reporter gene expression after *Phytophthora capsici*, *P. infestans*, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) but not *Botrytis cinerea* inoculation. **A**, Enhanced green fluorescent protein (EGFP) and shRLUC activity driven by a series of synthetic promoters. Reporter gene expressions were normalized EGFP and shRLUC driven by the Nos promoter. One-way analysis of variance and Dunn's multiple comparisons test was performed to compare expression driven by each synthetic promoter and pCaD. Four asterisks (****) indicate $P < 0.0001$, two (**) $P < 0.01$, and one (*) $P < 0.05$. **B** to **E**, Reporter gene expressions driven by pCaD and pMin change after *P. capsici*, *P. infestans*, *Pseudomonas syringae* pv. *tomato* DC3000, and *B. cinerea*. All relative expression was presented as fold changes based on mock-treated samples. Green and dark green bars mean EGFP fluorescence driven by pCaD and pMin. Blue and dark blue bars represent shRLUC activities driven by pCaD and pMin. A significant difference was detected using the Mann-Whitney test ($P < 0.05$). **B**, EGFP expression was measured at 12 h postinoculation (hpi). Error bar, $\pm$ standard error of the mean (SEM), $n = 64$. shRLUC activity was measured at 24 hpi. Error bar, $\pm$ SEM, $n = 40$. **C**, EGFP expression was measured at 12 hpi. Error bar, $\pm$ SEM, $n = 64$. shRLUC activity was measured at 24 hpi. Error bar, $\pm$ SEM, $n = 24$. **D**, EGFP expression was measured at 12 hpi. Error bar, $\pm$ SEM, $n = 24$. shRLUC activity was measured at 12 hpi. Error bar, $\pm$ SEM, $n = 24$. **E**, EGFP expression was measured at 24 hpi. Error bar, $\pm$ SEM, $n = 64$. shRLUC activity was measured at 6 hpi. Error bar, $\pm$ SEM, $n = 40$.

areas (Fig. 5C). In addition, a relatively decreased *Fv/Fm* ratio was detected in pCaDrev::Pvr4-CC and pMinrev::Pvr4-CC expressed areas (Fig. 5D). These results indicate that the pCaD and the pMin promoter could bidirectionally drive the transcription of the cell death-inducing Pvr4-CC and elicit plant cell death successfully.

DISCUSSION

Phytoalexins contribute to plant defense against pathogens (Kuc 1995; Li et al. 2015; Lee et al. 2017; Park et al. 2014; Piasecka et al. 2015). Capsidiol, the phytoalexin in *Nicotiana* and *Capsicum* species, is de novo synthesized by pathogen

Fig. 5. Pvr4-CC expressions driven by pCaD and pMin elicit more substantial cell death than that of 35S promoter. **A**, Scheme of Pvr4-CC and pCaD or pMin fusion construct. Pvr4-CC was fused to pCaD and pMin both directions to test whether pCaD and pMin elicit cell death. **B**, Pvr4-CC and pCaD or pMin construct induced cell death in *Nicotiana benthamiana*. As a negative control, 10-1-CC fused to pCaD and pMin was used. A total of 75 and 49 *N. benthamiana* leaves were used for infiltration of each construct, and 75 and 48 leaves showed the same results as presented. **C** and **D**, *Fv/Fm* value measurement after expression of each construct. Cell death intensity was normalized by the 10-1-CC expressed area. Significance of difference was detected using the Mann-Whitney test. Asterisks (****) indicate $P < 0.05$.

attack (Lee et al. 2017; Park et al. 2014; Stoessl et al. 1972). It is of interest that head-to-head orientation of capsidiol biosynthetic genes is a common configuration in the hot pepper genome because capsidiol biosynthetic genes *EAS* and *EAH* appear to be regulated simultaneously by a putative bidirectional promoter in response to biotic stimuli. In this study, we characterized a putative bidirectional promoter of capsidiol biosynthetic genes, named as pCaD, and created a synthetic promoter, pMin, by measuring the expression of two reporter genes, *shRLUC* and *EGFP*, after the inoculation of four different pathogens. We confirmed that pCaD and pMin have sustained the inducible properties following pathogen infection. Our results suggested that pCaD and a synthetic minimal promoter pMin have the potential for making an inducible expression system for plant biotech, such as developing disease-resistant crops using a gene of interest (Fig. 4B, C, D, and E).

The essential region of pCaD promoters was determined by deletion analyses (Figs. 2 and 3). Downstream region of the fourth W-box (W4) and GCC-box were critical for pathogen-induced gene expression in each direction. Especially in the case of pG_{only}, *shRLUC* activity driven by pG_{only} and pCaDrev showed similar intensity, even if it is composed of only 199 bp containing a GCC-box (Fig. 3). It is indicated that putative transcriptional factors, especially ERF, might be a major factor to control the adjacent gene expression. It is notable that treatment of the ethephon, an ethylene analog, also elevates the expression of *CA02g09520* (*EAS*) and *CA02g09530* (*EAH*) in *C. annuum* 'CM334' (Supplementary Fig. S3). These results imply that ethylene or ethylene-related factors might be involved in capsidiol biosynthesis in *C. annuum* 'CM334' and the activation of *CA02g09520* (*EAS*) and *CA02g09530* (*EAH*) en route to the capsidiol biosynthesis in *C. annuum* 'CM334'.

Based on the promoter deletion and recombination analysis, we generated a synthetic promoter set to maximize gene expressions in both directions. In Figure 4A, *shRLUC* activity was differentiated depending on the number of the W-box *cis* elements, while *EGFP* expression was elevated by the loss of the W-boxes. These results suggest that several WRKY transcription factors that bind to W-box elements in pCaD might well coordinate the pathogen-inducible and bidirectional properties of the pCaD promoter.

The results from inoculations of various pathogens concluded that expressions of both reporter genes under pCaD and minimal promoter pMin showed enhanced expression of the reporter genes following the inoculation of bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 as well as two oomycete pathogens, *P. capsici* and *P. infestans* (Fig. 4B, C, and D). Interestingly, increased *EGFP* expression induced by *B. cinerea* was observed at 24 hpi, whereas the other tested pathogens induce enhanced *EGFP* expression at 12 hpi (Fig. 4B, C, D, and E). As shown in Supplementary Fig. S2, the accumulation of *CA02g09520* (*EAS*) and *CA02g09530* (*EAH*) transcripts were also delayed following the inoculation of *B. cinerea* in *C. annuum* 'CM334' compared with the inoculation of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Supplementary Fig. S2). Considering the different responsiveness of the pCaD promoter to a necrotic fungal pathogen, *B. cinerea*, it may imply that the capsidiol biosynthetic pathway can respond differentially to the infection of necrotrophic pathogens. In fact, the previous study revealed that *B. cinerea* could detoxify capsidiol by converting to capsenone (Ward and Stoessl 1972), suggesting that *B. cinerea* may also have developed a distinctive strategy to overcome the toxicity of capsidiol.

Auto-death of host cells by an immediate response to pathogen invasion is an excellent strategy to prevent successful

colonization of pathogens. To test the feasibility of pCaD as a resource for making crops resistant against pathogens, the coiled-coil domain of *Pvr4* (Pvr4-CC) was used as a proof of concept. *Pvr4* was identified as a resistance gene against pepper mottle virus, pepper severe mosaic virus, and potato virus Y (Kim et al. 2017). The recognition of an avirulence factor, NIb, an RNA-dependent RNA polymerase of Potyviruses, by *Pvr4* induces cell death (Kim et al. 2015). Recently, it has been shown that the expression of the Pvr4-CC domain under the cauliflower mosaic virus (CaMV) 35S promoter is sufficient to induce cell death without NIb (Kim et al. 2018). To investigate whether Pvr4-CC can induce cell death under the control of pCaD and pMin, Pvr4-CC without its 5' UTR was linked to the promoters and was expressed in *N. benthamiana*. Interestingly, Pvr4-CC-mediated cell death under the control of pCaD and pMin was stronger than that driven by the CaMV 35S promoter (Fig. 5B) by transient expression using *Agrobacterium tumefaciens* without any challenge pathogen, suggesting that *Agrobacterium* itself could induce the expression of Pvr4-CC, triggering the cell death as a pathogen. Moreover, lower values of the relative *Fv/Fm* were detected in the Pvr4-CC expressed area driven by pCaD and pMin, confirming the Pvr4-CC-mediated cell death (Fig. 5C and D). Because cell death driven by pCaD and pMin is induced by *A. tumefaciens*, perhaps it is challenging to make transgenic plants via agrobacterium-mediated gene transfer. However, as shown in Supplementary Figure S3, *CA02g09520* (*EAS*) and *CA02g09530* (*EAH*) were not induced by wound stress, suggesting that gene transfer using particle bombardment would be an alternative way to produce a transgenic crop expressing the acute auto-cell death cassettes induced by pathogen invasion in the field (Supplementary Fig. S3). Taken together, the newly identified pCaD and synthetic pMin promoters could serve as powerful tools as bidirectional pathogen-inducible promoters for developing disease resistant crops against multiple pathogens including bacterial, oomycete, and filamentous fungal pathogens.

MATERIALS AND METHODS

Plant materials and growth conditions.

Nicotiana benthamiana was used for in planta expression. *N. benthamiana* seeds were germinated on the soil in controlled chambers with a 16-h light and 8-h dark photoperiod at 24°C. Ten days after germination, seedlings were transplanted to a pot (100 mm diameter). *Capsicum annuum* cultivar Criollo de Morelos-334 ('CM334') was used for cloning the intergenic region of *CA02g09520* and *CA02g09530*. Pepper seeds were germinated at 30°C in a dark incubator after sterilization with 0.2% sodium hypochlorite for 3 days. Pepper plants were transplanted to the 200-plug tray and were grown in a chamber with a 16-h light and 8-h dark photoperiod at 24°C. Young pepper plants were transplanted to a 50-plug tray after cotyledon expansion.

Cloning strategy.

In *C. annuum* 'CM334', there are 29 *EAS* orthologs and 28 *EAH* orthologs (Lee et al. 2017). Among a subset of *EAS* and *EAH*, *CA02g09520* and *CA02g09530* were most highly expressed after *P. infestans* inoculation in CM334 (Lee et al. 2017). The intergenic region (1,156 bp) of *CA02g09520* (*EAS*) and *CA02g09530* (*EAH*) was chosen as a putative pathogen-inducible promoter. Specific primers to amplify the region of pCaD were designed based on the v1.55 reference genome of pepper (Kim et al. 2014). To make a series of the chimeric cassette of promoters, deleted pCaD promoters were produced by PCR amplification. Promoter deletions were conducted to remove *cis* elements, including a GCC-box and four W-box

sequences in both directions. For generating a dual reporter cassette with deleted promoters, *shRLUC* (Woo et al. 2008) and *EGFP* genes were cloned in pCambia2300 (pC2300) vector using *KpnI*, *HindIII*, *PstI*, and *BamHI* (New England Biolabs, Ipswich, MA, U.S.A.). T4 ligases (New England Biolabs) were used for ligation of a series of promoters, except *GW₁₂* and *G_{only}* promoters. *EGFP* and *shRLUC* genes were fused to the direction of *EAS* transcription (pCaD::EGFP) and *EAH* transcription (pCaDrev::shRLUC), respectively. *GW₁₂* and *G_{only}* were linked with *shRLUC* and *EGFP* by PCR and were cloned using ligation-independent cloning (Oh et al. 2010). *shRLUC* and *EGFP* under the Nos promoter were used as expression controls. The sequences of all clones were confirmed by DNA sequencing at the National Instrumentation Center for Environmental Management (NICEM, Seoul, Republic of Korea). Each cloned vector was transformed into *A. tumefaciens* GV3101 for in planta transient expression assays. Pvr4-CC constructs with pCaD and pMin were amplified by PCR and were cloned in the pC2300 vector. To test bidirectional inducibility, Pvr4-CC was fused with pCaD and pMin in both directions.

In planta expression assay.

After transformation to *A. tumefaciens* (GV3101) using heat shock, cells were cultured at 28°C in a shaking incubator for 12 h. Cultured cells were centrifuged and resuspended by induction buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, and 150 µM acetosyringone) and were incubated at 28°C for 2 h before agroinfiltration. The concentration of cells was adjusted to 0.3 at an optical density at 600 nm (OD₆₀₀) for expression in *N. benthamiana* and were pressure-infiltrated using needleless syringes (Sparkes et al. 2006). After agroinfiltration, plants were stored in a controlled chamber with a 16-h light and 8-h dark photoperiod at 24°C for measuring expression of reporter genes or 21°C for the pathogen inoculation test.

Measurement of cell death intensity.

To measure cell death induced by pCaD and pMin with Pvr4-CC, constructs of Pvr4-CC-fused pCaD and pMin were transiently expressed in *N. benthamiana* via agroinfiltration. Two days after infiltration, detached *N. benthamiana* leaves were used to validate the inducible property of pCaD and pMin. 10-1-CC fused to 35S promoter, pCaD, and pMin were used as negative controls of Pvr4-CC driven by 35S, pCaD, or pMin. Cell death intensity was measured using FluorCAM (Photon System Instruments, Drasov, Czech) and the value of *Fv/Fm* was presented as an indicator of cell death intensity.

Fluorescence and luminescence expression assay.

To detect green fluorescence and luminescence derived by promoters, leaf discs were sampled after transient expression, using a cork borer (diameter = 4 mm). Sampled leaf discs were floated in black 96-well plates with 100 µl of distilled water. EGFP and shRLUC expression induced by agrobacterium was measured 2 days after infiltration. EGFP fluorescence and luminescence from shRLUC with coelenterazine (native) (Biotium, Fremont, CA, U.S.A.) was measured using a Synergy HTX plate reader (Biotek, Winooski, VT, U.S.A.). Coelenterazine concentration was adjusted to 2 µM in each well. To measure fluorescence and luminescence changed by pathogens, leaf discs from agroinfiltrated *N. benthamiana* leaves were sampled using a cork borer at 24 h after agroinfiltration, followed by the addition of pathogen suspensions to the 96-well plates. The expression of reporter genes was measured at 0, 6, 12 and 24 hpi. Change of gene expression was normalized with the expression of mock-treated samples.

Pathogen inoculation.

Two *Phytophthora* species, the potato late blight pathogen *P. infestans* and *P. capsici* were used. *P. capsici* (40476) was grown on V8 media at room temperature in the dark for 5 days. The mycelia were scraped using a spreader and were incubated under white light for 12 h. Dried mycelia were flooded with 5 ml of distilled water and were incubated on ice for releasing zoospores from sporangia. Zoospores were adjusted to 5×10^4 spores per milliliter for inoculation. *P. infestans* (T30-4) was grown on rye agar media at 17°C in dark conditions for 1 day. Zoospores were captured by flooding the plate with 7 ml of sterile distilled water and were incubated at 4°C until released from sporangia. The released zoospore was adjusted to 10×10^4 spores per milliliter using distilled water for inoculation. The bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 without HopQ1 effector (Wei et al. 2007) was grown in King's B media with proper antibiotics. The cultured cell was centrifuged and resuspended by buffer (10 mM MgCl₂) and was adjusted to 0.01 at OD₆₀₀. Necrotrophic fungi *Botrytis cinerea* isolate 05.10 was grown on potato dextrose agar media without antibiotics, for about 3 weeks, at room temperature in dark conditions. Gently rubbed mycelia with autoclaved distilled water was filtered by Mira cloths to harvest the conidiospores. Captured spores were diluted with 25% organic grape juice and were adjusted to 1×10^5 spores per milliliter for inoculation.

Quantitative reverse transcription (qRT)-PCR analysis.

To investigate whether CA02g09520 (*EAS*) and CA02g09530 (*EAH*) are activated after wound and ethylene treatment, CM334 was treated with the ethylene analog ethephon (ET) and with wounding stress. Ethephon solutions (25 ml) were sprayed on 5-week-old pepper leaves. Water with 0.01% Tween20 was prepared as a spraying buffer and 10% ethanol with buffer was used as a mock. Wounds were given to pepper leaves using needles. Five leaves were detached at each timepoint and whole leaves were ground with liquid nitrogen, using a glazed porcelain mortar with pestle. To confirm CA02g09520 (*EAS*) and CA02g09530 (*EAH*) are induced by *Pseudomonas syringae* pv. *tomato* DC3000 and *B. cinerea* infection, 5-week-old pepper leaves were used. Pathogen preparation was conducted the same as mentioned above. Five leaf discs were used for RNA extraction.

Total RNA was extracted from finely ground powders, using the TRIZOL method according to the manufacturer instructions (Invitrogen, Carlsbad, CA, U.S.A.). First-strand cDNA was synthesized using 2 µg of total RNAs with oligo (dT) and the Superscript II RT (Invitrogen) for qRT-PCR analysis. qRT-PCR was conducted using 25 ng of cDNA per microliter with premix from Applied Biosystems (Applied Biosystems, Foster city, CA, U.S.A.). To determine relative expression of CA02g09520 (*EAS*) and CA02g09530 (*EAH*) in each sample, specific primers were used (Supplementary Table 1). To normalize the expression level, the pepper ACTIN gene was used as an endogenous control.

Statistical analyses.

Statistical analysis and graphs were generated by Prism (Graphpad, San Diego, CA, U.S.A.) software. One-way analysis of variance and Dunn's multiple comparisons test was performed to compare expression driven by series of promoters. A Mann-Whitney test was performed to analyze variance between expressions induced by agroinfiltration and inoculation of four different pathogens.

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