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The barley powdery mildew effectors CSEP0139 and CSEP0182 suppress cell death and promote *B. graminis* fungal virulence in plants

Xue Li[1,2†], Cong Jin[3†], Hongbo Yuan[1,2†], Wanting Huang[1,4], Fang Liu[1,2], Renchun Fan[1,2], Jiankun Xie[3*] and Qian-Hua Shen[1,2*]

Abstract

The powdery mildew fungi secrete numerous Candidate Secreted Effector Proteins (CSEPs) to manipulate host immunity during infection of host plants. However, the function of most of these CSEPs in cell death suppression has not yet been established. Here, we identified several CSEPs from *Blumeria graminis* f. sp. *hordei* (*Bgh*) that have the potential to suppress BAX- and *Nt*MEK2^{DD}-triggered cell death in *Nicotiana benthamiana*. We further characterized two effector candidates, CSEP0139 and CSEP0182, from family six and thirty-two, respectively. CSEP0139 and CSEP0182 contain a functional signal peptide and are likely secreted effectors. Expression of either CSEP0139 or CSEP0182 suppressed cell death triggered by BAX and *Nt*MEK2^{DD} but not by the AVR_{a13}/MLA13 pair in *N. benthamiana*. Transient overexpression of CSEP0139 or CSEP0182 also inhibited BAX-induced cell death and collapse of cytoplasm in barley cells. Furthermore, overexpression of either CSEPs significantly increased *Bgh* haustorial formation in barley, whereas host-induced gene silencing (HIGS) of the CSEP genes reduced haustorial formation, suggesting both CSEPs promote *Bgh* virulence in barley. In addition, expression of CSEP0139 and CSEP0182 reduced size of the lesions caused by the necrotrophic *Botrytis cinerea* in *N. benthamiana*. Our findings suggest that CSEP0139 and CSEP0182 may target cell death components in plants to promote fungal virulence, which extends the current understanding of the functions of *Bgh* CSEPs and provides an opportunity for further investigation of fungal virulence in relation to cell death pathways in host plants.

Keywords: *Blumeria graminis*, Powdery mildew, Candidate for secreted effector, Fungal virulence, Cell death, Barley

Background

Powdery mildew fungi are widespread and important fungal pathogens that colonize many plant species, including economically important cereal crops, such as wheat and barley (Glawe 2008; Dean et al. 2012; Takamatsu 2013). Powdery mildew fungi are also obligate biotrophic pathogens that depend entirely on living host cells for growth and reproduction (Bourras et al. 2018). After penetration of the host cell wall, these fungi develop a specialized structure, called haustorium, inside host epidermal cells, mainly for nutrient uptake (Panstruga 2003; Both et al. 2005). These haustoria are also believed to be sites for signaling exchange between plants and pathogens, and for delivery of pathogen effector proteins into host cells to manipulate host immunity (Panstruga and Dodds 2009; Dou and Zhou 2012).

The barley powdery mildew pathogen (*Blumeria graminis* f. sp. *hordei*, *Bgh*), a specialized form of the *B.*

* Correspondence: qhshen@genetics.ac.cn; xiejiankun@jxnu.edu.cn
†Xue Li, Cong Jin and Hongbo Yuan contributed equally to this work.
[3]College of Life Sciences, Jiangxi Normal University, Nanchang 330022, China
[1]State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Innovation Academy for Seed Design, Chinese Academy of Sciences, Beijing 100101, China
Full list of author information is available at the end of the article

graminis species infecting crops of the grass family, normally colonizes barley as a host (Wyand and Brown 2003). Using integrated genomic, transcriptomic, and proteomic approaches, researchers have predicted and/or identified ~700 Candidates for Secreted Effector Proteins (CSEPs) from the *Bgh* genome, and investigated the functions of some of these CSEPs in fungal pathogenesis by host-induced gene silencing (HIGS) and overexpression analyses (Bindschedler et al. 2009; Godfrey et al. 2009; Nowara et al. 2010; Spanu et al. 2010; Pedersen et al. 2012; Pliego et al. 2013; Ahmed et al. 2015; Ahmed et al. 2016; Menardo et al. 2017; Frantzeskakis et al. 2018). It has been shown that ~63% of the identified *Bgh* CSEPs are predicted to contain an N-terminal signal peptide (SP), and share an N-terminal Y/F/WxC motif that is speculated to have a function in effector translocation to host cells (Godfrey et al. 2010; Spanu et al. 2010; Pedersen et al. 2012). In addition, a large portion (c. 25%) of the *Bgh* CSEPs are RNase-like proteins associated with haustoria (RALPH effectors) that may act as pseudoenzymes with critical function (Pedersen et al. 2012; Spanu 2017; Pennington et al. 2019). It is particularly interesting to note that most of the so far identified AVR_A effectors, each recognized by a cognate MLA receptor, are RALPHs that share structural similarity with fungal RNases but lack the residues critical for catalytic activity (Lu et al. 2016; Saur et al. 2019; Bauer et al. 2021). Furthermore, expression analyses have shown that many *CSEP* genes are differentially expressed during *Bgh* infection of barley, and many of them show predominant expression in haustoria (Spanu et al. 2010; Pedersen et al. 2012). Moreover, host targets that are involved in immunity and stress responses of some *Bgh* CSEPs have been identified, including pathogenesis-related (PR) proteins (Zhang et al. 2012; Pennington et al. 2016), small heat-shock proteins (HSPs) (Ahmed et al. 2015), and the ARF-GAP protein (Schmidt et al. 2014), among others (Pennington et al. 2016). However, the mechanisms by which *Bgh* CSEPs manipulate barley immune responses and enhance fungal virulence in host cells remain largely unknown.

Programmed cell death (PCD) plays an important role in a wide range of developmental processes and in responses to biotic and abiotic stresses in plants and animals (Dickman and Fluhr 2013). Although there are fundamental differences between plant and animal PCD, the basic morphological and biochemical features of PCDs are largely conserved (Das et al. 2009; Dickman and Fluhr 2013). BAX is a mouse pro-apoptotic protein with a conserved function of inducing cell death in plants. Expression of BAX in tobacco stimulates cell death, which closely resembles the hypersensitive responsive (HR) response induced by viral infection (Lacomme and Santa Cruz 1999). Cell death regulators have been identified in plants, using BAX-induced cell death. BAX inhibitor-1 (BI-1) homolog is one of the cell death suppressors isolated from different plant species, and has a conserved function in suppressing BAX-induced cell death (Huckelhoven 2004; Ishikawa et al. 2011; Xu et al. 2017). Similarly, using BAX and other types of cell death inducers, many pathogen effectors have been identified that can suppress cell death in plants (Lacomme and Santa Cruz 1999; Wang et al. 2011; Li et al. 2015; Xiang et al. 2016), such as Avr1b, Avr3b, Avh172, etc. from *Phytophthora sojae* (Dou et al. 2008; Dong et al. 2011; Wang et al. 2011), SNE1 from *Phytophthora infestans*, Six6 from *Fusarium oxysporum*, and Pst_8713 from *Puccinia striiformis* (Kelley et al. 2010; Gawehns et al. 2014; Zhao et al. 2018). Considering the existence of a large number of predicted effectors in *Bgh* and many other filamentous plant pathogens, it is envisaged that many of them should have the function in manipulating plant defense-related cell death (Panstruga 2003; Selin et al. 2016; Thordal-Christensen et al. 2018). However, so far only a few CSEPs, e.g. CSEP0264/BEC1011 and CSEP0064/BEC1054, have been reported or implicated in suppressing cell death in host plant (Pliego et al. 2013; Pennington et al. 2019).

In the present study, we coexpressed CSEP candidates with a cell death inducer, BAX or *Nt*MEK2^{DD}, and identified candidate *Bgh* effectors that may suppress HR-like cell death. *Nt*MEK2^{DD} is a constitutively active mutant of *Nt*MEK2 that activates defense responses and HR-like cell death in plants (Yang et al. 2001; Meng and Zhang 2013). We report here two CSEPs, CSEP0139 and CSEP0182 from family six and thirty-two respectively (Menardo et al. 2017), that could inhibit cell death responses in plants and enhance *Bgh* virulence during infection of barley. CSEP0139 and CSEP0182 suppressed BAX-induced cell death in both barley and *N. benthamiana*. Moreover, CSEP0139 and CSEP0182 suppressed cell death induced by *Nt*MEK2^{DD}. Interestingly, they did not suppress cell death induced by the AVR_{a13}-MLA13 pair in *N. benthamiana*. We employed transient gene expression and HIGS assays, and demonstrated that CSEP0139 and CSEP0182 contribute to *Bgh* virulence in barley. Our data suggest that CSEP0139 and CSEP0182 are likely secreted effectors with cell death-suppressing activity, and able to promote *Bgh* virulence during infection of barley.

Results

Identification of CSEPs with cell death suppressing activity and expression analysis of *CSEP0139* and *CSEP0182* during *Bgh* infection of barley

Previous studies have identified ~500 candidate secreted effector proteins (CSEPs) in the *Bgh* genome, and many of these *CSEP* genes are highly induced and differentially expressed during *Bgh* infection of barley

(Godfrey et al. 2010; Spanu et al. 2010; Pedersen et al. 2012; Hacquard et al. 2013). We selected some *CSEPs* for further study based on their relatively high expression in haustoria with respect to epiphytic tissues (Pedersen et al. 2012), and whether the *CSEPs* suppressed cell death in *N. benthamiana* triggered by BAX or *Nt*MEK2^{DD}, a constitutively active mutant of *Nt*MEK2 (Yang et al. 2001). We amplified ~ 100 *CSEP* genes, which are distributed into 34 effector families, from isolate *Bgh*A6 by PCR, and subcloned them into the vector pGR107 for *Agrobacterium tumefaciens*-mediated transient expression in *N. benthamiana* (Wang et al. 2011). Fifteen out of these ~ 100 CSEPs consistently suppressed BAX- and *Nt*MEK2^{DD}-trigged cell death, and one of them varied in cell death suppression in different experiments (Additional file 1: Table S1).

Here, we further characterized *CSEP0139* and *CSEP0182*, two effector genes from family six and thirty-two respectively (Menardo et al. 2017), which are highly expressed in haustoria but are not members of the RALPHs family, and expression of them suppressed cell death induced by BAX and *Nt*MEK2^{DD} in *N. benthamiana* (Additional file 1: Table S1). The expression profiles of *CSEP0139* and *CSEP0182* were then confirmed in a time-course experiment in compatible interaction by RT-qPCR, using total RNAs collected from the entire infected leaves before haustorium formation at 0, 4, 8, and 12 h post inoculation (hpi), and RNAs separately collected from epiphytic *Bgh* tissues on leaves (E) and the remaining leaf tissues containing only the haustoria (H) after haustorium formation at 24 and 48 hpi. Transcript levels of *CSEP0139* and *CSEP0182* remained almost unchanged from 0 to 12 hpi, but significantly increased in both H and E samples at 24 and 48 hpi, with particularly more abundance in the H samples (Additional file 2: Figure S1). Moreover, the expression of *CSEP0182* was significantly higher than that of *CSEP0139*, which is consistent with previously reported RNA-seq data (Pedersen et al. 2012). These results suggest that expression of both *CSEP0139* and *CSEP0182* genes is induced during haustorial formation and their transcripts are highly abundant in haustoria during *Bgh* infection of barley.

CSEP0139 and CSEP0182 contain a functional signal peptide

To examine if CSEP0139 and CSEP0182 are secreted proteins, we first analyzed if they contain an SP by using the Signal 4.0 software. Indeed, both CSEPs contain a putative N-terminal SP (Fig. 1a). In addition, a Y/FxC motif was also identified in both CSEPs, as previously indicated (Additional file 2: Figure S2) (Godfrey et al. 2010; Pedersen et al. 2012). We then examined if the SP was functional by using a yeast genetic assay, which is based on the requirement of invertase secretion for yeast cells to grow on a medium containing only raffinose as the carbon source (Gu et al. 2011). We constructed fusion plasmids in which the predicted SP sequences of CSEP0139 and CSEP0182 were fused in frame with a yeast invertase lacking its own SP in the pSUC2 vector. The first 25 amino acids from the *M. oryzae* protein Mg87 and the SP of the oomycete effector *Ps*Avr1b were used as a negative and a positive control, respectively (Gu et al. 2011). The yeast cells of the YTK12 mutant were transformed separately with each of these plasmids to evaluate yeast growth on the YPRAA medium with raffinose as the carbon source. The invertase fusion plasmid with the SP from PsAvr1b enabled yeast to grow on the medium, while the plasmid with the 25 N-terminal amino acids of Mg87 did not, as expected. Importantly, plasmids with the SP from either CSEP0139 or CSEP0182 enabled the yeast to grow on the YPRAA medium (Fig. 1b). These results indicate that both CSEP0139 and CSEP0182 carry a functional SP and are likely secreted effectors.

Subcellular localization of CSEP0139 and CSEP0182 in barley and *N. benthamiana*

To examine the subcellular localization of CSEP0139 and CSEP0182, we generated two sets of constructs to express YFP fusion of the mature form of the CSEPs (lacking SP) in barley and *N. benthamiana*, respectively. The CSEP0139-YFP and CSEP0182-YFP fusions were individually coexpressed with a free CFP marker in barley epidermal cells by particle bombardment. Confocal imaging revealed that CSEP0139-YFP and CSEP0182-YFP were localized to both the cytosol and the nucleus of barley cells (Additional file 2: Figure S3a). CSEP0139-YFP and CSEP0182-YFP were then individually expressed in *N. benthamiana* by agroinfiltration-mediated transient gene expression, and were also found to localize in the cytosol and the nucleus (Additional file 2: Figure S3b). These results suggest that CSEP0139 and CSEP0182 localize to both the cytosol and the nucleus upon overexpression in barley or *N. benthamiana*.

CSEP0139 and CSEP0182 suppress BAX- and *Nt*MEK2^{DD}-induced cell death in *N. benthamiana*

Our initial screen of *Bgh* CSEPs identified sixteen CSEPs, including CSEP0139 and CSEP0182 that suppressed cell death in *N. benthamiana* (Additional file 1: Table S1). To further confirm the activity of CSEP0139 and CSEP0182 in cell death suppression, we included CSEP0340 as a negative control that did not suppress cell death induced by BAX and *Nt*MEK2^{DD} (this study), and the Oomycete effector Avh328 as a positive control that consistently suppressed BAX-induced cell death (Wang et al. 2011). The CSEPs and the effector controls were infiltrated and expressed 12 h prior to that of the

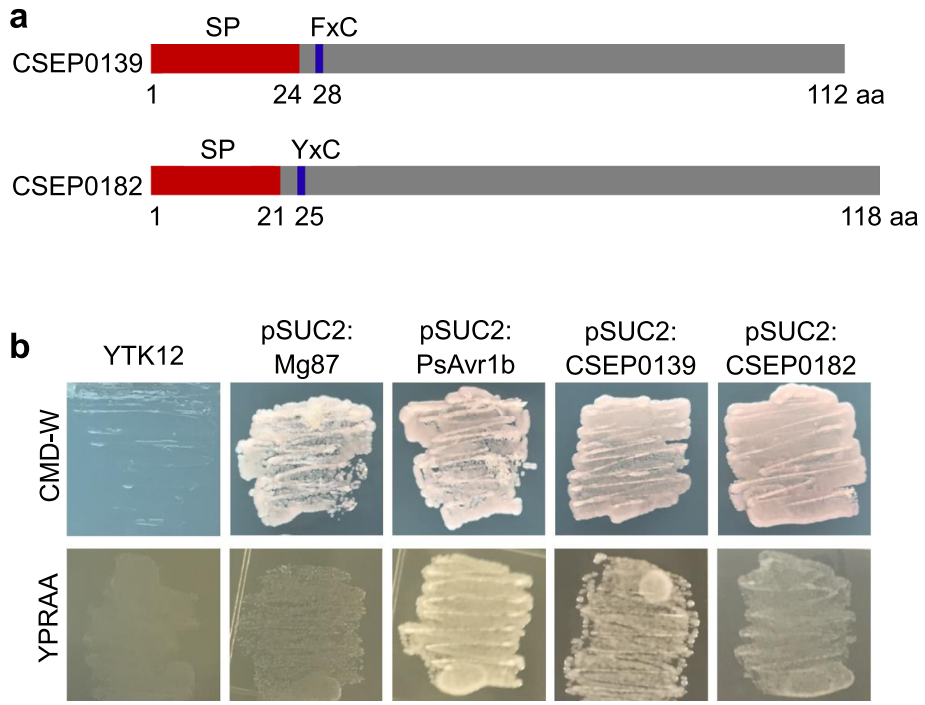

Fig. 1 CSEP0139 and CSEP0182 contain a functional signal peptide. **a** Schematic representation of CSEP0139 and CSEP0182, the N-terminal signal peptide (SP) was predicted by Signal 4.0 (http://www.cbs.dtu.dk/services/SignalP), highlighted in red. Shades of blue indicate F/YxC motif. **b** Yeast invertase secretion assay for the verification of SP. The SP sequences of CSEP0139, CSEP0182, and *P. sojae Ps*Avr1b and the N-terminal sequence of *M. oryzae* Mg87 were individually fused to yeast invertase lacking its own SP in the pSUC2 vector. CMD-W (minus Trp) media were used to select positive transformants of yeast mutant strain YTK12 carrying the pSUC2 vector. Yeast growth on YPRAA media indicates secretion of invertase. The pictures were taken at 3 days after plating

cell-death inducers, with the effector and cell-death inducer infiltrated next to each other but with an overlapping area in *N. benthamiana*. Indeed, the expression of either CSEP0139 or CSEP0182, or the positive control Avh328 consistently suppressed BAX-induced cell death (Fig. 2a) and *Nt*MEK2^{DD}-induced cell death (Fig. 2b) in the overlapping area. By contrast, the expression of CSEP0340 did not suppress BAX-induced or *Nt*MEK2^{DD}-induced cell death in the overlapping area (Fig. 2a, b, bottom panels). These results indicate that CSEP0139 and CSEP0182 can suppress BAX- and *Nt*MEK2^{DD}-induced cell death in *N. benthamiana*.

CSEP0139 and CSEP0182 do not suppress the AVR_{a13}-MLA13 pair induced cell death

To further test the activity of the two CSEPs in suppressing HR-like cell death induced by plant proteins, we coexpressed the barley immune receptor MLA13 with its cognate AVR effector AVR_{a13} (also known as *Bgh* CSEP0372 from family 34, and recently shown to adopt a predict common RNase-like fold) to trigger HR cell death in *N. benthamiana* (Lu et al. 2016; Saur et al. 2019). Similarly, CSEP0139 and CSEP0182 or the control effectors were expressed 12 h prior to the expression of the AVR_{a13}/MLA13 pair (Fig. 3). Unexpectedly, the expression of CSEP0139 or CSEP0182 did not suppress AVR_{a13}/MLA13-triggered cell death (Fig. 3, upper two panels), and similarly, the expression of Avh328 or CSEP0340 did not suppress AVR_{a13}/MLA13-triggered cell death in *N. benthamiana*, either (Fig. 3, bottom panel), although partial suppression of AVR_{a13}/MLA13-triggered cell death was sometimes observed for the CSEPs and the controls (Fig. 3). These results indicate that CSEP0139 and CSEP0182 most likely do not suppress the AVR_{a13}-MLA13 pair triggered cell death in *N. benthamiana*.

CSEP0139 and CSEP0182 inhibit BAX-induced cell death in barley

To further test whether these two CSEPs suppress cell death in host barley plant, we adopted a BAX-induced cell death assay in barley (Eichmann et al. 2006). In this assay, GFP expression in transformed barley epidermal cells displays normal cytoplasmic strands in the living cells, and coexpression of BAX induces cell death and causes cytoplasmic collapse of the transformed cells, whereas the expression of barley BAX inhibitor-1 (*Hv*BI-1) can delay or block this cell-death response (Eichmann et al. 2006). In our experiments, coexpression of GFP and BAX and EV typically induced cell death and led to the disappearance of cytoplasmic strands and nucleus,

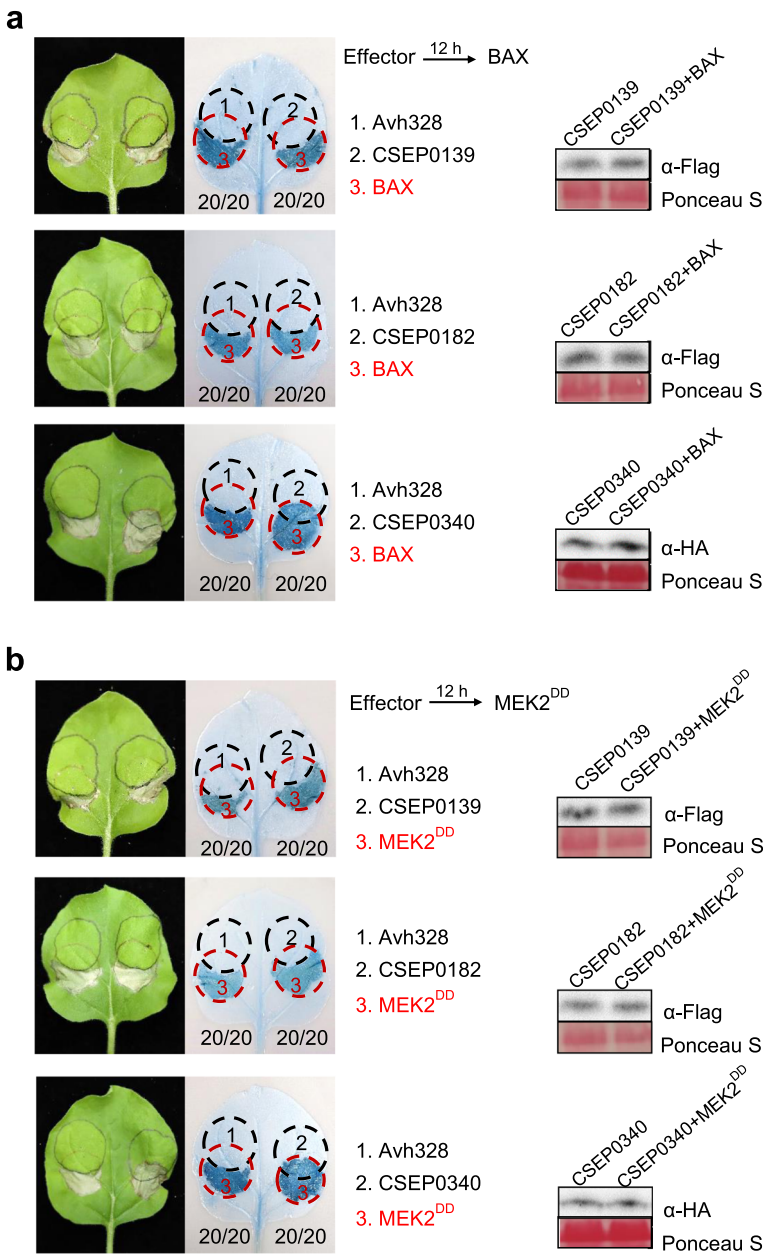

Fig. 2 CSEP0139 and CSEP0182 inhibit BAX- and *Nt*MEK2^{DD}-induced cell death in *N. benthamiana*. **a** Suppression of BAX-triggered cell death by CSEP0139 and CSEP0182 in *N. benthamiana*. **b** Suppression of *Nt*MEK2^{DD}-induced cell death by CSEP0139 and CSEP0182 in *N. benthamiana*. The Oomycete effector Avh328 was used as a positive control, and CSEP0340 as a negative control. Expression of CSEPs, BAX and *Nt*MEK2^{DD} was done by agroinfiltration in different but overlapping areas in leaves of *N. benthamiana*, with 12 h time lapse between the agroinfiltration of the effector and the cell death inducer. Cell death was visualized by trypan blue staining, and photographs were taken at 5 days after infiltration. Protein accumulation of CSEP fusions were detected at 2 days after infiltration by Western blotting

and accumulation of some irregular patches in most of the transformed cells (Fig. 4a, 2nd cell), thus the percentage of transformed cells showing collapse of the cytoplasm was set to 100% (Fig. 4b, 2nd bar). By contrast, coexpression of GFP and EV did not typically result in cell death in the transformed cells (Fig. 4a, 1st cell), with a relative low rate of cells showing collapse of the cytoplasm (~ 32%) (Fig. 4b, 1st bar). Importantly, the coexpression of GFP and BAX plus CSEP0139 or CSEP0182 significantly reduced the rate of cells with collapse of cytoplasm by ~ 30%, as compared to the coexpression of GFP and BAX and EV (Fig. 4a, b, 3rd & 4th bar). These results indicate that expression of CSEP0139 and CSEP0182 can inhibit or alleviate BAX-induced cell death in barley cells.

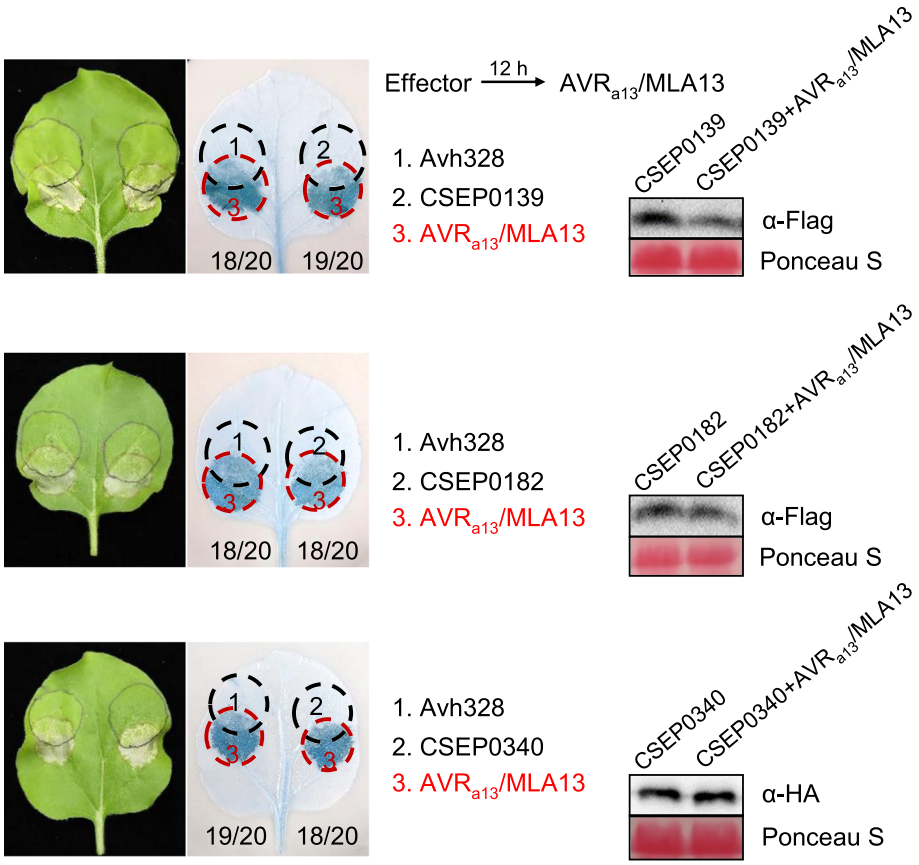

Fig. 3 CSEP0139 and CSEP0182 do not inhibit AVR$_{a13}$/MLA13-induced cell death in *N. benthamiana*. Oomycete Avh328 and CSEP0340 were used as a positive and a negative control, respectively. The experimental procedures are same as those in Fig. 2 except that the AVR$_{a13}$/MLA13 pair, instead of BAX or *Nt*MEK2^{DD}, was expressed 12 h after the expression of CSEP0139 or CSEP0182

CSEP0139 and CSEP0182 promote *Bgh* virulence in barley

To investigate the function of CSEP0139 and CSEP0182 in *Bgh* virulence, we transiently overexpressed CSEP0139 or CSEP0182 in barley epidermal cells by particle bombardment in both compatible and incompatible interactions (Shen et al. 2007; Bai et al. 2012). A GUS reporter was also coexpressed with the CSEPs to mark the transformed barley cells by GUS staining. The leaves of barley line P01 were used for bombardment and followed by inoculation with a virulent or an avirulent isolate, *Bgh*A6 or *Bgh*K1 respectively, and then fungal haustorium formation rate (haustorium index, HI%) was scored microscopically. Significantly, the expression of CSEP0139 or CSEP0182 increased *Bgh* haustorium index from ~ 50% to ~ 63% or ~ 65% respectively in the compatible interaction, and from ~ 16% to ~ 24% or 26% respectively in the incompatible interaction (Fig. 5a and Additional file 1: Table S2). These results suggest that both CSEP0139 and CSEP0182 can promote *Bgh* virulence during early stages of barley infection.

We further took advantage of the HIGS approach in verifying the function of *Bgh* effectors (Nowara et al. 2010; Pliego et al. 2013; Ahmed et al. 2015). Silencing of *CSEP0139* or *CSEP0182* gene through HIGS was achieved by particle bombardments of an RNAi vector harboring sense and antisense fragments of the corresponding effector gene (Himmelbach et al. 2007). Silencing of either *CSEP0139* or *CSEP0182* significantly reduced the *Bgh* haustorium index by ~ 50% or ~ 40%, respectively, compared to the empty vector (EV) control (Fig. 5b and Additional file 1: Table S3). Silencing of barley *Mlo* significantly reduced haustorium index by ~ 75%, similar as previously reported (Himmelbach et al. 2007). Thus, these HIGS data further support that CSEP0139 and CSEP0182 contribute to *Bgh* virulence in barley infection.

Expression of CSEP0139 and CSEP0182 reduces *Botrytis* virulence in *N. benthamiana*

Since necrotrophic fungi induce cell death for successful infection, we tested if the expression of CSEP0139 or CSEP0182 may affect the infection of a necrotrophic fungus, such as *Botrytis cinerea*. We individually expressed CSEP0139, CSEP0182 and GFP (used as a

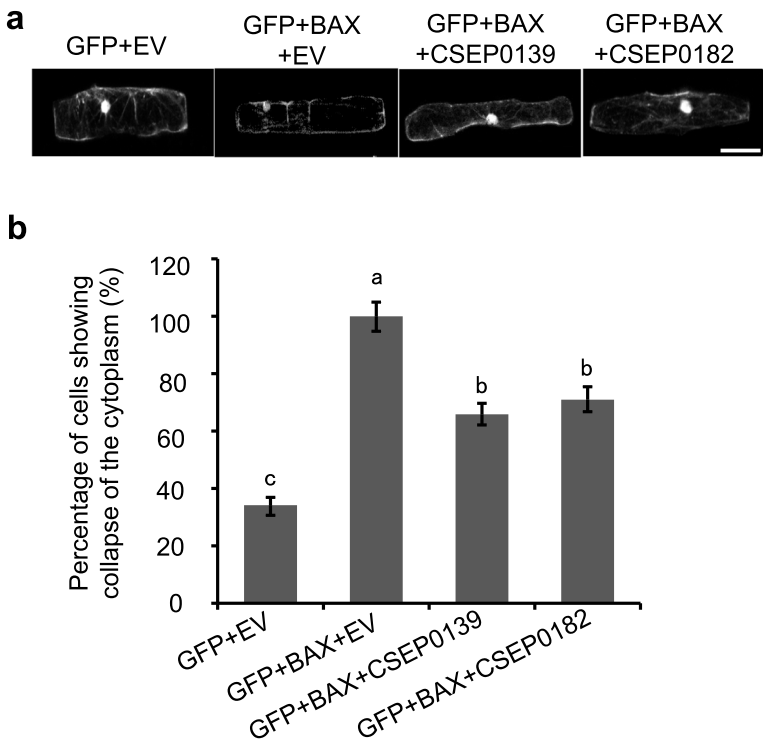

Fig. 4 CSEP0139 and CSEP0182 inhibit BAX-induced cell death in barley. **a** Suppression of BAX-triggered cell death by CSEP0139 and CSEP0182 in barley. Transient gene overexpression assay was conducted in barley epidermal cells by particle bombardment with combinations of plasmid expressing GFP and empty vector (EV), GFP and BAX and EV, GFP and BAX and CSEP0139, and GFP and BAX and CSEP0182, respectively. Representative pictures indicate that GFP fluorescence shows normal cytoplasmic strands in cells expressing GFP + EV, or GFP + BAX + CSEP0139 or GFP + BAX + CSEP0182, but shows collapsed cytoplasm strands in cell expressing GFP + BAX + EV. Photographs were taken using Nikon A1 confocal laser scanning microscope at 10 h after bombardment. Scale bar = 50 μm. **b** Quantification of BAX-induced cell death suppressed by CSEP0139 or CSEP0182. The percentage of cells showing collapse of the cytoplasm was shown in various plasmid combinations. The combinations expressing GFP + EV and GFP + BAX + EV were set as controls. The coexpression of GFP and BAX and EV typically induced cell death, thus the percentage of transformed cells showing collapse of the cytoplasm in GFP + BAX + EV was set to 100%. Data shown are means of three replicates ±SE. Means with different letters are significantly different ($P < 0.05$). Duncan's multiple range test was used to compare all the means. At least 100 cells were counted per experiment

control) in *N. benthamiana* by agroinfiltration and inoculated *B. cinerea* spores in the middle of infiltrated area 24 h later, and scored disease symptoms at 2 and 3 days post inoculation (dpi) of *B. cinerea* (Fig. 6). Interestingly, pre-expression of CSEP0139 or CSEP0182 significantly reduced the size of disease lesions caused by *B. cinerea* by ~ 22–29% at 3 dpi, as compared to the GFP control (Fig. 6). This result indicates that expression of CSEP0139 and CSEP0182 can reduce the virulence of *B. cinerea* in *N. benthamiana*, possibly by affecting cell death processes during *B. cinerea* infection.

Discussion

Many filamentous fungi are predicted to encode numerous secreted effector proteins, some of which have been shown to contribute to fungal virulence (Franceschetti et al. 2017). More than 500 CSEPs have been identified via genome mining in powdery mildew fungi, such as *Bgh* and *Bgt* (*Blumeria graminis* f. sp. *tritici*), however, only a fraction of them have been demonstrated to contribute to fungal virulence on plant hosts (Pliego et al. 2013; Menardo et al. 2017; Bourras et al. 2018; Thordal-Christensen et al. 2018). As obligate biotrophs, *Bgh* and *Bgt* depend entirely on living host tissues for their survival, and therefore, it is essential for them to manipulate host defense responses that may trigger PCD. These fungi may utilize multiple CSEPs to target components of host cell death pathways, however, the functions of such CSEPs have rarely been reported so far. Previously, a study conducted using 50 *Bgh* candidate effectors identified one effector (BEC1011/CSEP0264) that interferes with host cell death in barley (Pliego et al. 2013). Here, we report that CSEP0139 and CSEP0182, two *Bgh* effectors from two different CSEP families that do not belong to the RALPHs superfamily, suppress BAX-induced cell death, as well as *Nt*MEK2^{DD}-triggered cell death in plants. These two effectors also contribute to *Bgh* virulence during infection of barley. Based on these data, we

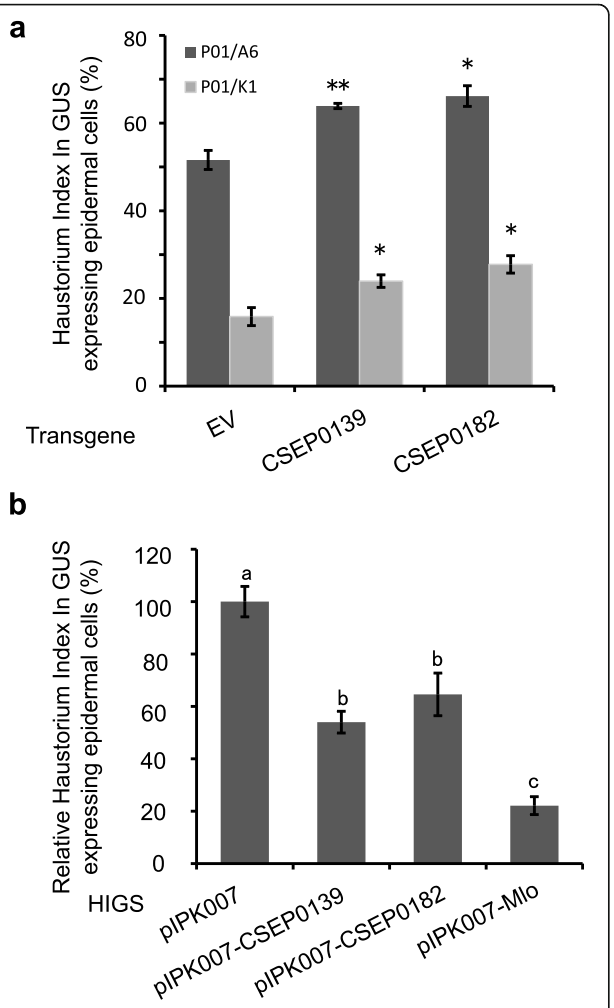

Fig. 5 CSEP0139 and CSEP0182 promote *Bgh* virulence in barley. **a** Overexpression of CSEP0139 and CSEP0182 increased *Bgh* haustorium formation in barley. One-week-old barley leaves (P01) were bombarded with GUS reporter along with pUBi-CSEP0139 or pUBi-CSEP0182 construct, respectively. Leaves were inoculated with spores of virulent isolate *Bgh*A6 or avirulent isolate *Bgh*K1 at 4 h after bombardment. *Bgh* haustorium index (%) was microscopically scored at 48 hpi. EV: empty vector. Error bar indicates SD, and similar experiments were repeated three times. The asterisk represents significant differences from EV at $P < 0.01$, determined by Student's *t* test. **b** Silencing of *CSEP0139* and *CSEP0182* reduced *Bgh* haustorium formation in barley. The experimental setting was the same as in (a), with pIPK007 as the empty vector; HIGS constructs, pIPK007-CSEP0139 and pIPK007-CSEP0139, were constructed and used for silencing of *CSEP0139* and *CSEP0182*, respectively. Inoculation of virulent isolate *Bgh*A6 spores was carried out at 48 h after bombardment. *Bgh* haustorium index of each transformation was normalized to that of the empty vector pIPK007 (set to 100%). pIPK007-Mlo was used as a positive control. Data show the average values and SD, similar experiments were repeated three times. Means with different letters indicate significant difference ($P < 0.05$). Duncan's multiple range test was used to compare all the means

envisage that there are more CSEPs from powdery mildew fungi having redundant functions in suppressing cell death and promoting fungal virulence in the host.

CSEP0139 was selected as one of the candidate *Bgh* genes for silencing in a previous study (Aguilar et al. 2016), and knock-down of *CSEP0139* expression was found not to affect fungal aggressiveness during early stages of infection. However, in this study we observed the reduction of haustorial formation upon silencing of *CSEP0139*. One possible explanation for this might be the variation in silencing efficiency in different cells as well as in different experiments. Furthermore, overexpression of CSEP0139 significantly increased haustorial formation in barley cells, which was consistent with the silencing data, and expression analyses in both studies indicated that *CSEP0139* expression was induced by *Bgh* infection and its transcripts were highly abundant in haustoria at 24–48 hpi (Fig. 5 and Additional file 2: Figure S1) (Aguilar et al. 2016).

Although BAX-like homologs may not exist in plants, BAX can stimulate cell death in plants (Dickman and Fluhr 2013). In mammals, BAX-induced cell death is associated with mitochondria, and BAX translocation from cytosol to the mitochondrial membranes results in the release of cytochrome c and stimulates the formation of an apoptosome complex, subsequently triggering cell death (Ishikawa et al. 2011). In plants, it has been shown that BAX-induced cell death and certain types of PCD are associated with mitochondria and release of cytochrome c (Lam et al. 2001; Yao et al. 2004; Tateda et al. 2009; Li et al. 2017). Expression of BAX in barley epidermal cells induces apparent cell death responses that can be clearly visualized through microscope (Eichmann et al. 2006). Using this expression system, we successfully identified the cell death-suppressing function of effectors CSEP0139 and CSEP0182. Here, overexpression of CSEP0139 and CSEP0182 delayed or affected BAX-induced cell death and increased barley susceptibility to *Bgh* isolates (Figs. 4 and 5a). It is noteworthy that BI-1 proteins, which are conserved cell death suppressors in animals and plants, efficiently suppressed BAX-induced cell death (Ishikawaet al. 2011). Previously, overexpression of barley *Hv*BI-1 had clarified different aspects of its cell death-related function, such as weakening a cell-wall-associated local hydrogen peroxide burst in a resistant *mlo*-mutant barley, delaying *Mla12*-mediated race-specific resistance responses to *Bgh*, and enhancing barley susceptibility to *Bgh* fungus (Eichmann et al. 2006; Babaeizad et al. 2009; Eichmann et al. 2010). However, the question of how to relate the function of the two CSEPs to that of *Hv*BI-1 in the context of BAX-induced cell death still remains unanswered. Animal and plant BI-1 proteins are ER-resident transmembrane proteins that are believed to act downstream of the BAX-induced mitochondrial

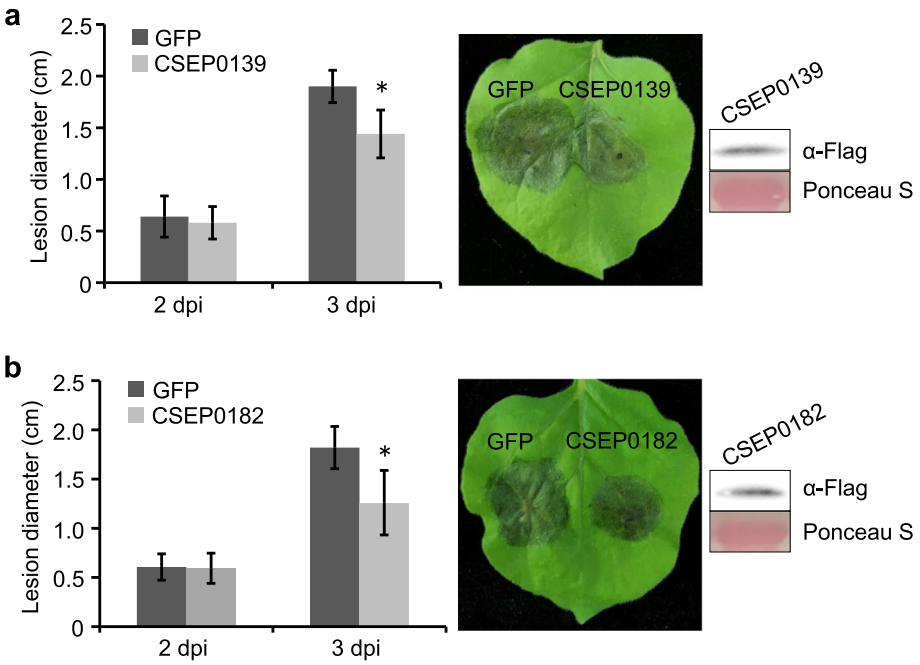

Fig. 6 Expression of CSEP0139 and CSEP0182 reduces the size of lesions caused by *Botrytis cinerea* in *N. benthamiana*. CSEP0139 **(a)** or CSEP0182 **(b)** was expressed in *N. benthamiana* by agroinfiltration, with GFP construct as control. *B. cinerea* was inoculated at 24 h after agroinfiltration with GFP or indicated CSEP in the middle of pre-infiltrated areas, and pictures were taken at 3 dpi. The lesion size caused by *B. cinerea* infection was measured as the diameter of infected area at 2 and 3 dpi, and shown as the average values and SE from three independent experiments, each with 10 leaves. The asterisk indicates significant difference at $P < 0.05$ by Student's *t* test

membrane modification and to regulate ER-stress responses (Xu et al. 2008). As both CSEP0139 and CSEP0182 can localize to both cytosol and nucleus upon overexpression, whether or not these two CSEPs may directly interact with barley *Hv*BI-1 protein remains to be an open question. Since *HvBI-1* expression is also induced in epidermal tissues after *Bgh* infection (Eichmann et al. 2010), it will be of interest to examine if *Hv*BI-1 and CSEP0139/CSEP0182 act independently in barley cells, and if they show any similarities in inhibiting BAX-induced cell death. Based on our data, we can only speculate that these two effectors act downstream of BAX-induced cell death. Future experiments may reveal if these two CSEPs act downstream of mitochondria and on the release of cytochrome c, as well as ER-stress responses in plant cells.

Recent studies have identified several barley powdery mildew AVRa effectors, which are recognized by the cognate MLA receptors, and the expression of the matching AVR_a-MLA pair triggers cell death in barley and *N. benthamiana* (Lu et al. 2016; Saur et al. 2019; Bauer et al. 2021). Here, we found that the AVR_{a13}-MLA13 pair induced visible cell death in *N. benthamiana*, however, pre-expression of CSEP0139 and CSEP0182 did not show clear suppression of this type of cell death (Fig. 3). This result may indicate that cell death triggered by the AVR_{a13}-MLA13 pair may be somewhat different from that induced by BAX or $NtMEK2^{DD}$, while BAX and $NtMEK2^{DD}$ might induce cell death through a potentially shared cell death signaling and/or similar mechanism. Nevertheless, we cannot fully rule out the possibility that CSEP0139 and CSEP0182 may partially suppress the AVR_{a13}/MLA13 pair triggered cell death in *N. benthamiana* (Fig. 3). Therefore, examining the activity of CSEP0139 and CSEP0182 in suppressing ETI-related cell death in host barley remains to be an interesting topic in the future. Since host cell death signaling pathways are probably the primary targets of biotrophic fungi, these CSEPs might be used as useful tools in probing the components of cell death signaling pathways in barley. To further elucidate the underlying mechanisms of CSEP0139 and CSEP0182 in suppressing cell death in host plant, their host targets need to be identified using integrated genetic and biochemical approaches in the future. Further experiments are required to reveal if CSEP0139 and CSEP0182 target different or same components/pathways of cell death signaling in host cells.

Conclusions

We provide evidence for the key functions of two *Bgh* effectors CSEP0139 and CSEP0182 in promoting fungal virulence and suppressing cell death in host plant. Both CSEPs carry a functional signal peptide and are localized

in the cytosol and nucleus when overexpressed in plant cells. Both CSEPs have the potential to suppress cell death induced by BAX and/or *Nt*MEK2^{DD} in barley and *N. benthamiana*. On the contrary, pre-expression of CSEP0139 or CSEP0182 reduces the virulence of the necrotrophic fungus *B. cinerea* in *N. benthamiana*. These findings provide bases for further understanding the virulent strategies of the biotrophic fungal pathogens and will facilitate the development of efficient strategies for combatting these pathogens in the field.

Methods

Plant and fungal materials

Barley (*Hordeum vulgare* L.) cultivars Golden Promise and 'P01' (isogenic line from cv Pallas containing *Mla1*) were grown in a growth chamber under the conditions of 20 °C with light for 16 h and 18 °C in darkness for 8 h. *N. benthamiana* plants were grown at 24 ± 1 °C under a 16 h light/8 h darkness photoperiod in a greenhouse.

The barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*) isolate A6 (*AvrMla6*, *AvrMla10*, *AvrMla12*, *virMla1*) and K1 (*AvrMla1*, *virMla6*, *virMla10*, *virMla12*) were used in this study. For maintenance and experimental use, one-week-old barley seedlings were inoculated with *Bgh* spores and maintained in a growth chamber under a 16 h light (20 °C)/8 h darkness (18 °C) cycle and 70% relative humidity.

RNA isolation and RT-qPCR

Total RNA was isolated using Trizol solution (Invitrogen), and digested with RNase-free DNase I (Takara) to eliminate potential DNA contamination. The first-strand cDNA was synthesized with reverse transcriptase M-MLV (Invitrogen) and used as template. To analyze the expression patterns of *CSEPs*, one-week-old first barley leaves (P01) were inoculated with the virulent *Bgh* isolate A6, and total RNA was isolated from leaves at 0, 3, 6, 12, 24, and 48 hpi. In addition, at 24 and 48 hpi, *Bgh* epiphytic tissues from the leaf surface and leaf tissues containing *Bgh* haustoria were separately collected. The epiphytic tissues were collected by dipping *Bgh*-infected leaves in 10% cellulose acetate (Ahmed et al. 2015). qPCR was performed with specific primers (Additional file 1: Table S4), using an ABI step-one real time PCR system and Gotaq qPCR Master Mix (Promega). Relative expression was determined by comparing with expression at 0 hpi, arbitrarily set to 1. Expression of the *Bgh* glyceraldehyde 3-phosphate dehydrogenase gene (*G3PDH*) was used to normalize the *CSEP* expression of each sample, using the delta deltact method (Ahmed et al. 2015). Three biological repetitions were carried out at each observation time point, and two independent experiments were performed.

Validation of the predicted SP of CSEPs

The predicted SPs of CSEP0139 and CSEP0182 were validated using the invertase secretion-deficient yeast strain YTK12, as previously described (Gu et al. 2011). The predicted SP sequences of CSEPs and oomycete effector Avr1b and the first 25 amino acids of *M. oryzae* Mg87 (Gu et al. 2011) were fused in frame with the yeast invertase lacking its own SP in the pSUC2 vector. The pSUC2-derived constructs (Additional file 1: Table S5) were transformed into the invertase secretion-deficient yeast strain YTK12, which were then plated on CMD-W medium (0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar). Yeast positive clones were transferred onto YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, 2 μg/L antimycin, and 2% agar) for invertase secretion assay.

Subcellular localization analysis

For the subcellular localization assay in barley, CSEP0139 and CSEP0182 (without SP) were cloned into the destination vector, pUbi-GW-YFP, by gateway technology, and the constructs thus obtained, i.e., pUbi-CSEP0139-YFP and pUbi-CSEP0182-YFP, were co-transformed with the pUbi-CFP marker construct into barley epidermal cells by particle bombardment (Shen et al. 2007). Two days after bombardment, the fluorescent signal was monitored, and pictures were taken using a Nikon A1 confocal laser-scanning microscope. GFP/YFP and CFP were excited at 488 nm and 405 nm, respectively.

For the subcellular localization assay in *N. benthamiana*, CSEP0139 and CSEP0182 (without SP) were cloned into the CaMV 35S promoter-driven destination vector, i.e., CTAPi-GW-YFP, using gateway technology (Bai et al. 2012), to obtain the constructs CTAPi-CSEP0139-YFP and CTAPi-CSEP0182-YFP. These constructs were then delivered into *A. tumefaciens* strain GV3101 for transient expression in *N. benthamiana*, as described in the following section. The leaves were used for fluorescence detection 2 days after infiltration.

Single-cell transient gene expression and HIGS in barley

Transient gene expression assay in individual epidermal cells of barley was performed by particle bombardment, as previously described (Shen et al. 2007). *CSEP* (without SP) sequence was cloned into the pUbi-GW vector to generate pUbi-CSEP constructs, which together with a ß-glucuronidase (GUS) reporter vector, were delivered into barley leaf epidermal cells. The leaves were inoculated with the virulent *Bgh* isolate A6 or the avirulent *Bgh* isolate K1 at 4 h after bombardment, and were stained with GUS staining solution (0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Na_2EDTA, 5 mM $K_4Fe[CN]_6$, 5 mM $K_3Fe[CN]_6$, 0.1% Triton X-100, 20% methanol, 1 g/L X-

gluc, pH 7.0) at 48 hpi. Fungal haustoria were observed and evaluated in the GUS-expressing cells after the leaves were stained with 0.6% Coomassie solution and cleared with water. Haustorium index was calculated using the number of GUS-expressing cells with developed haustorium divided by total number of GUS-expressing cells with germinated but aborted spores (incompatible) and with developed haustorium (compatible). Significant differences between the constructs and empty vector were determined using Student's *t* test.

For transient silencing of *CSEP* genes of *Bgh* by HIGS, the *CSEP0139* and *CSEP0182* fragments were cloned into the 35S promoter-driven hairpin destination vector pIPK007, as previously described (Himmelbach et al. 2007). The following experimental procedure was the same as that for the transient gene expression assay, except that the leaves were inoculated with *Bgh* spores at 48 h after bombardment (Nowara et al. 2010). Silencing of *HvMlo* was used as a positive control (Himmelbach et al. 2007). Relative haustorium index was calculated as the haustorium index of each construct divided by that of the EV (pIKP007) construct. Significant differences between constructs and EV (pIKP007) were determined using ANOVA and Duncan's multiple range test.

Inhibition of BAX-induced cell death in barley

Expression of mouse BAX can lead to cell death in yeast, tobacco, *Arabidopsis* and barley (Lacomme and Santa Cruz 1999; Kawai-Yamada et al. 2001). We tested the inhibition of BAX-induced cell death by CSEP0139 and CSEP0182 in barley using the transient transformation method with particle bombardment, as described in a previous study (Eichmann et al. 2006). In brief, 0.05 μg of pUbi-BAX plasmid DNA and 0.5 μg of pUbi-GFP plasmid DNA per shot were transferred into one-week-old barley epidermal cells, and GFP fluorescence signal was used to detect the intact scaffolds or collapsed cytoplasmic strands in living cells, using a Nikon A1 confocal laser-scanning microscope, 10–14 h after transformation. For assaying the inhibition of BAX-induced cell death in barley, 0.05 μg of pUbi-BAX plasmid DNA, 0.5 μg of pUbi-GFP plasmid DNA, and 1.6 μg of pUbi-CSEP0139, pUbi-CSEP0182 or pUbi-EV plasmid DNA were delivered into barley epidermal cells by particle bombardment. The percentage of cells showing collapse of the cytoplasm (%) was scored as the number of GFP-expressing cells with collapsed cytoplasm divided by the total number of GFP-expressing cells. For each experiment, at least 100 GFP-expressing cells were scored.

Agroinfiltration-mediated transient gene expression in *N. benthamiana*

CSEP0139, CSEP0182 and CSEP0340 sequences without SP were cloned into the PVX vector pGR107 and the sequence of Flag or HA tag was added to the genes in frame using suitable restriction enzyme cutting sites to obtain constructs pGR107-CSEP0139, pGR107-CSEP0182 and pGR107-CSEP0340 (Wang et al. 2011). All constructs were confirmed by sequencing, and then introduced into *A. tumefaciens* strain GV3101 (pJIC SA_Rep) for transient expression in *N. benthamiana*. For this, agrobacteria carrying the aforementioned constructs were cultured overnight, washed with 10 mM $MgCl_2$ three times, and then resuspended with 10 mM $MgCl_2$ to achieve a final OD_{600} of 0.5. After incubation for 3 h at room temperature, the resuspended solution was infiltrated into 4-week-old *N. benthamiana* leaves, using a needleless syringe. For further expression of BAX, $NtMEK2^{DD}$, and AVR_{a13}/MLA13 (mixed agrobacteria transformed with AVR_{a13} or MLA13 expressing constructs), agrobacteria were infiltrated 12 h later into a different but with overlapping area expressing CSEPs on the leaves. pGR107-CSEP0340 was used as a negative control, and pGR107-Avh328 from *P. sojae* was used as a positive control suppressing BAX-induced cell death (Wang et al. 2011).

Trypan blue staining for assessing cell death in *N. benthamiana*

Agroinfiltration-mediated gene expression was performed following the same procedure as mentioned in the previous section. Cell death symptoms were monitored at 3–5 days after infiltration, and the photographs were taken at 5 days after infiltration. For trypan blue staining, the leaves were boiled with a 1:1 mixed solution of trypan blue staining solution (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue, dissolved in 10 mL distilled water) and ethanol for 5 min, and maintained overnight at room temperature. The leaves were then de-stained in chloral hydrate solution (2.5 g/mL) until the color was completely faded.

Botrytis cinerea infection assay

Four to five-week-old *N. benthamiana* leaves were used for transient gene expression and inoculated with *Botrytis cinerea*. In brief, one half of a leaf was used for infiltration of GFP constructs and the other half for CSEP0139 or CSEP0182 constructs, by agroinfiltration-mediated transient expression. One day later, in the middle of infiltrated area, 10 μL conidial suspension (10^6 conidia/mL) of *B. cinerea* was inoculated. Three experiments were conducted for each CSEP, and 10 to 15 leaves were used per experiment. Lesion diameters of *B. cinerea* were measured for ~ 10 representative leaves at 2 and 3 dpi, and photographs were taken at 3 dpi.

Western blotting

Total proteins, expressing the indicated constructs, were extracted from *N. benthamiana* leaves at 2 days after

infiltration. Leaf samples (0.1 g) were ground and resuspended in 250 μL of 2 × loading buffer (1 M Tris·HCl, 5% SDS, 25% glycerol and 0.25 mg/mL bromophenol blue). Suspensions were boiled in a water bath for 5 min and then centrifuged at 12,000 rpm for 10 min. Twenty microlitre of the supernatant was used for SDS-PAGE electrophoresis, following which the proteins were transferred onto a nitrocellulose membrane, for 90 min at 200 mA. The membrane was stained with Ponceau solution to give equal loading, and then incubated with anti-Flag or anti-HA antibody. Super pierce ECL western blotting substrate was used for detection under a cooled CCD camera (Tanon 5200).

Gene accession numbers

CSEP0139 (BLGH_07004), CSEP0182 (BLGH_06939), CSEP0340 (BLGH_06995), AVR_{a13}/CSEP0372 (BLGH_02099), as available in the EnsemblFungi database (http://fungi.ensembl.org/index.html). *Bgh* G3PDH (X99732.1), BAX (L22472.1), *Nt*MEK2 (AB264547.1), MLA13 (AF523678.1) as available in the GenBank database (https://www.ncbi.nlm.nih.gov/).

Abbreviations

Avr: Avirulence; *Bgh*: *Blumeria graminis* f. sp. *hordei*; *Bgt*: *Blumeria graminis* f. sp. *tritici*; CSEPs: Candidate Secreted Effector Proteins; ETI: Effector-triggered immunity; EV: Empty vector; G3PDH: Glyceraldehyde 3-phosphate dehydrogenase; GUS: ß-glucuronidase; HI: Haustorium Index; HIGS: Host-induced gene silencing; HR: Hypersensitive responsive; *Hv*: *Hordeum vulgare* L; MEK2: Mitogen-activated protein kinase kinase 2; MLA: Mildew locus A; *Nb*: *Nicotiana benthamiana*; *Nt*: *Nicotiana tabacum*; PCD: Programmed cell death; PR: Pathogenesis-related; PVX: Potato virus X; RALPH: RNase-like proteins associated with haustoria; SP: Signal peptide

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-021-00084-z.

Additional file 1: Table S1. CSEPs suppressing cell death triggered by BAX and $MEK2^{DD}$ in *N. benthamiana*. **Table S2.** Scoring of haustorium index after overexpression of CSEP0139 or CSEP0182 in barley P01 in compatible (P01/*Bgh*A6) and incompatible (P01/*Bgh*K1) interaction. **Table S3.** Scoring of haustorium index after silencing of *CSEP0139* or *CSEP0182* by HIGS approach. **Table S4.** List of primers used in this study. **Table S5.** List of constructs used in this study.

Additional file 2: Figure S1. Expression of *CSEP0139* (**a**) and *CSEP0182* (**b**) is induced during *Bgh* infection of barley. The barley isogenic line P01 was inoculated with the compatible isolate *Bgh*A6 for the time course experiments. Total RNA was isolated from *Bgh*-infected barley leaves at 0, 3, 6, 12, 24, and 48 hpi. H and E denote haustorium containing leaf tissues and epiphytic *Bgh* tissues, respectively. Relative expression of *CSEP0139* or *CSEP0182* was determined by comparing with expression at 0 hpi, arbitrarily set to 1. *Bgh glyceraldehyde 3-phosphate dehydrogenase* was used as the reference gene. Error bars indicate SD of three biological repetitions. Means with different letters indicate significant difference ($P < 0.05$). Duncan's multiple range test was used to compare all the means. Two independent experiments were performed with similar results. **Figure S2.** Nucleotide and amino acid sequences of CSEP0139 and CSEP0182. Shaded sequence is the predicted signal peptide (SP). Red box represents the F/YxC motif. The sequences underlined are used in the HIGS constructs for gene silencing. **Figure S3.** CSEP0139 and CSEP018 are localized to the cytoplasm and nucleus. **a** One-week-old barley leaves were bombarded with pUBi-CSEP0139-YFP or pUBi-CSEP0182-YFP, together with pUBi-CFP construct. **b** CSEP0139-YFP or CSEP0182-YFP was expressed by agroinfiltration in *N. benthamiana* leaves. Confocal images were taken 48 h after bombardment in barley or agroinfiltration in *N. benthamiana*, with excitation at 405 nm (CFP) or 488 nm (YFP) channel, using Nikon A1 confocal microscope. Scale bar = 50 μm.

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Authors' contributions

XL, CJ, HY, WH and FL performed the experiments. RF and JX contributed project management. XL, CJ, HY, RF and QHS analyzed data. QHS, JX and RF supervised the students. QHS and HY conceived the project, and designed the research. QHS, HY and XL wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Innovation Academy for Seed Design, Chinese Academy of Sciences, Beijing 100101, China. [2]CAS Center for Excellence in Biotic Interactions, University of Chinese Academy of Sciences, Beijing 100049, China. [3]College of Life Sciences, Jiangxi Normal University, Nanchang 330022, China. [4]School of Life Sciences, Yunnan University, Kunming, China.

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