Identification of effector CEP112 that promotes the infection of necrotrophic
Alternaria solani

Chen Wang¹, Dai Zhang¹, Jianing Cheng¹, Dongmei Zhao¹, Yang Pan¹, Qian Li¹, Jiehua Zhu¹,²*, Zhihui Yang¹,²* and Jinhui Wang¹,²*

Abstract

Background: Alternaria solani is a typical necrotrophic pathogen that can cause severe early blight on Solanaceae crops and cause ring disease on plant leaves. Phytopathogens produce secretory effectors that regulate the host immune response and promote pathogenic infection. Effector proteins, as specialized secretions of host-infecting pathogens, play important roles in disrupting host defense systems. At present, the role of the effector secreted by A. solani during infection remains unclear. We report the identification and characterization of AsCEP112, an effector required for A. solani virulence.

Result: The AsCEP112 gene was screened from the transcriptome and genome of A. solani on the basis of typical effector signatures. Fluorescence quantification and transient expression analysis showed that the expression level of AsCEP112 continued to increase during infection. The protein localized to the cell membrane of Nicotiana benthamiana and regulated senescence-related genes, resulting in the chlorosis of N. benthamiana and tomato leaves. Moreover, comparative analysis of AsCEP112 mutant obtained by homologous recombination with wild-type and revertant strains indicated that AsCEP112 gene played an active role in regulating melanin formation and penetration in the pathogen. Deletion of AsCEP112 also reduced the pathogenicity of HWC-168.

Conclusion: Our findings demonstrate that AsCEP112 was an important effector protein that targeted host cell membranes. AsCEP112 regulated host senescence-related genes to control host leaf senescence and chlorosis, and contribute to pathogen virulence.

Keywords: Alternaria solani, Effector, Chlorosis, Senescence, Gene knockout, Pathogenicity
photosynthetic pigment contents, resulting in a general reduction in plant growth and a decline in yield [4, 5].

Necrotrophic pathogens can directly kill host tissues, and they obtain nutrients mainly from dead cells. Cell death is also an important early-stage signal of the successful infection by necrotrophic pathogens [6–8]. Necrotrophic pathogens may thrive by subverting the resistance mechanisms acquired by plants to combat other pathogens [9, 10]. They can be divided into two categories on the basis of their host range. One category contains pathogens that are host-specific necrotrophs, which usually produce host-selective toxins [11, 12]. The other category contains pathogens, including A. solani, that are broad host-range necrotrophs. Compared with biotrophs, the interaction mode of necrotrophs with a large range of host plants remains to be explored. In plant defense responses to attacking pathogens, salicylic acid (SA) dependent defenses act against biotrophs, and jasmonic acid (JA) and ethylene (ET) dependent responses act against necrotrophs [13, 14]. However, an increasing number of studies have found that SA signaling, and not JA signaling, is required for host defenses against certain necrotrophic pathogens [15, 16].

Many necrotrophs enhance pathogenicity using plant defense responses, and they induce host-cell death by secreting phytotoxic secondary metabolites [17, 18]. Some low molecular weight metabolites and secreted proteins are the basic determinants of pathogenicity or virulence [19]. Effectors enable successful pathogens to overcome MAMP-triggered immunity, leading to effector-triggered susceptibility. These effectors can be recognized by intracellular receptors (R proteins) to activate effector-triggered immunity (ETI), a major source of qualitative resistance to biotrophs [20–22]. However, the effectors are co-opted by necrotrophs to promote disease [23]. Until now, no effector had been cloned from A. solani. Thus, the identification of effector proteins is of great significance for understanding the pathogenic mechanism of necrotrophic A. solani.

Based on the transcriptome and genome results of Alternaria solani, we selected a novel effector, AsCEP112, which localized to the host cell membrane and was highly expressed during infection. Transient expression assays in Nicotiana benthamiana and tomato showed that AsCEP112 regulated host senescence-related genes to promote host chlorosis. Moreover, we demonstrated that the deletion of the AsCEP112 gene did not affect pathogen vegetative growth or development, but it reduced the melanin content and penetrability. It also significantly reduced virulence. These results suggest that AsCEP112 is critical for the virulence of A. solani and provide insights into the molecular interactions between A. solani and its host.

Materials and methods
Plants, fungal and bacterial strains.
The wild-type strain HWC-168 of A. solani was collected from leaves infected with potato early blight in Weichang County, Hebei Province, China. All the plants were grown on the campus of Hebei Agricultural University (Baoding, China). Nicotiana benthamiana and tomato were grown in a greenhouse at 23±1 °C with a 16-h light period. Potato 'Favorita' was grown in a greenhouse at 20±1 °C. The WT A. solani HWC-168 strain was cultured on Potato Dextrose Agar (PDA) medium in the dark at 25 °C. Escherichia coli strain DH5α was cultured in Luria–Bertani (LB) medium at 37 °C and used for plasmid amplification. Agrobacterium tumefaciens strain EHA105 was cultured in LB medium at 28 °C and used for Agrobacterium-mediated transient expression in plant leaves.

Sequence analysis
The Online tools SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/), SMART (http://smart.embl-heidelberg.de/) and ProParam (https://web.expasy.org/protparam/) were used to predict protein signal peptides, structural domains, molecular masses and theoretical isoelectric points.

RNA isolation and cDNA synthesis
In this study, the hyphae of A. solani and the leaves of potato infected at different time points were selected for gene expression analyses. The hyphal and plant total RNAs were extracted using an Easy Pure Plant RNA Kit (TransGen Biotech, Beijing, China) in accordance with the manufacturer’s instructions. The total RNA concentrations were determined by spectrophotometric analysis and adjusted to be uniform. Using the Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech), 400 ng to 500 ng of total RNA was used to synthesize first-strand cDNA.

Quantitative real-time PCR analysis
Based on previous studies on A. solani, we selected a relatively stable housekeeping gene "Actin" as an internal reference gene [24, 25]. Specific primers for Actin and AsCEP112 were designed using the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) (Supplementary Table S2). The SEN4, SAG12 and DHAR1 sequences were obtained from Uniport (https://www.uniprot.org/), and Primer 3 Plus (https://www.primer3plus.com/) was used to design specific primers for each gene. PP2A was selected as a housekeeping gene in N. benthamiana [26]. The cDNAs of the hyphae growing on potato and N. benthamiana leaves were serial diluted to detect
primer specificity. The qRT-PCR was performed using the Bio-Rad CFX384 Touch Real-Time System (Bio-Rad, USA), and the total reaction volume was 20 μL, containing 2 μL gene specific primers, 6 μL of cDNA and 10 μL of 2 × Magic SYBR Mixture (CWBiO, Jiangsu, China). The qRT-PCR was performed under the following conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 59 °C for 30 s. The melting curve analysis was performed at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s, with a final step at 50 °C for an additional 30 s. The threshold cycle (CT) values were used to determine the fold change in transcript accumulation with the \[\frac{\Delta \Delta CT\text{(Sample)}}{\Delta \Delta CT\text{(Control)}}\] method [27]. Six biological and two technical replicates were performed for each sample in all the qRT-PCR reactions.

Subcellular localization and hypersensitive response assay
The transient expression of AsCEP112 was performed using an Agrobacterium-mediated transient gene expression system [28, 29]. The full-length open reading frame (referred to as FL), or the truncated coding region with or without the signal peptide sequence but with an engineered ATG start codon (referred to as NSP), of AsCEP112 was PCR amplified using specific primers and integrated independently into the binary vector pCAMBIA1301::EGFP at a position downstream of the CaMV35S promoter. Then, the fusion vectors were transformed independently into the A. tumefaciens strain EHA105 [30]. The amplification product and pCAMBIA1301 vector digested with restriction enzymes SalI and BamHI (TaKaRa, Beijing, China) were ligated using a pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech).

INF1, an elicitor secreted by Phytophthora infestans, which induces defense responses including the hypersensitive response (HR), was used as the positive control in Solanaceae family infiltration tests, specifically Nicotiana species [31]. Recombinant Agrobacterium was cultured at 28 °C and resuspended in an infiltration medium containing 10 mmol L\(^{-1}\) MgCl\(_2\), 10 mmol L\(^{-1}\) 2-(N-morpholino) ethanesulfonic acid (pH 5.6) and 200 μmol L\(^{-1}\) acetosyringone. The recombinant strains were tested on N. benthamiana leaves at an optical density (OD)\(_{600}\) of 0.6–0.8. Different infiltration groups were injected into the leaves of N. benthamiana. At 72 h after incubation, the treated leaves were collected, and subcellular localization was observed under a confocal laser microscope (TCS SP8, Leica, Germany) with excitation at 488 nm and emission at 507 nm.

For the detection of HR, the strains expressing the AsCEP112 protein were infiltrated into the right side of each leaf, and the empty vector (EV) was infiltrated into the left side. The N. benthamiana and tomato were cultured for 5 days at 23 °C until the disease symptom appeared. Whole N. benthamiana and tomato leaves were colored using a DAB Kit (CWBiO) at room temperature. After 12 h, the leaves were discolored by boiling in 95% ethanol for 30 min and photographed. Each treatment was replicated six times.

Signal peptide secretion assay
A yeast secretion system was used to validate the function of predicted signal peptides. The signal peptide of AsCEP112 was cloned into vector pSUC2 using specific primers, and the recombinant plasmid was transformed into yeast strain YTK12. The positive colonies were screened on a CMD–W medium (0.075% tryptophan dropout supplement, 0.67% yeast nitrogen base without amino acids, 2% sucrose, 0.1% glucose and 2% agar). To test for invertase secretion, successfully transformed yeast strains were grown on YPRA agar (1% yeast extract, 2% peptone, 2% raffinose, 2 mg/mL antimycin A and 2% agar).

Deletion and restoration of AsCEP112 in A. solani
A gene knockout strategy with homologous recombination technology was used for A. solani. The hygromycin B resistance gene (Hyg) was used to mark the knockout AsCEP112 gene. Using specific primers, approximately 1-kb upstream and downstream fragments from the WT HWC-168 genomic DNA and the split hygromycin B resistant gene fragments from the pUC-HYG plasmid were amplified for collective transformation into the pUC19 vector using restriction enzymes EcoRI and BamHI and the pEASY-Uni Seamless Cloning and Assembly Kit. The PCR products were purified from the gels using a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China). Mixtures of upstream- and downstream-hygromycin fusion fragments were used for transforming protoplasts of A. solani.

The WT HWC-168 hyphae were inoculated into 200-mL flasks containing 100 mL Potato Dextrose Broth (PDB) medium, incubated at 25 °C and shaken at 120 rpm min\(^{-1}\) for 36 h. The mycelia were filtered through three layers of gauze and rinsed 2–3 times thoroughly with 0.7 mol L\(^{-1}\) NaCl solution. The hyphae were transferred to a 50-mL centrifuge tube using tweezers, and the protoplasts were digested with a mixture of enzymes, 0.1 mg of snailase (Solarbio, Beijing, China), 0.1 mg of driselase (Biotopped, Beijing, China) and 0.1 mg of lysing enzyme (SIGMA, Shanghai, China) in 10 mL of 0.7 mol L\(^{-1}\) NaCl. The protoplasts were resuspended in STC buffer (1.168 M D-Sorbitol, 10 mM Tris–HCl and 4.96 mM CaCl\(_2\)) and the fusion fragments mixtures were added to the PTC buffer (15 mM
PEG4000, 10 mM Tris–HCl and 10 mM CaCl₂). The transformants were screened on Regeneration PDA medium containing ampicillin (50 µg mL⁻¹) and hygromycin (50 µg mL⁻¹), and PCR was performed using specific primers to confirm the transformants.

After successfully obtaining the AsCEP112 gene mutant strain, the mutant strain was used to produce AsCEP112 protoplasts. The AsCEP112-FL gene was amplified with specific primers, and it and the vector KN containing the neomycin resistance gene (Neo) were digested with restriction enzymes EcoRI and BamHI. The fragments were ligated using the pEASY-Uni Seamless Cloning and Assembly Kit, and the constructed plasmid was transformed into AsCEP112 mutant protoplasts using PEG-mediated protoplast transformation technology. Finally, neomycin resistance was used to screen for revertant strains.

Melanin, penetration and pathogenicity tests
The colonies of the WT, AsCEP112 mutant and revertant strains having diameters greater than 5 mm were inoculated independently into 350-mL flasks containing 100 mL PDB medium, incubated at 25 °C and shaken at 150 rpm min⁻¹ for 5 d. Then, the media of the WT, AsCEP112 mutant and revertant strains were filtered through gauze, and the absorbance levels of the solutions were measured at 400 nm.

Sterile cellophane was spread on the PDA medium, and then, the WT, deletion mutant and revertant strains were placed on the PDA medium and incubated at 25 °C for 3 d in the dark. Then, the cellophane was removed and the strains were cultured for 4–5 d.

Alternaria solani strain HWC-168 was inoculated into potato agar medium. After the hyphae were overgrown, mechanical damage was performed, and sporulation was stimulated by ultraviolet light for 15 min. Spore suspensions of A. solani WT, AsCEP112 mutant and revertant strains were prepared. The final spore concentrations were adjusted to 10⁵ mL⁻¹ per suspension. Five-week-old potato leaves were sterilized with 70% alcohol, washed with sterile water, and then, the petioles were inserted into water agar medium. Approximately 20 µL of each suspension was inoculated into potato leaves in vitro. The leaves were incubated at 25 °C under a 16-h photoperiod. The accumulation of H₂O₂ was detected by DAB staining [32]. In vitro potato leaves were harvested at 5 dpi and immersed in DAB solution (CWBIO) at room temperature. After 12 h, the leaves were discolored and photographed by boiling them in 95% ethanol for 30 min. The tests were conducted in three replicates.

Results
Bioinformatics identification of AsCEP112
By comparing the genome and transcriptome information of A. solani, we identified the AsCEP112 gene, which had effector characteristics, and analyzed its function. The signal peptide of the AsCEP112 gene corresponded to amino acids (aa) 1 to 18, and no signal domain was identified using online tools (Supplementary Table S1). To determine the phylogenetic relationship of the identified AsCEP112, the amino acid sequences of complete proteins were aligned in NCBI using MEGA, and a Maximum-Likelihood phylogenetic tree was constructed (Supplementary Figure S1). However, no CEP112 homologs were found in genera other than Alternaria. The results indicated that the AsCEP112 sequence was more closely related to other Alternaria spp. Thus, it may have formed by vertical gene transfer in A. solani.

Expression patterns of AsCEP112 in the infection of A. solani
To identify the role of AsCEP112 in the infection of A. solani, its temporal expression pattern was assessed by qRT-PCR of infected potato leaves compared with mycelia. The expression of AsCEP112 in the infection stage continued to increase compared with in the hyphal stage and the log₂ fold change of AsCEP112 expression was up-regulated by 13.18 fold at 72 hpi (Fig. 1).

Subcellular localization of AsCEP112 in N. benthamiana
SignalP 5.0 predicted the N-terminal 18 amino acids of AsCEP112 as a specific signal peptide sequence [33]. The secretory function of the putative signal peptide was confirmed using a yeast invertase secretion assay. When the predicted signal peptide of AsCEP112 was fused to the yeast invertase sequence in the vector pSUC2, it mediated the complementation of yeast YTK12 mutant strains (invertase-deficient) grown on raffinose or YPRA agar. These results indicated that the signal peptide of AsCEP112 had a secretory function (Supplementary Fig. S2).

Secreted effector proteins enter different host cell locations depending on their roles. Effector proteins regulate functions by targeting different organelles and ultimately destroy host defense signaling, which increases susceptibility [34]. When fungal effectors are secreted, they function either in the apoplastic spaces or inside host cells after translocation. Therefore, to detect the localization of AsCEP112 in the host, we performed a subcellular localization assay with the green fluorescent protein (GFP) fusion. It indicated that
the overexpressed AsCEP112-NSP (lacking the N-terminal SP) in *N. benthamiana* eventually acted on the membranes of host cells (Fig. 2).

**Fig. 1** Expression patterns of the AsCEP112 gene during the infection of potato leaves. The expression level at the hyphal stage was used as a control to analyze the specific expression at the infection stage. Actin expression was used as an internal reference for normalizing within the samples. Student’s t-test was used as a significance test of differences (n = 6). *, indicates a difference (0.01 < p < 0.05) compared with the control (hyphal stage); **, indicates a significant difference (0.001 < p < 0.01) compared with the control; ***, indicates an extremely significant difference (p < 0.001) compared with the control. Bars represent standard deviations (SDs).

**Fig. 2** Subcellular localization of AsCEP112 proteins in *N. benthamiana*. The Agrobacterium strain EHA105 containing the pCAMBIA1301 vector, as the control, and the AsCEP112 gene were independently transiently expressed in *N. benthamiana* leaves. Bar = 20 μm.

AsCEP112 promotes chlorosis of *N. benthamiana* and Tomato
Fungal pathogens manipulate host immune responses
to promote infection by secreting specific effector proteins. To determine whether AsCEP112 had the function of inducing host-cell death, suppressing host-immune response or promoting host-cell death, we performed transient expression assays in *N. benthamiana*. As the positive control, INF1, which induces programmed cell death in *N. benthamiana*, was used.

When AsCEP112 (FL and NSP) was injected alone, the leaves showed no symptoms of cell death. When INF1 was co-injected with AsCEP112 (FL and NSP), host immune responses were not suppressed (Supplementary Fig. S3). In addition, we determined whether AsCEP112 promoted infection. The *N. benthamiana* leaves infiltrated with *Agrobacterium* AsCEP112-NSP alone showed chlorotic symptoms. At the same time, we also repeated this experiment in tomato plant leaves, and found that *Agrobacterium* AsCEP112-NSP also caused chlorotic symptoms on tomato leaves. In addition, we investigated the ability of AsCEP112-NSP to activate hydrogen peroxide (H$_2$O$_2$) in *N. benthamiana* and tomato leaves. Compared with the control, H$_2$O$_2$ was activated by AsCEP112-NSP at 5 dpi (Fig. 3).

Many genes that regulate plant growth during senescence are defined as senescence-related genes [35]. The transcription levels of three senescence-related genes (*SEN4, SAG12* and *DHAR1*) were independently determined in the *N. benthamiana* leaves infiltrated with EV and AsCEP112-NSP. After treatment with ASCEP112-NSP, the expression levels of senescence-related genes were significantly up-regulated compared with after the EV treatment. *SAG12* expression was upregulated the most, being 14.16-fold higher than in the EV treatment. These results suggested that AsCEP112 induces plant chlorotic symptoms by regulating senescence-related genes.

**Construction and confirmation of AsCEP112 deletion and revertant strains**

Homologous recombination technology was used to obtain AsCEP112 deletion mutants through PEG-mediated transformation of protoplasts. Hyg-specific primers were used to verify the mutant strain by RT-PCR. A band was detected in the transformants, but no band was detected in the WT strains (Fig. 4). Thus, AsCEP112 was replaced by a single copy of Hyg in the genomic DNA of *A. solani*. In this study, two knockout mutants of AsCEP112 were obtained.

In the same manner, the Hyg gene in each AsCEP112 deletion strain was replaced with the AsCEP112 gene to obtain two corresponding revertant strains. AsCEP112 gene-specific primers were used to detect mutant strains (Supplementary Fig. S4).

**AsCEP112 affects colony melanin and penetration, but not the growth and sporulation rates**

To verify the role of AsCEP112 in the growth of *A. solani*, we compared the morphology, growth rate, sporulation rate and penetration of the WT, AsCEP112 mutant and revertant strains. The colony growth and sporulation rates of the WT, AsCEP112 mutant and revertant strains were roughly the same (Supplementary Fig. S5).

The melanin area in the inner circles of the AsCEP112 mutant strain colonies were smaller compared with those...
Fig. 4 AsCEP112 gene replacement and RT-PCR screening of mutants. A Diagram of the homologous recombination of AsCEP112 and Hyg genes. B Verification of the Hyg gene in the genomes of WT and AsCEP112 mutant strains.

Fig. 5 Determination of the phenotypes and melanin levels of the wild-type, AsCEP112 mutant and revertant strains. A The solutions of wild-type, AsCEP112 mutant and revertant strains for the determination of the melanin contents. B The absorbance values of the wild-type, AsCEP112 mutant and revertant strain solutions at 400 nm. *, indicates a difference ($p < 0.05$) compared with the control (HWC-168); ***, indicates an extremely significant difference ($p < 0.001$) compared with the control (HWC-168). Bars represent standard deviations (SDs).

Fig. 6 Penetration of the WT, AsCEP112 mutant and revertant strains. The colony morphology of wild-type, AsCEP112 mutant and revertant strains filtered through three layers of sterile cellophane.
of the WT and revertant strains (Supplementary Fig. S6). Therefore, the melanin content of the WT, AsCEP112 mutant and revertant strains were determined. The solutions of the AsCEP112 mutant strains were discolored. After measuring the OD400 of the solutions, it was determined that the absorbance levels of the AsCEP112 mutant strains decreased significantly compared with those of the other strains (Fig. 5).

A comparison of the mechanical penetrations of the WT, AsCEP112 mutant and revertant strains revealed that when three layers of cellophane were spread on the PDA medium, the cellophane was penetrated by the WT and the revertant strains. However, the AsCEP112 mutant strains did not penetrate the cellophane although they left melanin on the PDA medium (Fig. 6). The results indicated that the absence of AsCEP112 reduced the penetration capability of A. solani.

**Susceptibility of the AsCEP112 knockout mutants to pathogenicity**

To determine whether the deletion of AsCEP112 contributed to the full pathogenicity of A. solani, the isolated leaves of potato were inoculated with the spore suspensions of the WT, AsCEP112 mutant and revertant strains. The average diameter of the lesions of the AsCEP112 mutant strain was approximately 62.86% that of the WT strain. The average diameter of lesions of the WT strain was approximately 0.99 cm. The average diameters of lesions of the mutant strains were approximately 0.61 and 0.64 cm, and the average diameters of lesions of the revertant strains were approximately 0.94 and 0.98 cm. Compared with the WT and revertant strains, the AsCEP112 mutant strains produced smaller lesion areas, and no chlorotic symptoms were obvious on the leaves. Moreover, after the leaves were stained with DAB solution, the accumulations of H2O2 at the inoculation sites of the AsCEP112 mutant strains were less than those of the WT and revertant strains (Fig. 7). Thus, the deletion of AsCEP112 caused a decrease in the pathogenicity of A. solani infecting potato leaves. The statistical analysis showed a significant difference (p < 0.001) in the sizes of the lesions produced by WT and mutant strains.

**Discussion**

*Alternaria solani* is a kind of necrotrophic pathogen that can cause early blight disease of tomato, potato, tobacco, and many other vegetables and crops, and lead to huge losses in agricultural production [36]. The changes in the JA- and SA-dependent defenses pathways in the host during an *A. solani* infection are different from those that occur during common necrotroph infections [37]. To date, no in-depth studies on the factors released by *A. solani* during the potato infection process have been reported. Thus, it is urgent to explore the pathogenic mechanism of *Alternaria solani* involved in infecting potatoes.

Specific effectors are expressed during different pathogenic stages, indicating that specific effectors play critical roles in the corresponding infection stages [38]. The expression of the AsCEP112 gene continued to increase for 72 h after *A. solani* infected the potato. The first 72 h of *A. solani* infection is also the most intense stage of host-cell damage. *Alternaria solani* invades host cells through hyphae by 8 hpi, and then destroys the host cells by 24 hpi. At 72 hpi, the host cells are basically eliminated [39]. The changes in host cells during the entire infection process corresponded to the

![Fig. 7 Detecting the pathogenicity of the AsCEP112 gene.](image-url)
changes in AsCEP112 expression. Additionally, the expression levels of defense-related genes and defense-related enzymes (SOD, POD and PAL) in the host also increase significantly in 72 h, indicating that the substances secreted by A. solani cause serious damage to defense-related responses of the host [40]. The change in the expression of AsCEP112 probably indicates that it plays a crucial pathogenic function in the A. solani infection process.

Phytopathogen effectors are usually overexpressed in plants to induce phenotypes, reflecting their virulence activity levels. Effectors with different functions may play roles in inhibiting host-cell death or inducing host-cell death during the infection [41]. Sclerotinia sclerotiorum, a typical necrotrophic pathogen, induces significant cell death by secreting the SscP1 effector, and it inhibits the host immune responses by secreting the SsITL effector [42, 43]. Using the yeast signal trap assay system, we found that the AsCEP112 signal peptide was fused to the invertase gene, resulting in the secretion of invertase by yeast. This indicates that AsCEP112 was probably secreted into the extracellular space during host plant infection. For the transient expression of AsCEP112, there were no symptoms of inducing or inhibiting cell death. The expression symptoms and fluorescence quantitative detection of AsCEP112 in N. benthamiana and tomato leaves showed that AsCEP112 promoted leaf chlorosis by regulating host senescence-related genes. As a main host defense line against pathogens, the cell membrane has many recognition receptors, including receptor-like kinases and proteins. The pattern recognition receptors located on the cell membrane play an important role in this process [44, 45]. The localization of AsCEP112 on the cell membrane indicated that AsCEP112 tends to mimic pathogen-associated molecular patterns rather than specific domains. The disruption of an important defense pathway between plant cell membranes and chloroplasts ultimately disturbs SA-dependent defences, and intact SA signaling is required for potato defenses against the necrotrophic pathogen A. solani [37, 46]. These results further strengthen the research value of the AsCEP112 gene located on the cell membrane.

Melanin is ubiquitous in fungi, and it greatly improves the tolerance of fungi to the external environment, including ultraviolet radiation, enzymatic hydrolysis and extreme temperatures [47, 48]. Although the production of melanin is not necessary for pathogenic fungi, it has a protective function against external stress [49–51]. The deletion of AsCEP112 reduced the melanin areas of the A. solani mutant strains. In addition, the disease incidence on detached leaves of inoculated potato showed that the absence of AsCEP112 also decreased the incidence area. CfEc92 of Colletotrichum fructicola, F12 of Fusarium graminearum and AvrPtoB of Pseudomonas all play important roles in promoting the virulence of the pathogen itself [52–54]. The transient expression of AsCEP112 and the determination of the phenotype and pathogenicity of the AsCEP112 mutant strain, confirmed the importance of AsCEP112 in the A. solani infection process. It confirmed that we should further study the pathogenic mechanism of AsCEP112 in host plants.

Conclusion

In summary, we investigated gene functions of AsCEP112, a candidate effector selected from A. solani. The AsCEP112 gene was highly expressed during potato infection and promoted host leaf chlorosis by regulating host senescence-related genes. The AsCEP112 mutant strains reduced melanin production, penetration and ultimately host virulence. However, the molecular mechanism of AsCEP112 in the host remains unclear. We will explore further the role of chlorosis caused by AsCEP112 in infection and possible targets in the potato host to elucidate the molecular basis of the interaction between this necrotrophic pathogen and its host.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03845-w.
Acknowledgements
We thank the anonymous reviewers for constructive suggestions that helped to improve the manuscript.

Authors’ contributions
C.W. J.W and J.Z. designed the project. C.W. performed the experiments with help from D. Z. J.Z. and Z.Y. supervised the project. All authors contributed to the article and approved the submitted version.

Funding
This work was financially supported by China Agriculture Research System of MOF and MARA (CARS-09-P18), Hebei key research and development program (21326515D).

Availability of data and materials
All datasets generated for this study are included in the manuscript/Supplementary Files. (Accession number of gene sequence: NCB1 OM735616) (https://www.ncbi.nlm.nih.gov/).

Declarations

Ethics approval and consent to participate
Not applicable. All experimental studies on plants were compiled with relevant institutional, national, and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

Author details
1 College of Plant Protection, Hebei Agricultural University, Baoding 071001, People’s Republic of China. 2 Technological Innovation Center for Biological Control of Crop Diseases and Insect Pests of Hebei Province, Baoding 071001, People’s Republic of China.

Received: 2 February 2022 Accepted: 12 September 2022 Published online: 29 September 2022

References
1. Nehela Y, Taha NA, Elzaawely AA, et al. Benzonic Acid and Its Hydroxylated Derivatives Suppress Early Blight of Tomato (Alternaria solani) via the Induction of Salicylic Acid-Biosynthetic and Enzymatic and Nonenzymatic Antioxidant Defense Machinery. J Fungi (Basel). 2021;7(6):663.
2. Biliaev DV, Yuonieva NO, Tereshonok DV, et al. High Resistance of Tomato to Early Blight Is Achieved by Expression of the Pro-SmAMP1 Gene for S. marcescens. Plant Mol Biol. 2021;10(7):1395.
3. Adhikari P, Oh Y, Pantheer V, et al. Current Status of Early Blight Resistance in Tomato: An Update. Int J Mol Sci. 2017;18(10):2019.
4. González-García Y, Cadenas-Pliego G, Alpuche-Solís ÁG, Cabrera RI, Juárez-Maldonado A. Carbon Nanotubes Decrease the Negative Impact of Alternaria solani in Tomato Crop. Nanomaterials (Basel). 2021;11(5):1080.
5. Atta MS, El-Sayyd GS, Abd Elkodous M, El-Batal AI. The effective antagonistic potential of plant growth-promoting rhizobacteria against Alternaria solani—causing early blight disease in tomato plant. Sci Hortic. 2020;266:101928.
6. Govrin EM, Rachmilevitch S, Tiwari BS, Solomon M, Levine A. An Elicitor from Botrytis cinerea Induces the Hypersensitive Response in Arabidopsis thaliana and Other Plants and Promotes the Gray Mold Disease. Phytopathology. 2006;96(3):299–307.
7. van Kan JA. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 2006;11(5):247–53.
8. Mengiste T. Plant immunity to necrofactors. Annu Rev Phytopathol. 2012;50:267–94.
9. Faris JD, Zhang Z, Lu H, Lu S, Reddy L, Cloutier S, Fellers JR, Meinhardt SW, Rasmussen JB, Xu SS, et al. A unique wheat disease-resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. Proc Natl Acad Sci U S A. 2010;107(30):15344–9.
10. Dickman MJ, Flurh R. Centrality of host cell death in plant-microbe interactions. Annu Rev Phytopathol. 2013;51:543–70.
11. Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagiy E, Egusa M, Yamamoto M, Otani H. Host-selective toxins produced by the plant pathogenic fungus Alternaria alternata. FEMS Microbiol Rev. 2013;37(1):44–66.
12. Wolpert JT, Dunkle LD, Cuffeit L. M. Host-selective toxins and avirulence determinants: what’s in a name? Annu Rev Phytopathol. 2002;40:251–85.
13. Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol. 2005;43:205–27.
14. Robert-Stephan I, Grant M, Jones JD. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annu Rev Phytopathol. 2011;49:31–43.
15. Tang M, St. Améne A, Wang S, Qiu S, Zheng X, Zhang J, Bhdaviaur V, Sun W. Versatile effectors of phytopathogenic fungi target host immunity. J Integr Plant Biol. 2021;63(11):1856–73.
16. Angulo C, de la Leya M, Finetti I, Lépiz-Cruz J, Fernández-Crespo E, García-Agüstein G, González-Bosch C. Role of dihydrogenase o-DQO and SA in basal resistance and in hexanoyl acid-induced resistance of tomato (Solanum lycopersicum) plants against Botrytis cinerea. J Plant Physiol. 2015;175:163–73.
17. Novakova M, Sasek V, Dobrev P, Valenta O, Burketo L. Plant hormones in defense response of Brassica napus to Sclerotinia sclerotiorum—revisiting the role of salicylic acid in the interaction with a necrotroph. Plant Physiol Biochem. 2014;80:308–17.
18. Tanigawara M, Mateen A, Wang S, Qi S, Zheng X, Zhang J, Bhdaviaur V, Sun W. Versatile effectors of phytopathogenic fungi target host immunity. J Integr Plant Biol. 2021;63(11):1856–73.
19. Torres MF, Cuadros DF, Vaillancourt LJ. Evidence for a diffusible factor that induces susceptibility in the Colletotrichum-maize disease interaction. Mol Plant Pathol. 2014;15(1):80–93.
20. Liu Z, Zhang Z, Faris JD, Oliver RP, Syme R, McDonald MC, McDonald BA, Solomon PS, Lu S, Sheller WL, et al. The cysteine rich necrotrophic effector SnTox1 produced by Stagonospora nodorum triggers susceptibility of wheat lines harboring Sm1n. PLoS Pathog. 2012;8(1):e1002467.
21. Cook DE, Maraisch CH, Thomma BP. Understanding plant immunity as a surveillance system to detect invasion. Annu Rev Phytopathol. 2015;53:541–63.
22. Lo Presti L, Lanver D, Schweizer G, Tanaka S, Liang L, Tollot M, Zuccaro A, Reissmann S, Kahmann R. Fungal effectors and plant susceptibility. Annu Rev Plant Biol. 2015;66:513–45.
23. Stergiopoulos I, de Wet PJ. Fungal effector proteins. Annu Rev Phytopathol. 2009;47:233–63.
24. Lai Z, Mengiste T. Genetic and cellular mechanisms regulating plant responses to necrotrophic pathogens. Curr Opin Plant Biol. 2013;16(4):505–12.
25. Wolters PJ, Faino L, McDonald BA, McDonald MC, Bouwmeester K, Govers F. Alternaria solani to Early Blight Is Achieved by Expression of the Pro-SmAMP1 Gene for S. marcescens. Plant Mol Biol. 2018;19(8):1956–70.
26. Liu D, Sand L, Han C, Yu J, Li D, Zhang Y. Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. PLoS One. 2012;7(9):e46451.
27. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45.
28. Du Y, Mipina MH, Birch PR, Bouwmeester K, Govers F. Phytophthora infestans RKLX Effector AvR1 Interacts with Exocyst Component Sec5 to Manipulate Plant Immunity. Plant Physiol. 2015;169(3):1975–90.
29. Shih PR, Chou SJ, Muller C, Hakier BA, Deenen R, Lai EM. Differential roles of glucosinolates and camalexin at different stages of Agrobacterium-mediated transformation. Mol Plant Pathol. 2018;19(8):1956–70.
30. Krenke P, Samajova O, Luptovciak I, Doskocilova A, Komis G, Samaj J. Transient plant transformation mediated by Agrobacterium tumefaciens: Principles, methods and applications. Biotechnol Adv. 2015;33(6):Pt 2:1024–42.
31. Kamoun S, van West PAA. Qualitative and quantitative resistance against early blight introgressed in potato. Biology. 2021;10(9):892.
32. Liu D, Shi L, Han C, Yu J, Li D, Zhang Y. Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. PLoS One. 2012;7(9):e46451.
33. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45.
34. Du Y, Mipina MH, Birch PR, Bouwmeester K, Govers F. Phytophthora infestans RKLX Effector AvR1 Interacts with Exocyst Component Sec5 to Manipulate Plant Immunity. Plant Physiol. 2015;169(3):1975–90.
32. Chen W, Li Y, Yan R, Xu L, Ren L, Liu F, Zeng L, Yang H, Chi P, Wang X, et al. Identification and Characterization of Plasmodiophora brassicae Primary Infection Effector Candidates that Suppress or Induce Cell Death in Host and Nonhost Plants. Phytopathology. 2019;109(10):1689–97.
33. Liu L, Wang Z, Li J, Wang Y, Yuan J, Zhan J, Wang P, Lin Y, Li F, Ge X. Verticillium dahliae secreted protein Vd424Y is required for full virulence, targets the nucleus of plant cells, and induces cell death. Mol Plant Pathol. 2021;22(9):1109–20.
34. Gong A-d, Jing Z-y, Zhang K, Tan Q-q, Wang G-l, Liu W-b. Bioinformatic analysis and functional characterization of the CFEM proteins in maize anthracnose-fungus Colletotrichum graminicola. J Integr Agric. 2019;18(2):541–50.
35. Jiang Y, Liang G, Yang S, Yu D. Arabidopsis WRKY57 functions as a node of convergence for jasmonic acid- and auxin-mediated signaling in jasmonic acid-induced leaf senescence. Plant Cell. 2014;26(1):230–45.
36. Zhang D, Yu S, Yang Y, Zhang J, Zhao D, Pan Y, Fan S, Yang Z, Zhu J. Anti-fungal effects of volatiles produced by bacillus subtilis against Alternaria solani in potato. Front Microbiol. 2020;11:1196.
37. Brouwer SW, Odelaye F, Bunra DD, Lennman M, Hedley PE, Grenville-Briggs L, Alexandersson E, Liljeroth E, Andreasson E. Intact salicylic acid signalling is required for potato defence against the necrotrophic fungus Alternaria solani. Plant Mol Biol. 2020;104(1–2):1–19.
38. Toruno TY, Stergiopoulos I, Coaker G. Plant-pathogen effectors: cellular probes interfering with plant defenses in spatial and temporal manners. Annu Rev Phytopathol. 2016;54:419–41.
39. Dita MA, Brommonschenkel SH, Matsuoka K, Mizubuti ESG. Histo-pathological study of the Alternaria solani infection process in potato cultivars with different levels of early blight resistance. J Phytopathol. 2007;155(7–8):462–9.
40. Song W, Ma X, Tan H, Zhou J. Abscisic acid enhances resistance to Alternaria solani in tomato seedlings. Plant Physiol Biochem. 2011;49(7):693–700.
41. Wang Q, Han C, Ferreira AO, Yu X, Ye W, Tripathy S, Kale SD, Gu B, Sheng Y, Sui Y, et al. Transcriptional programming and functional interactions within the Phytophthora sojae RXLR effector repertoire. Plant Cell. 2011;23(6):2064–86.
42. Zhu W, Wei W, Fu Y, Cheng J, Xie J, Li G, Yi X, Xiang Z, Dickman MB, Jiang D. A secretory protein of necrotrophic fungus Sclerotinia sclerotiorum that suppresses host resistance. PLoS One. 2013;8(1):e53901.
43. Yang G, Tang L, Gong Y, Xie J, Fu Y, Jiang D, Li G, Collinge DB, Chen W, Cheng J. A cerato-platanin protein ScsCP1 targets plant PR1 and contributes to virulence of Sclerotinia sclerotiorum. New Phytol. 2018;217(2):739–55.
44. Zhang H, Chen C, Li L, Tan X, Wei Z, Li Y, Li J, Yan F, Chen J, Sun Z. A rice LRR receptor-like protein associates with its adaptor kinase OsSOBIR1 to mediate plant immunity against viral infection. Plant Biotechnol J. 2021;19(2):541–50.
45. Albert I, Hua C, Numbereger T, Pruitt RN, Zhang L. Surface sensor systems in plant immunity. Plant Physiol. 2020;182(4):1582–96.
46. Medina-Puche L, Tan H, Dogra V, Wu M, Rosas-Diaz T, Wang L, Ding X, Zhang D, Fu X, Kim C, et al. A defense pathway linking plasma membrane and chloroplasts and co-opted by pathogens. Cell. 2020;182(5):1109–24 e112.
47. Cho Y, Srivastava A, Ohm RA, Lawrence CB, Wang KH, Grigoriev IV, Marahatta SP. Transcription factor Amr1 induces melanin biosynthesis and suppresses virulence in Alternaria brassicicola. PLoS Pathog. 2012;8(10):e1002974.
48. Fetzner R, Seither K, Wenderoth M, Herr A, Fischer R. Alternaria alternata transcription factor CmrA controls melanization and spor development. Microbiology (Reading). 2014;160(Pt 9):1845–54.
49. Zeng F, Meng Y, Hao Z, Li F, Zhai W, Shen S, Cao Z, Dong J. Setosphaeria turcica ATR turns off appressorium-mediated maize infection and triggers melanin-involved self-protection in response to genotoxic stress. Mol Plant Pathol. 2020;22(3):401–14.
50. Zhu S, Yan Y, Qu Y, Wang J, Feng X, Liu X, Lin F, Lu J. Role refinement of melanin synthesis genes by gene knockout reveals their functional diversity in Pyricularia oryzae strains. Microbiol Res. 2021;242:126620.
51. Krishnan P, Meile L, Pliosonneau C, Ma X, Hartmann FE, Croll D, McDonald BA, Sanchez-Vallet A. Transposable element insertions shape gene regulation and melanin production in a fungal pathogen of wheat. BMC Biol. 2018;16(1):78.