Phytophthora capsici CBM1-containing protein CBP3 is an apoplastic effector with plant immunity-inducing activity

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Funding information
Fundamental Research Funds for the Central Universities, Grant/Award Number: KYLH201703; National Natural Science Foundation of China, Grant/Award Number: 32072507

Abstract
Carbohydrate-binding module family 1 (CBM1) is a cellulose-binding domain that is almost exclusively found in fungi and oomycetes. CBM1-containing proteins (CBPs) have diverse domain architectures and play pivotal roles in the plant–microbe interaction. However, only a few CBPs have been functionally investigated. In this study, we identified PcCBP3 in an oomycete pathogen, Phytophthora capsici. PcCBP3 contains two tandem CBM1 domains and its orthologs from other Phytophthora species exhibit diversity including gene loss, pseudogenization, variations in sequences, and domain structures. PcCBP3 is upregulated during infection and knockout of PcCBP3 results in significantly decreased virulence. Moreover, PcCBP3 requires signal peptide to induce BAK1-dependent cell death in Nicotiana benthamiana. Further studies indicate that PcCBP3-triggered cell death and plant immunity require its N-terminal region, which is conserved in CBM1-containing proteins and other small, secreted, cysteine-rich protein from oomycetes. These results suggest that PcCBP3 is an apoplastic effector and could be perceived by the plant immune system.

KEYWORDS
BAK1, cellulose binding domain, oomycete pathogen, pathogen-associated molecular pattern

1 | INTRODUCTION

Carbohydrate-binding modules (CBMs) play a central role in enzymic degradation of insoluble polysaccharides, such as components of plant cell walls. The noncatalytic CBM domains can promote the association of the enzymes with insoluble substrates (Boraston et al., 2004). CBMs are grouped into 87 families in the carbohydrate-active enZymes (CAZy) database (Lombard et al., 2014), among which the CBM1 family with cellulose-binding function is found almost exclusively in fungi and oomycetes (Larroque et al., 2012). Most of the fungal CBM1-containing proteins (CBPs) contain one CBM1 appended to a catalytic domain, whereas oomycete CBPs generally contain multiple CBM1s without a catalytic domain (Larroque et al., 2012). The distinct domain architectures of oomycete CBPs suggest their different roles and evolution.

The number of CBPs in plant-interacting microbes with necrotrophic or hemibiotrophic lifestyles is remarkably large compared with biotrophs and saprophytes (Larroque et al., 2012), suggesting that CBPs are involved in plant–microbe interaction. For instance, the swollenin gene from Trichoderma reesei encodes a CBP that contains an N-terminal CBM1 and C-terminal expansin-like domain. The swollenin-silenced transformants show reduced ability to colonize...
domains and triggers BAK1-dependent cell death in P. capsici (et al., 2015; Lamour et al., 2012b). Moreover, phytopathogens and infects a wide range of crop plants (Kamoun et al., 2013). Well-known examples of MAMPs include bacterial flagellin (Gomez-Gomez & Boller, 2000), fungal chitin (Eckardt, 2008), oomycete INF1 (Kamoun et al., 1997), and XEG1 (Ma et al., 2015). Intriguingly, all the four CBPs mentioned above are also recognized by plants as MAMPs (Gaulin et al., 2006; Brotman et al., 2008; Gui et al., 2017, 2018), reflecting their important roles during plant-microbe interactions. Oomycete CBPs have variable domain architectures, whereas only CBEL from Phytophthora parasitica has been functionally determined.

Phytophthora capsici is one of the most notorious oomycete phytopathogens and infects a wide range of crop plants (Kamoun et al., 2015; Lamour et al., 2012b). Moreover, P. capsici also infects the model plants Nicotiana benthamiana and Arabidopsis thaliana, presenting a model pathogen for understanding Phytophthora-plant interactions (Lamour et al., 2012b; Wang et al., 2013). In this study, we identified a virulence-essential PcCBP3 that contains two CBM1 domains and triggers cell death in N. benthamiana. The Ser24 site at the conserved N-terminal region is essential for PcCBP3-induced cell death.

2 | RESULTS

2.1 | PcCBP3 is a CBM1-containing protein that triggers cell death in N. benthamiana

To identify the potential CBM1-containing proteins in P. capsici, we searched its genome sequence (Lamour et al., 2012a) using the hmmsearch program, and obtained nine candidates containing CBM1 domains (Table S1). Among them, one shares 88.4% protein sequence similarity with PpCBEL, a known Phytophthora MAMP (Gaulin et al., 2006). The eight others exhibit different domain architectures and were named as PcCBP1 to PcCBP8 accordingly (Figure 1a). All the nine CBPs contain a N-terminal signal peptide, suggesting that they are probably secreted proteins. Intriguingly, all of them contain no catalytic domain, which is distinct from most of the fungal CBPs. PcCBPs possess different numbers of CBM1 domains that are separated by low-complexity regions.

Previous reported CBPs such as PpCBEL, VdCUT11, and VdEG3 could activate plant immunity and trigger cell death in N. benthamiana (Gaulin et al., 2006; Gui et al., 2017, 2018). Therefore, to reveal roles of P. capsici CBPs during interactions with plants, all the nine CBPs were cloned from P. capsici strain LT263 and transiently expressed in N. benthamiana by agroinfiltration. PcCBP3 induced cell death in N. benthamiana leaves, whereas all other CBPs failed to trigger cell death (Figure 1c). Immunoblot assays showed that all the CBP proteins were normally expressed in N. benthamiana (Figure 1d). Interestingly, we noticed that PcCBEL could not induce cell death and its activity was distinct from its close homolog from P. parasitica, PpCBEL (Gaulin et al., 2006). We inferred that the sequence divergence may possibly account for the difference of these two proteins (Figure S1). Therefore, we focused on PcCBP3 for further study.

2.2 | CBP3 is moderately conserved in Phytophthora species

We investigated PcCBP3 orthologs from 37 Phytophthora species with available genome sequences. In total, 24 CBP3 orthologs were identified in 23 species (Figure 2a). Ten species lacked a CBP3 ortholog and three species had only pseudogenes. Moreover, the 24 CBP3 orthologs were divided into six variants based on the domain architecture (Figure 2b and Table S2). Most of these orthologs showed a similar domain architecture to PcCBP3. Multiple sequence alignment of these orthologs showed that the CBM1 domain is highly conserved with >90% similarity, whereas the region linking two CB1Ms is highly variable with only 38.94% similarity (Figure 2c). Transient expression of four CBP3 orthologs from Phytophthora species that cause major crop diseases led to the identification of PpCBP3 from P. parasitica that also induced cell death in N. benthamiana, while PsCBP3, PiCBP3, and PcmCBP3 did not (Figure 2d). Immunoblot analysis showed that all the proteins were expressed in N. benthamiana leaves (Figure 2e). These results suggest that CBP3 from different Phytophthora species are divergent and probably have different roles in the Phytophthora-plant interaction. Interestingly, we found that PcCBP3 and PpCBP3 showed similar western blot bands that contained ladder-like larger bands, whereas PsCBP3, PiCBP3, and PcmCBP3 did not have these larger bands (Figure 2e).

2.3 | PcCBP3 is a virulence factor of P. capsici

To determine the function of PcCBP3 in the P. capsici virulence, CRISPR/Cas9-mediated genome editing was performed to construct PcCBP3 knockout lines (Figure 3a). PCR detection identified two knockout mutants (ΔPcCBP3-2 and ΔPcCBP3-4) (Figure 3b). In addition, a negative transformant (NT) was selected as a control because PcCBP3 was intact (Figure 3b). Knockout of PcCBP3 did not affect the growth and mycelial morphological characteristics of P. capsici. The virulence of knockout mutants was evaluated on N. benthamiana. The results showed that the average lesion sizes caused by both ΔPcCBP3-2 and ΔPcCBP3-4 were significantly smaller than that caused by NT (Figure 3c,d). Moreover,
quantitative reverse transcription PCR (RT-qPCR) analysis showed that PcCBP3 transcripts were induced at the early stage during infection of N. benthamiana (Figure 3e). Previously reported transcriptomes of P. capsici–A. thaliana interaction (Ma et al., 2018) were also used to examine the expression profile of PcCBP3. The data showed that PcCBP3 was upregulated during infection with A. thaliana (Figure S2). Among the nine CBP genes of P. capsici, seven genes were induced during interaction with the host plant (Figure S2), suggesting their potential important roles in virulence. These results indicate that PcCBP3 is required for virulence during infection.

2.4 | PcCBP3 is an apoplastic protein and induces BAK1-dependent cell death

To determine whether PcCBP3 possess a functional signal peptide (SP), we fused the SP to yeast invertase using the yeast secretion system. SP<sup>PCBP3</sup> and the positive control SP<sup>Avr1b</sup> could lead to the secretion of invertase, which reduced triphenyltetrazolium chloride (TTC) to red formazan. In contrast, no colour change was observed for negative control SP<sup>Mgk7</sup> or empty vector (Figure 4a). Moreover, deletion of SP<sup>PCBP3</sup> failed to induce cell death in N. benthamiana. VdEG3 is an apoplastic CBP whose signal peptide is required for cell death-inducing activity (Gui et al., 2017). We thus replaced the signal peptide of VdEG3 by SP<sup>PCBP3</sup>. As shown in Figure 4b, deletion of SP<sup>VdEG3</sup> abolished VdEG3-induced cell death, which could be rescued by SP<sup>PCBP3</sup>. Immunoblot analysis showed that all the proteins were expressed in N. benthamiana leaves (Figure 4c). Furthermore, we investigated the subcellular localization of mCherry tagged-PcCBP3 in N. benthamiana. The results showed that the mCherry signal was mainly observed at the cell edge (Figure 4d). To distinguish the mCherry signal of apoplast and plasma membrane, N. benthamiana cells were plasmolysed with 30% sucrose. PcCBP3 accumulated mainly in the apoplast region, as shown in Figure 4d. These results indicate that PcCBP3 is an apoplastic protein.

The plant cell surface-localized receptor-like kinases BAK1 and SOBIR1 are essential for many apoplastic effector-triggered cell death and/or immunity (Heese et al., 2007; Liebrand et al., 2013). To determine whether they are required for PcCBP3-induced cell death, NbBAK1 and NbSOBIR1 were silenced by tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS). The P. infestans elicitor
INF1 was used as positive control because INF1 induces cell death in a BAK1- and SOBIR1-dependent manner (Domazakis et al., 2018). PcCBP3 still triggered cell death in GFP- and SOBIR1-silenced plants (Figure 4e). However, silencing BAK1 completely abolished PcCBP3-triggered cell death (Figure 4e). Immunoblot analysis showed that PcCBP3 was expressed in VIGS N. benthamiana leaves (Figure 4f). The transcripts levels of BAK1 or SOBIR1 in BAK1- or SOBIR1-silenced plants was c.70% lower than that in GFP-silenced plants, which was confirmed by RT-qPCR analysis (Figure 4g). These results suggest that PcCBP3 acts in extracellular space and is perhaps perceived by a BAK1-dependent receptor complex.

Flexible larger bands are required for PcCBP3-induced cell death and plant resistance

Plants mount a series of defence responses after perception of apoplastic effectors (Yu et al., 2017). Consistently, lesion areas caused by P. capsici...
YIN et al. (Figure 5a,b) in N. benthamiana leaves pre-expressing PcCBP3 are significantly smaller than that pre-expressing empty vector. However, Escherichia coli-expressed PcCBP3 failed to induce cell death and promote plant resistance to *P. capsici* (Figure 5c,d). Immunoblot analysis showed that *E. coli*-expressed PcCBP3 had a single band (Figure 5e), lacking the larger bands present in *N. benthamiana*-expressed PcCBP3. Likewise, when PcCBP3 was expressed in *N. benthamiana* leaves without signal peptide, only a single band with the expected size could be detected (Figure 4c). PsCBP3, PICBP3, and PcmCBP3, which did not induce cell death, also lacked larger bands, as revealed by immunoblot analysis (Figure 2d,e). Furthermore, *E. coli*-expressed PcCBP3 did not induce reactive oxygen species (ROS) production in *N. benthamiana* (Figure 5f). These findings indicate that larger bands are indispensable for PcCBP3-triggered cell death and plant resistance.

We noticed that the larger bands of PcCBP3 were stable in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE),
even when the Laemmli sample buffer contained the reducing agent β-mercaptoethanol (Figures 1d, 2e and 4c). To explore the larger bands of PcCBP3, we first predicted potential N-linked glycosylation sites using the NetNGlyc server. However, no glycosylation site was predicted in PcCBP3. The ladder-like immunoblot bands resemble covalent dimer and polymer, which are also SDS-resistant (Xiang et al., 2015). To verify the self-association of PcCBP3, a coimmunoprecipitation (CoIP) assay was performed to assess PcCBP3 polymer formation in planta. Hemagglutinin (HA)-tagged and FLAG-tagged PcCBP3 were coexpressed in N. benthamiana leaves. However, HA-tagged PcCBP3 did not coimmunoprecipitate with FLAG-tagged PcCBP3 (Figure S3), suggesting that PcCBP3 does not associate with itself.
2.6 | Ser24 is required for cell death-inducing activity of PcCBP3

PcCBP3 is a small, secreted protein that contains two CBM1 domains and two small linkers L1 and L2 (Figure 6a). To map the region responsible for PcCBP3-induced cell death, four deletion mutants lacking CBM1 or linker were generated and expressed in N. benthamiana. As shown in Figure 6a, deletion of either CBM1 or L2 did not affect the death-inducing activity of PcCBP3, whereas deletion L1 abolished PcCBP3-induced cell death. Intriguingly, L1 mutant expressed in N. benthamiana leaves lacked the larger bands, while other mutants all contained larger bands confirmed by immunoblot
analysis (Figure 6b). We further generated a mutant without both CBM1 domains (SP + L1 + L2), which contains no cysteine but still induced cell death (Figure S4). However, a mutant containing only SP and L1 regions (SP + L1) failed to induce cell death (Figure S4). These findings indicate that CBM1 domains are not necessary for PcCBP3-induced cell death.

Because the L1 motif has unknown annotation in public databases, including Pfam and SMART, we examined the distribution of the L1 motif. Of the nine CBPs from P. capsici, PcCBP2, PcCBP3, and PcCBP7 contain an L1 motif after the signal peptide. To explore whether non-CBM1 proteins also contain an L1 motif, we performed Blastp searches on the GenBank database. In total, 19 proteins were identified from Phytophthora and other oomycete species (Figure S5). All of these are small, secreted cysteine-rich (SCR) proteins. Multiple sequence alignment of representative sequences showed that most of these proteins are unrelated (Figure S5). Based on the multiple sequence alignment of CBP3 orthologs, we generated point mutants in the conserved sites by site-directed mutagenesis. First, the four most conserved basic amino acids (H26, R28, H30, and K32) were changed to alanine. However, none of these mutants failed to induce cell death, whereas S31A did not affect the cell death-inducing activity (Figure 6c). Cell death was confirmed by viewing under UV light (Figure 6d). Immunoblot analysis showed that both S24A and S31A exhibited remarkable reduction of larger bands (Figure 6e). These findings suggest that Ser24 in the L1 motif is indispensable for the cell death-inducing activity of PcCBP3.

3 | DISCUSSION

CBM1 is a noncatalytic domain with cellulose-binding function. CBM1 is found almost exclusively in fungi and oomycetes (Larroque et al., 2012). The CBM1 domain contains four conserved cysteines that form two disulphide bonds and are required for cellulose-binding activity (Gilkes et al., 1991). CBPs are widely distributed in plant-interacting microbes, suggesting their important roles for pathogens (Larroque et al., 2012). The fungal pathogen V. dahliae contains 28 CBPs and at least two of them (VdCUT11 and VdEG3) are required for cotton infection and are also recognized by plants (Gui et al., 2017, 2018). In this study, nine CBPs were found in the oomycete phytopathogen P. capsici, among which PcCBP3 was shown to be involved in the P. capsici-plant interaction. PcCBP3 is required for virulence and triggers

FIGURE 6 Ser24 is required for cell death-inducing activity of PcCBP3. (a) Diagram of PcCBP3 deletion mutants. SP, signal peptide; L1, linker 1; L2, linker 2. Representative photographs showing the cell death-inducing activity are on the right. (b) Immunoblot analysis of PcCBP3 mutants expressed in Nicotiana benthamiana leaves. PcCBP3 mutants were tagged by C-half of luciferase (LUC). Ponceau S staining of Rubisco (RBC) was used to indicate the protein loading control. (c) Representative N. benthamiana leaves 3 days after transient expression the indicated genes by agroinfiltration. (d) The same leaves shown in (c) viewed under UV light. (e) Immunoblot analysis of PcCBP3 mutants expressed in N. benthamiana
plant cell death and resistance. Compared to the reported CBPs involved in plant–microbe interaction, PcCBP3 is a different CBP that consists of two tandem CBM1 domains.

Several CBPs are recognized by plants and activate plant resistance, including PcCBP3. However, their modes of action seem to be largely different. For swollenin from T. reesei and CBEL from *P. parasitica*, chemically synthesized CBM1 peptides are sufficient to induce defence responses (Brotman et al., 2008; Gaulin et al., 2006). VdCut11 probably activates plant immunity indirectly by degrading the plant cell wall because mutagenesis of its activity sites in the cutinase domain abolishes elicitor activity (Gui et al., 2018). A 63 amino acid region in the glycoside hydrolase domain of VdEG3 is sufficient for cell death-inducing activity (Gui et al., 2017). The region required for elicitor function of PcCBP3 was mapped to a c.10 amino acid linker after the signal peptide (L1 motif). However, the L1 motif is required but not sufficient for PcCBP3-induced cell death.

Posttranslational modification (PTM) of proteins is a versatile regulatory process that is pivotal for many pathogen effectors and plant immune signalling proteins (Tahir et al., 2019; Withers & Dong, 2017). Examples of PTMs are phosphorylation, ubiquitination, sumoylation, glycosylation, oligomerization, and proteolytic cleavage (Tahir et al., 2019). PTMs of some apoplastic effectors are also essential for their perception by plants. For instance, glycosylation of *P. sojae* XEG1 is dispensable because XEG1 is glycosylated when expressed in *N. benthamiana*, and *Pichia pastoris*-expressed but not *E. coli*-expressed XEG1 is active (Ma et al., 2015). The small cysteine-rich secreted protein PC2 from *P. infestans* is cleaved by apoplastic subtilisin-like proteases, which releases an immunogenic peptide and activates plant immunity (Wang et al., 2021). PcCBP3 might undergo PTM, as indicated by the ladder-like larger bands in immunoblot analysis, which is indispensable for triggering cell death and plant resistance. However, what kind of PTM is responsible for the larger bands of PcCBP3 remains obscure. The conserved L1 motif after the signal peptide was required for PcCBP3-induced cell death, and immunoblot analysis revealed that the ΔL1 mutant probably lacked these larger bands. By site-directed mutagenesis, the conserved Ser24 in the L1 motif was found to be required for the larger bands and indispensable for PcCBP3-induced cell death.

Besides different immunogenic regions of CBPs, the receptor-like kinases BAK1 and SOBIR1 also play different roles in the perception of CBPs. CBEL-triggered defence responses but not cell death required BAK1, while the roles of SOBIR1 in CBEL detection remain undetermined (Larroque et al., 2013). Both BAK1 and SOBIR1 are required for VdCut11-triggered cell death (Gui et al., 2018), while PcCBP3- and VdEG3-triggered cell death only required BAK1 but not SOBIR1 (Gui et al., 2017). However, the defence responses stimulated by VdEG3 are probably SOBIR1-dependent because silencing of SOBIR1 impairs defence responses but not cell death induced by PsXEG1, a homolog of VdEG3 (Wang et al., 2018). These findings together suggest that plants can perceive diverse microbial CBPs via different mechanisms.

In summary, we identified a CBP from *P. capsici* (PcCBP3) consisting of two CBM1 domains. PcCBP3 is an apoplastic effector and triggers BAK1-dependent cell death in *N. benthamiana*. Ser24 in the conserved L1 motif is indispensable for the detection of PcCBP3 by plants.

## 4 EXPERIMENTAL PROCEDURES

### 4.1 Bioinformatics analyses

To identify the CBPs in *P. capsici* we retrieved the aligned CBM1 sequences from the Pfam database (PF00734) as a query. The hmmsearch program in HMMER v. 3.2 package (Eddy, 2009) was used to search the predicted proteome of *P. capsici* strain LT1534 downloaded from the Department of Energy (DOE) Joint Genome Institute (Grigoriev et al., 2011). The identified CBPs were subjected to domain annotation. The N-terminal signal peptide was predicted using web servers SignalP v. 4.1 (Petersen et al., 2011) and Phobius (Käll et al., 2007). The domain architectures of CBPs were annotated by SMART (a Simple Modular Architecture Research Tool) (Letunic & Bork, 2018) and NCBI CDD (Conserved Domain Database) (Marchler-Bauer et al., 2015), and were displayed by IBS (Illustrator for Biological Sequences) (Liu et al., 2015).

To identify PcCBP3 orthologs from other *Phytophthora* species, we performed tblastn searches to genomes of 37 *Phytophthora* species deposited in the NCBI genome database. The maximum-likelihood species tree of these 37 *Phytophthora* species were constructed using IQ-TREE (Nguyen et al., 2015) and were displayed by IBS (Illustrator for Biological Sequences) (Liu et al., 2015).

### 4.2 Plant growth conditions and inoculation assays

*N. benthamiana* plants were grown in soil in a growth room at 25 °C with 60% relative humidity and a 16 hr day/8 hr night photoperiod. *P. capsici* strain LT263 and knockout mutants were maintained on 20% (vol/vol) V8 juice agar in the dark. For the inoculation assay, *P. capsici* was grown on V8 medium for 2 days and mycelial plugs from the colony edge were taken by a 5-mm diameter corkbore. The zoospores were prepared as described above. The N-terminal signal peptide was predicted using the online tool SignalP v. 4.1 (http://www.cbs.dtu.dk/services/SignalP-4.1/). A potential N-linked glycosylation site was predicted by the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/).

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4.3 | Plasmid construction

The CBP genes were amplified from genomic DNA of the cognate Phytophthora species by PCR and cloned into the binary vector pSuper with a 3 × FLAG tag. Truncated versions and point mutations of PcCBP3 were generated by overlap PCR. The pTRV2 constructs used for silencing NbBAK1 or NbSOBIR1 were generated as described before (Nie et al., 2019). The primers used in this study are listed in Table S3.

4.4 | Transient expression and virus-induced gene silencing in N. benthamiana

Agrobacterium-mediated transient expression and TRV-based gene silencing were performed as described previously (Nie et al., 2019; Zhang et al., 2020). The levels of PcCBP3 were determined by RT-qPCR using NbAct as the reference gene. The primers are listed in Table S3.

4.5 | RT-qPCR analysis of PcCBP3 during infection

P. capsici hyphae inoculating on N. benthamiana leaves were collected at 0, 1.5, 6, 12, 24 and 36 hr postinoculation. Total RNA was extracted using a Plant Total RNA Kit (ZomanBio) and cDNA was synthesized by PrimeScript RT Master Mix (Takara) according to the manufacturer’s instructions. Real-time PCR was performed using TB Green Premix Ex Taq II (Takara) on the ABI QuantStudio 6 Flex system (Thermo Fisher). The gene-specific primers used for RT-qPCR are listed in Table S3.

4.6 | CRISPR/Cas9-mediated knockout of PcCBP3

The gene knockout in P. capsici by the CRISPR/Cas9 system was described previously (Li et al., 2019). Briefly, the sgRNA of PcCBP3 was designed using EuPaGDT (Peng & Tarleton, 2015) and the potential off-target was assessed by performing a Blastn search to the genome of P. capsici LT1534. The secondary structure of sgRNA was predicted using RNAstructure (Reuter & Mathews, 2010). The sgRNA and flanking sequences of PcCBP3 were cloned into pYF2.3G-Ribo-sgRNA and pBluescript SKII+ vectors, respectively. Protoplast transformation was performed as previously described (Fang & Tyler, 2016). The transformants were selected by G418 antibiotic, PCR detection, and sequencing of target genomic DNA. The primers are listed in Table S3.

4.7 | Yeast secretion trap assay

The yeast secretion trap assay was used for functional evaluation of the signal peptide of PcCBP3 according to a protocol described previously (Yin et al., 2018). Briefly, SP\textsuperscript{PCBP3} was fused to the invertase gene in the pSU2 vector and then transformed into the yeast strain YTK12. Positive transformants were confirmed by growth on CMD−W medium. To detect invertase secretion, yeast cultures grown in YPAD liquid medium were used for TTC assay.

4.8 | Immunoblot analysis

To detect proteins expressed in N. benthamiana leaves, a 7-mm diameter leaf disc was taken by corkborer. After adding 70 μl of Tris-buffered saline, the leaf disc was homogenized and then 20 μl 5× Laemmli sample buffer was added. The sample was boiled for 5 min and centrifuged at room temperature for 5 min at 16,873 × g. The supernatant was used for SDS-PAGE and western blot with α-FLAG or α-HA antibodies (Abcam). Immunoprecipitations were performed using anti-FLAG M2 Affinity Gel (Sigma-Aldrich) according to instructions.

4.9 | Purification of E. coli-expressed PcCBP3

To express PcCBP3 in E. coli, PcCBP3 without signal peptide was cloned into pET28a and then transformed into E. coli BL21 (DE3). E. coli was cultured in LB medium containing 50 μg/ml kanamycin at 28 °C to an OD\textsubscript{600} of 0.6. Then, 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium and protein expression was induced at 16 °C overnight. E. coli cells were collected by centrifuging at 4 °C for 1 min at 12,396 × g, and washed by phosphate-buffered saline (PBS, pH 7.4) three times. Cells were resuspended using 10 ml PBS and disrupted by sonication. His-tagged proteins were purified by affinity chromatography using HisPur Ni-NTA Resin (Thermo Scientific) according to the manufacturer’s instructions.

4.10 | Luminol-based chemiluminescence assay

ROS production induced by E. coli-expressed PcCBP3 was measured as described previously (Albert et al., 2015). In brief, 0.125 cm\textsuperscript{2} leaf discs from N. benthamiana leaves were taken by corkborer and incubated in a 96-well plate with 200 μl of water overnight. The water was replaced by a buffer containing 1 μM protein or flg22 peptide, 20 μM L-012 (Waco), and 20 μg/ml horseradish peroxidase (Sigma-Aldrich). The chemiluminescent signal was measured immediately using a luminometer (Tecan F200).

4.11 | Subcellular localization

Imaging of the mCherry signal was performed as previously described (Wang et al., 2021a). Briefly, PcCBP3-mCherry was transiently expressed in N. benthamiana leaves for 48 hr and then imaged
by a confocal laser-scanning microscope (DM18; Leica). Plasmolysis was performed by treating leaves with 30% sucrose for 10 min.

ACKNOWLEDGEMENTS
We thank Dr Xiangxiu Liang of the China Agricultural University for helpful suggestions and revisions of the manuscript. This study was supported by the Fundamental Research Funds for the Central Universities (KYLH201703) and the National Natural Science Foundation of China (32072507).

AUTHOR CONTRIBUTIONS
Z.Y. and D.D. designed the project. Z.Y., N.W., W.D., and L.P. performed the experiments. Z.Y. and D.S. performed the bioinformatics analyses. Z.Y. and D.D. wrote the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT
The genes used in this study are deposited in the GenBank database at https://www.ncbi.nlm.nih.gov/gencode/ with the following accession numbers: PpCBEL (MT774123), PpCBP1 (MT774124), PpCBP2 (MT774125), PpCBP3 (MT774126), PpCBP4 (MT774127), PpCBP5 (MT774128), PpCBP6 (MT774129), PpCBP7 (MT774130), PpCBP8 (MT774131), PpCBP3 (XP_008897813), PICBP3 (AC8L0756), PsCBP3 (XP_009522362), and PcmCBP3 (RAW27988).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Yin, Z., Wang, N., Duan, W., Pi, L., Shen, D. & Dou, D. (2021) *Phytophthora capsici* CBM1-containing protein CBP3 is an apoplastic effector with plant immunity-inducing activity. *Molecular Plant Pathology*, 22, 1358–1369. [https://doi.org/10.1111/mpp.13116](https://doi.org/10.1111/mpp.13116)