

SCR96, a small cysteine-rich secretory protein of *Phytophthora cactorum*, can trigger cell death in the Solanaceae and is important for pathogenicity and oxidative stress tolerance

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SUMMARY

Peptides and small molecules produced by both the plant pathogen *Phytophthora* and host plants in the apoplastic space mediate the relationship between the interplaying organisms. Various *Phytophthora* apoplastic effectors, including small cysteine-rich (SCR) secretory proteins, have been identified, but their roles during interaction remain to be determined. Here, we identified an SCR effector encoded by *scr96*, one of three novel genes encoding SCR proteins in *P. cactorum* with similarity to the *P. cactorum* phytotoxic protein PcF. Together with the other two genes, *scr96* was transcriptionally induced throughout the developmental and infection stages of the pathogen. These genes triggered plant cell death (PCD) in the Solanaceae, including *Nicotiana benthamiana* and tomato. The *scr96* gene did not show single nucleotide polymorphisms in a collection of *P. cactorum* isolates from different countries and host plants, suggesting that its role is essential and non-redundant during infection. Homologues of SCR96 were identified only in oomycetes, but not in fungi and other organisms. A stable protoplast transformation protocol was adapted for *P. cactorum* using green fluorescent protein as a marker. The silencing of *scr96* in *P. cactorum* caused gene-silenced transformants to lose their pathogenicity on host plants and these transformants were significantly more sensitive to oxidative stress. Transient expression of *scr96* partially recovered the virulence of gene-silenced transformants on plants. Overall, our results indicate that the *P. cactorum scr96* gene encodes an important virulence factor that not only causes PCD in host plants, but is also important for pathogenicity and oxidative stress tolerance.

Keywords: oxidative stress, pathogenicity, *Phytophthora cactorum*, small cysteine-rich protein, stable protoplast transformation, transcriptional profile, transient expression.

INTRODUCTION

Phytophthora species are notorious oomycete pathogens that attack a wide range of agriculturally and ornamentally important

plants. The best known potato late blight disease, which is caused by *P. infestans* and was responsible for the Great Irish Famine in the 1840s, remains a serious problem worldwide (Kamoun *et al.*, 2015). *Phytophthora cactorum*, a destructive and widespread soil-borne pathogen, has been reported to affect an extremely wide range of hosts that span several plant families. It often causes root, collar and crown rots, as well as foliar and fruit infections, limiting the production of many economically important crops, such as strawberry, apple, pear, tomato and rhododendron, worldwide (Erwin and Ribeiro, 1996). Despite the importance of *Phytophthora* diseases in agriculture and ecosystems, it is difficult to combat them because of a lack of understanding of their mechanisms of pathogenesis.

Plant survival in the presence of pathogen threats requires both effective perception of, and appropriate responses to, pathogenic attack. To do so, plants have developed two distinct layers of immunity: pathogen- or microbe-associated molecular pattern (PAMP/MAMP) and damage-associated molecular pattern (DAMP)-triggered immunity (PTI), and effector-triggered immunity (ETI) (Dodds and Rathjen, 2010; Jones and Dangl, 2006). PTI, known as the first layer of plant defence, is often achieved through the recognition of PAMPs or DAMPs by membrane-located pattern recognition receptors (Boller and Felix, 2009; Monaghan and Zipfel, 2012). PAMPs or DAMPs are found in molecules previously designated as 'general elicitors'. Their recognition initiates the induction of various antimicrobial responses, including the reactive oxygen species (ROS) burst, cell wall reinforcement and the expression of defence genes, without signs of programmed cell death (Boller and Felix, 2009). Although PTI restricts the growth of the vast majority of potential pathogens, successful pathogens have evolved mechanisms to effectively suppress this basal defence by the secretion of an array of effectors (Bozkurt *et al.*, 2012). Plants have, in turn, evolved nucleotide-binding leucine-rich repeat resistance proteins, which allow for the direct or indirect recognition of pathogen effectors. This secondary immunity, namely ETI, is often accompanied by the hypersensitive response (HR), corresponding to a localized programmed cell death, and by systemic acquired resistance (Dou and Zhou, 2012; Giraldo and Valent, 2013; Jones and Dangl, 2006).

So far, several types of necrosis-inducing 'general elicitor' have been characterized in plant pathogens and, in particular, in

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Phytophthora pathogens (Hein *et al.*, 2009). These include elicitors, NLPs (Nep1-like proteins), CBEL (cellulose-binding elicitor lectin) glycoproteins and small cysteine-rich (SCR) proteins, such as *P. cactorum* PcF (Kamoun, 2006). Little is known about the roles of the hypersensitive-like response induced by such necrosis-inducing elicitors. They are predicted to be located at the interface between pathogens and hosts, and to execute functions outside of the host cell, and are therefore called apoplastic effectors (Kamoun, 2006). NLPs appear to function as phytotoxins to induce cell death as a result of their structural homology to pore-forming toxins produced by sea anemones (i.e. actinoporins) (Ottmann *et al.*, 2009). The recognition of elicitors and the inability of pathogens to inhibit this recognition could limit the host range of the pathogens (Kamoun *et al.*, 1998). Plant receptor-like kinase, such as *Nicotiana benthamiana* BAK1/SERK3, contributes to elicitor-mediated necrosis, and kinase gene silencing leads to enhanced plant susceptibility to *P. infestans* (Chaparro-Garcia *et al.*, 2011). Furthermore, elicitor-, NLP- and CBEL-induced cell death can be suppressed by host-translocated effectors, demonstrating that oomycetes have evolved delicate mechanisms to counteract the host cell death triggered by necrosis-inducing elicitors (Bos *et al.*, 2006; Chen *et al.*, 2013, 2014; Gilroy *et al.*, 2011; Liu *et al.*, 2011). Based on these results, it has been suggested that responses to these proteins could overlap with ETI and play an important role in the outcome of *Phytophthora*–plant interactions (Thomma *et al.*, 2011).

Mature PcF, secreted after N-terminal signal processing, is an acidic 52-amino-acid protein comprising a 4-hydroxyproline and six cysteine residues bridged intramolecularly (Orsomando *et al.*, 2001). PcF can trigger HR symptoms of localized cell death on the leaves of both strawberry and tomato, and induction of the key defence-related enzyme PAL21 and other pathogenesis-related (PR) genes in tomato (Orsomando *et al.*, 2001, 2003). It has been suggested that PcF may mimic the effects of a plant signalling protein because it seemingly has structural homology to the pollen protein Ole-e6 with allergenic activity (Nicastro *et al.*, 2009). Oomycetic SCR proteins can be related to PcF mainly on the basis of their conserved sequences, cysteine patterns and N-terminal secretory signals (Bos *et al.*, 2003; Orsomando *et al.*, 2011). The expansion of PcF/SCR gene families in oomycetes has been noted in whole-genome analyses and the size of the family in several species, such as *P. infestans* ($n = 16$), *P. sojae* ($n = 19$), *P. ramorum* ($n = 4$), *Pythium ultimum* ($n = 3$) and *Hyaloperonospora arabidopsidis* ($n = 2$), has been estimated through annotation (Baxter *et al.*, 2010; Haas *et al.*, 2009; Lévesque *et al.*, 2010; Tyler *et al.*, 2006). In our recent work, three SCR genes plus PcF were detected in the *P. cactorum* transcriptome (Chen *et al.*, 2014). Comparative structural and functional characterization of PcF and several homologues from *P. infestans* highlighted a surface-exposed conserved amino acid stretch SK(E/C)C as a possible structural determinant responsible for the differential phytotoxicity (Orsomando *et al.*, 2011).

Although a 'PcF/SCR' family has been proposed, it has been studied much less than other effectors (Stassen and Van den Ackerveken, 2011). To date, PcF remains the only characterized member within the family. The other family members include proteins encoded by polymorphic genes, and are often identified by the abbreviation 'SCR' followed by their residue number, such as SCR70, SCR74 and SCR91 from *P. infestans* (Bos *et al.*, 2003; Liu *et al.*, 2005). Although PcF/SCR proteins share with fungal avirulence proteins (e.g. Avr2 and Avr4 of *Cladosporium fulvum*) the feature that they are secreted and are SCR proteins, the oomycete counterparts are generally considered to function as extracellular toxins (Bos *et al.*, 2003; Stergiopoulos and de Wit, 2009; Wawra *et al.*, 2012). However, their molecular action and biological functions have not been identified to date.

The main objective of this study was to investigate the roles of newly identified *P. cactorum* PcF/SCR effectors (hereafter referred to as 'SCR' followed by their residue number) in pathogen–plant interactions using transcriptional profiles, transient expression, sequence analysis and gene silencing. Overall, the results show that these genes differentially expressed during the infection phase can trigger plant cell death (PCD) in *N. benthamiana* and tomato, and one of them, SCR96, is important for the pathogenicity and oxidative stress tolerance of *P. cactorum*. This study demonstrates that PCD caused by these small molecules is not a PTI response and that these effectors are probably virulence determinants. The work described herein provides a crucial foundation for further determination of the roles of PcF/SCR effectors in the virulence of *Phytophthora* pathogens.

RESULTS

PcF/SCR gene expression profiling of *P. cactorum*

Recently, we have assembled the transcriptome of *P. cactorum* life cycle stages containing 21 662 unique genes (Chen *et al.*, 2014). About 620 effectors, including PcF and three SCR proteins (SCR96, SCR99 and SCR121), were identified (Chen *et al.*, 2014) (Table 1). To understand the transcriptional profiles of these unique genes during life cycle stages, three cDNA libraries representing mycelia (MY), swimming zoospores (ZO) and germinating cysts with germ tubes (GC) were separately constructed and sequenced in this study using the Illumina Genome Analyzer Ix platform (Illumina, San Diego, CA, USA). Each sequenced sample yielded 100-bp reads from single-end sequencing of cDNA fragments. Most of the distinct reads were matched to *P. cactorum* genes (Table S1, see Supporting Information), and the transcript level of each expressed gene was calculated and normalized to the reads per kilobase of exon model per million mapped reads (RPKM) (Mortazavi *et al.*, 2008). RPKM values showed that, among the effector genes, PcF and three SCR genes were differentially expressed during these developmental stages

Table 1 *Phytophthora cactorum* PcF/small cysteine-rich (SCR) effectors analysed in this study.

Gene*	GenBank accession no.	Gene length (bp)	SignalP HMM#	SignalP NN#	SignalP length (amino acids)#	Number of cysteine residues†
<i>PcF</i>	KT215392	222	0.999	0.940	21	6
<i>scr96</i>	KT215393	291	0.999	0.851	23	8
<i>scr99</i>	KT215394	300	1.00	0.964	19	6
<i>scr121</i>	KT215395	366	0.994	0.916	22	6

*The gene sequences were amplified from the isolate 10 300 (Chen *et al.*, 2014).†Only cysteine residues in the mature peptide sequences were counted.

#Hidden Markov model (HMM) probability, Neural Network (NN) mean S score, and signal peptide length were predicted using SignalP v3.0.

(Fig. 1a). *scr96* and *scr121* were highly expressed in MY versus GC and ZO, whereas the other two genes (*scr99* and *PcF*) showed increased expression in GC versus MY and ZO (Fig. 1a).

To determine whether SCR genes were induced during plant invasion by *P. cactorum*, we inoculated *N. benthamiana* with zoospores of the pathogen and investigated their expression using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). All the tested genes were differentially expressed during plant invasion (Fig. 1b). In particular, *scr96* was highly expressed during the very early infection stage (3 h post-inoculation, hpi) (Fig. 1b). The expression patterns during the life cycles profiled by RT-PCR are consistent with the results obtained by RNA-Seq analysis (Fig. 1).

Normally, *Phytophthora* pathogens invade plants by mycelia or germinating cysts, which develop into mycelia during the penetration process. As shown in Fig. 1, the transcript up-regulation suggests that SCR proteins play important roles during the pre-infection (cyst germination) and infection stages.

SCR96 and other SCR proteins trigger cell death in solanaceous plants

To investigate the biological function of effectors, transient expression assays were performed on solanaceous plants using the infiltration of *Agrobacterium tumefaciens* cells with *Potato virus X* (PVX) carrying *scr96* or other homologous genes. At 6 days post-treatment, negative control leaves (pGR107-*gfp* and 10 mM MgCl₂) remained green and healthy, but infiltration with SCR96 conferred symptoms of necrosis as vigorously as the positive control INF1 on *N. benthamiana* (Fig. 2a). No visible symptoms were observed from the transient expression of *scr99*, *scr121* and *PcF* during the tests (Fig. 2b–d). We then performed similar experiments on *Solanum lycopersicum* and found that the expression of *scr96*, *scr121* and *scr99* resulted in leaf cell death at 4 days post-treatment (Fig. 2e–g). No visible symptoms were observed when *inf1* and *PcF* were transiently expressed in tomato leaves (Fig. 2h). Similar experiments were also conducted on *N. tabacum*, but no changes were observed with the tested PcF/SCR proteins, except INF1 (Fig. 2i). Notably, we cloned PcF from the isolate 10300 and performed parallel tests. Like the negative controls, PcF cannot induce PCD in these three tested plant species (Fig. 2d,h,i).

The sequences of *scr96* were subsequently examined in a limited collection of *P. cactorum* isolates from different countries and host plants (Table S2, see Supporting Information) to determine whether sequence polymorphisms may exist. However, it was found that full-length DNA sequences of *scr96* were identical among all the tested *P. cactorum* isolates.

Homologues of SCR96 exist only in oomycetes, but not in fungi

The aforementioned SCR genes plus *PcF* were successfully full length cloned and sequenced, yielding the same sequences as transcriptomic data (Chen *et al.*, 2014). These genes do not contain any introns and are typically short in length, ranging from 222 to 366 bp. SignalP analysis identified a putative signal peptide at the N-terminus of each protein. Conserved domain analysis revealed nothing but an even number of cysteine residues in the mature peptide sequences (Table 1; Fig. S1, see Supporting Information).

As a result of its strong expression in MY, GC and early infection stages, and its PCD-triggering activity in solanaceous plants, we investigated SCR96 in more detail. To determine the presence of its homologues in other organisms, we performed a BLASTX search of the FungiDB and genome databases for various oomycetes. Homologues of SCR96 were identified only in oomycetes, including *P. capsici*, *P. parasitica*, *P. sojae*, *P. cinnamomi*, *H. arabidopsidis* and *Pythium* spp., but not in fungi or other organisms. Notably, two homologues (PHYCA_130237 and PHYCA_511621) of SCR96 were identified in *P. capsici*. The alignment of SCR96 homologues demonstrated a high conservation of SCR96 among different species of oomycete (Fig. 3a). Phylogenetic analysis revealed that SCR96 and all of its homologues, except PcF, from other *Phytophthora* spp. form a clade distinct from that of other oomycetes, with the homologue from *Pythium vexans* as the closest relative. Intriguingly, like one of the secretory SCR proteins of *C. fulvum*, AVR2 (GenBank accession no. AI211404), *P. cactorum* PcF forms a clade distant from that of other oomycete SCRs (Fig. 3b).

Protoplast transformation and *gfp* (green fluorescent protein) expression in *P. cactorum*

It has been shown that the transformation of *Phytophthora* species with gene constructs results in gene silencing of both the

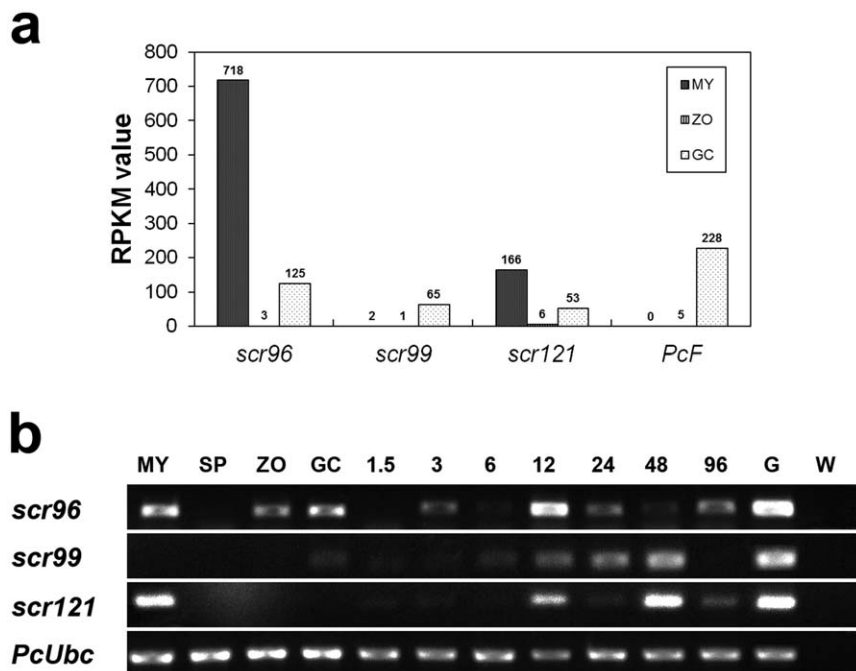


Fig. 1 Expression profiles of *Phytophthora cactorum* PcF/small cysteine-rich (SCR) effector-encoding genes. (a) The gene expression level was determined by calculating the number of reads for each gene and then normalizing this to the reads per kilobase of exon model per million mapped reads (RPKM). The RPKM values of *scr96*, *scr99*, *scr121* and *PcF* were calculated in this study using the RNA-Seq data (GenBank SRA accession no. SRP059996). (b) The expression of *scr96*, *scr99* and *scr121* was profiled using reverse transcription-polymerase chain reaction (RT-PCR) throughout the *P. cactorum* life cycle, including mycelia (MY), sporangium (SP), zoospore (ZO) and germinating cyst (GC), and during infection stages, including 1.5, 3, 6, 12, 24, 48 and 96 h post-inoculation (hpi) of *Nicotiana benthamiana*. W, water used as PCR template; G, *P. cactorum* genomic DNA; *PcUbc*, internal control *PcUbc* gene of *P. cactorum*. Three independent biological experiments were performed and yielded similar results.

transgenes and the endogenous gene (van West *et al.*, 1999). As stable transformation of *P. cactorum* has not been attempted, to analyse the gene functions, the standard transformation protocol using protoplasts and a polyethylene glycol/calcium chloride (PEG/CaCl₂)-mediated method was adapted from the literature (McLeod *et al.*, 2008). The main modification is that the geneticin (G418) concentration used for transformant selection was 17.5 µg/mL based on a geneticin sensitivity test of the wild-type (WT) isolate 10 300 (Fig. S2, see Supporting Information).

Using these procedures, the binary transformation vector pGFPN, containing both the *gfp* and selectable marker (*neomycin phosphotransferase II*, *nptII*) gene expression cassettes, was transformed into *P. cactorum* WT isolate 10 300 and 25 G418-resistant transformants were obtained. After screening visually for fluorescence using an epifluorescent Olympus BX53 microscope (Tokyo, Japan) and later single zoospore isolation, the brightest transformants (GFP1 and GFP2) were retained. The transformants fluoresced green in the life stages including mycelia, sporangia, germinating cysts and oospores (Fig. S3, see Supporting Information). The WT isolate did not fluoresce at all. There were no significant differences in the tested characteristics (mycelia growth rate, yields of sporangia and oospores, and pathogenicity) between transgenic GFP isolates and the WT (data not shown).

SCR96 is important for the virulence of *P. cactorum*

To determine whether SCR96 was required for *P. cactorum* pathogenicity, stable *scr96*-silenced strains of *P. cactorum* (10 300) were generated using the above established transformation-

mediated gene silencing method. We obtained three *scr96*-silenced lines, S3, S10 and S21, in which *scr96* expression was reduced by 65%, 80% and 95%, respectively, compared with the WT strain, whereas *scr96* expression was not affected in the transformation control strain (*gfp*-expressing transformant GFP1) (Fig. 4a). Inoculation assays were performed by dropping zoospore suspensions of the WT and the silenced transformants onto healthy leaves of 5-week-old *N. benthamiana* plants. At 2 days post-inoculation (dpi), the leaves inoculated with the WT isolate zoospores showed typical disease symptoms and water-soaked lesions (Fig. 4b,c). In contrast, the silenced transformants (S10 and S21 shown here) produced no visible lesions at all (Fig. 4b,c). S3, the third transformant, behaved overall in a similar manner to S10 and S21. We then tested whether transient expression of *scr96 in planta* could recover *Phytophthora* virulence on *N. benthamiana* plants. At 48 h after infiltration of *A. tumefaciens* cells harbouring *scr96*, the agroinfiltration zone was inoculated with *scr96*-silenced transformants (S10 and S21). Intriguingly, transient expression of *scr96* partially compensated for the virulence of gene-silenced transformants on *N. benthamiana* (Fig. 4d–f). Together, these results indicate that SCR96 is required for *Phytophthora* virulence.

SCR96 is related to oxidative stress tolerance

To gain further insight into the role of SCR96 in the infection process, two *scr96*-silenced lines (S10 and S21), the WT strain 10 300 and the transformation control GFP1 were cultured on Plich's agar medium supplemented with different concentrations of H₂O₂. The *scr96*-silenced lines showed no significant reduction

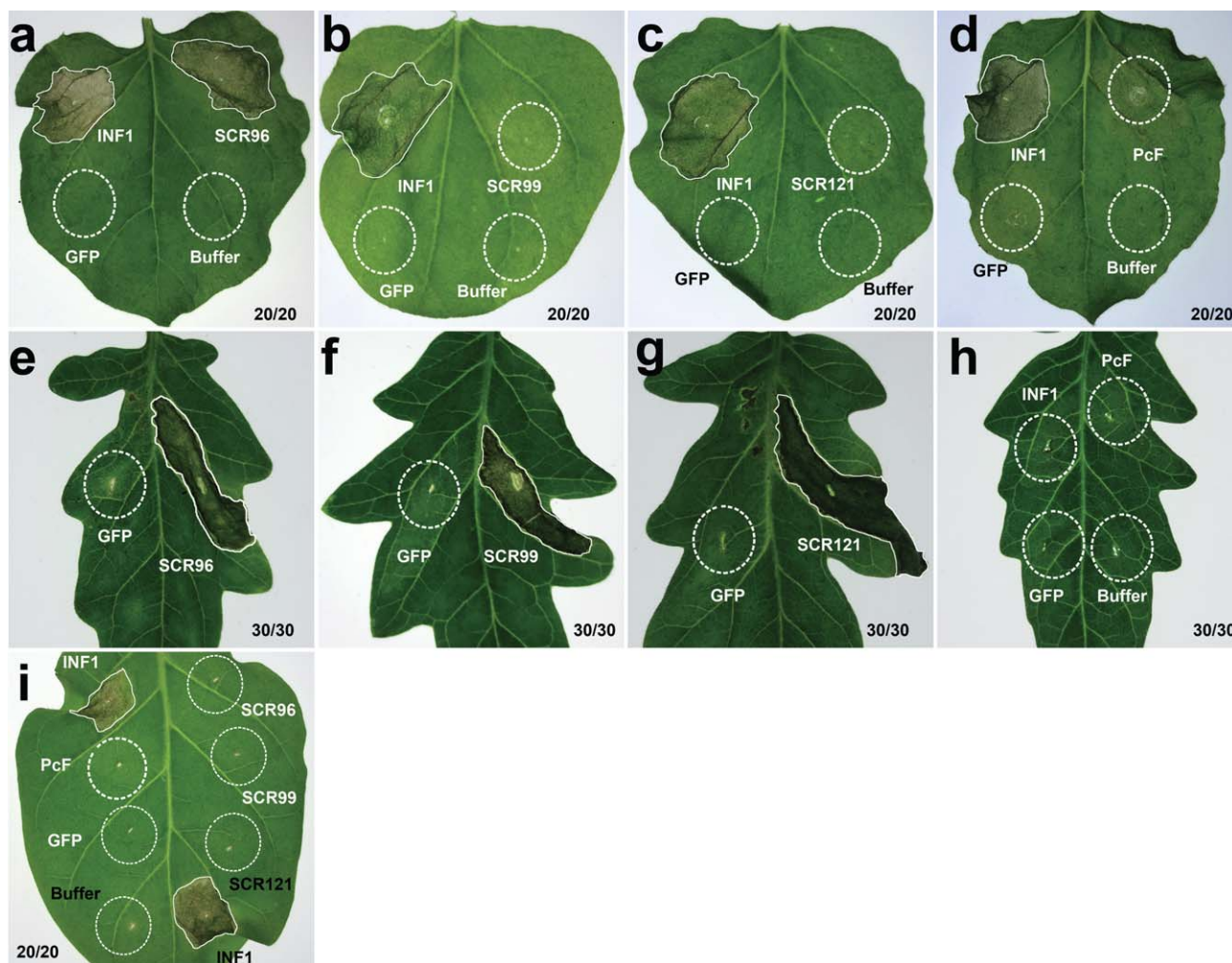


Fig. 2 Transient assays of *Phytophthora cactorum* PcF/small cysteine-rich (SCR) effectors in Solanaceae plants. The leaves of three species of Solanaceae plants, *Nicotiana benthamiana* (a–d), tomato (e–h) and *N. tabacum* (i), were infiltrated with *Agrobacterium tumefaciens* (strain GV3101) cells to express the effectors. The expression of *scr96* triggered plant cell death (PCD) in both *N. benthamiana* (a) and tomato (e), whereas *scr99* (f) and *scr121* (g) induced PCD only in tomato. SCR99 (b) and SCR121 (c) did not induce PCD in *N. benthamiana*. Together with SCR96, these three cannot trigger PCD in *N. tabacum* (i). The *P. cactorum* protein PcF cannot cause PCD in any type of plant (d, h, i). The *P. infestans* elicitor INF1 served as a positive control in both *N. benthamiana* and *N. tabacum* (a–d, i), but not in tomato (h). The marker gene *gfp* and 10 mM MgCl₂ buffer were used as negative controls in all tested plants. The broken circles indicate the agroinfiltration areas without PCD, whereas irregular shaped areas are tissues in which PCD occurs. Photographs were taken 6 days (a–d) or 4 days (e–i) after infiltration.

in mycelial growth on Plich's agar medium in the absence of H₂O₂ when compared with the WT and control transformant GFP1 (Fig. 5). In contrast, treatment with 4 or 6 mM H₂O₂ completely suppressed the mycelial growth of *scr96*-silenced transformants, whereas the WT and GFP1 lines still proliferated (Fig. 5). At 8 mM H₂O₂, all the strains were unable to grow (data not shown). Together, these results indicate that SCR96 is essential for oxidative stress tolerance of *P. cactorum*.

DISCUSSION

Since the discovery of the PcF/SCR family, little has been determined about its biological functions and roles in the virulence of

Phytophthora pathogens. Apart from PcF, all other members have not been characterized and their functions have not been verified experimentally. In this study, we demonstrated that a putative *P. cactorum* PcF/SCR protein, SCR96, is one of the 'general elicitors' triggering PCD in *N. benthamiana* and tomato, and silencing of its expression affects its pathogenicity on host plants and its tolerance to oxidative stress.

Our previous transcriptomic study detected three novel PcF/SCR effectors (SCR96, SCR121 and SCR99) and PcF from *P. cactorum* isolate 10 300 (Chen *et al.*, 2014). Based on transcriptomic profiling and RT-PCR data, it was found that these genes were transcribed differentially throughout the *P. cactorum* life cycle and infection phase on *N. benthamiana* (Fig. 1). Among them, *scr96*

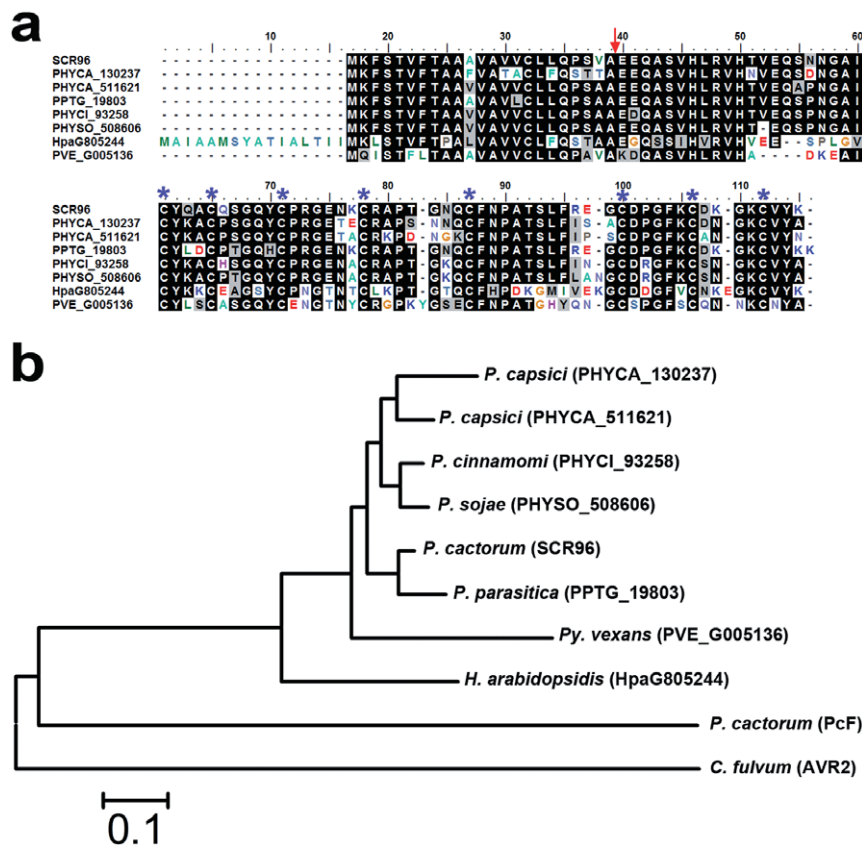


Fig. 3 Protein sequence alignment and phylogenetic analysis of *Phytophthora cactorum* SCR96 and its homologues in different oomycete species. (a) Protein sequences were aligned and shaded for consensus (50% threshold for shading) using BioEdit. The homologues of SCR96 compared here include PHYCA_130237 and PHYCA_511621 from *P. capsici*, PPTG_19803 from *P. parasitica*, PHYCI_93258 from *P. cinnamomi*, PHYSO_508606 from *P. sojae*, HpaG805244 from *Hyaloperonospora arabidopsidis* and PVE_G005136 from *Pythium vexans*. The predicted cleavage site for signal peptides is indicated by a red arrow. The conserved cysteine sites are marked at the top by blue asterisks for visualization. (b) Phylogeny of SCR96 and homologues from different species. The tree was constructed by the neighbour-joining (NJ) algorithm implemented in MEGA 5 with 1000 bootstrap replicates. The IDs, except for SCR96, PcF and AVR2, in parentheses after the species names are from the FungiDB database. *Cladosporium fulvum* AVR2 served as an outgroup in the NJ tree.

was highly expressed during mycelium, cyst germination and early infection stages (Fig. 1a,b). Similarly, the expression of *scr74* was significantly up-regulated during *P. infestans* colonization stages of potato and tomato (Liu *et al.*, 2005). The expression patterns of the PcF/SCR effector genes are consistent with their expected roles in assisting the colonization of plant tissues by the pathogen. To explore their predicted roles further, we tested whether the effectors could cause phenotypic reactions when expressed in host plants, and found that three effectors (SCR96, SCR121 and SCR99) could elicit PCD in *N. benthamiana* and/or tomato (Fig. 2). Purified PcF from culture filtrate induced leaf necrosis on tomato and strawberry (Orsomando *et al.*, 2001). Two PcF homologues from *P. infestans*, SCR74 and SCR91, together with PcF, after recombinant expression in bacteria and purification, triggered leaf withering and phenylalanine ammonia lyase induction on tomato (Orsomando *et al.*, 2011). PCD induced by phytotoxins is thought to play a central role in providing a source of nutrition for necrotrophic and hemibiotrophic pathogens (Dou and Zhou, 2012; Mott *et al.*, 2014). However, such a response caused by different PcF/SCR effectors shows various degrees of effectiveness (Orsomando *et al.*, 2011). In our case, we observed no changes visible to the naked eye when some of the PcF/SCRs, e.g. SCR96 and PcF, were expressed in leaves of solanaceous plants (Fig. 2). Whether such effectors are host species or even variety selective, or are required for infection on susceptible host plants that have

the corresponding dominant receptor, requires further study. More work needs to be performed to determine whether these observations in transient expression assays are also a result of poor protein expression or stability.

As fungal pathogen virulence factors, phytotoxins contribute to successful infection by causing direct damage to cell membranes, alteration of gene expression, inhibition of plant protein function, mimicking of plant hormones and induction of cell death through the production of ROS (Möbius and Hertweck, 2009). In oomycetes, it is presumed that two families of toxic proteins act in an offensive manner by triggering host cell death that could favour the necrotrophic phase of development. NLPs have been suggested to be involved in the disruption of plant plasma membranes and subsequent cytolysis on the basis of their similarities to the pore-forming toxins of sea anemones and from *in planta* experiments (Ottmann *et al.*, 2009). In contrast, the nature of PcF/SCRs remains to be resolved. It has been assumed previously that they are possibly oomycete avirulence proteins because of the characteristics they share with fungal avirulence proteins, i.e. SCR secretory proteins (Bos *et al.*, 2003). Unlike fungal avirulence proteins, such as Avr2 of *C. fulvum* (Rooney *et al.*, 2005), however, plant targets of PcF/SCR effectors are still unknown. PcF/SCRs are assumed to function in the plant apoplast as phytotoxins (Stassen and Van den Ackerveken, 2011). It is within this space that the fate of many host-

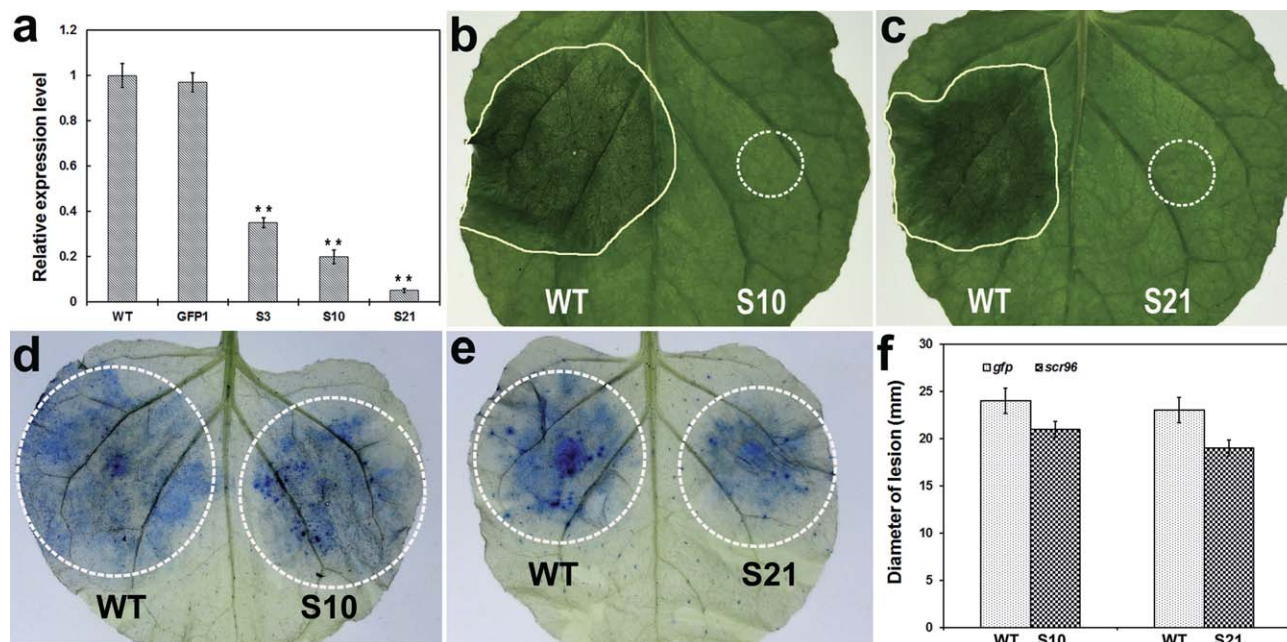


Fig. 4 The role of *Phytophthora cactorum scr96* in the infection of *Nicotiana benthamiana*. (a) Silencing of *scr96*. The gene *scr96* mRNA abundances in three stably silenced *P. cactorum* transformants (S3, S10 and S21) were determined by quantitative polymerase chain reaction (PCR) 3 days after mycelial growth in V8 medium relative to *PcUbc* mRNA abundances, and then normalized to the wild-type isolate 10 300 (WT). GFP1 is a transformation control expressing green fluorescent protein. Standard errors from three technical replicates are shown. Statistical significance was analysed using Student's *t*-test between the WT/GFP1 and WT/transformant comparisons (** $P < 0.01$). (b, c) Two representative gene-silenced transformants (S10 and S21, respectively) show a loss in virulence on *N. benthamiana* compared with the WT. Photographs were taken at 48 h post-inoculation (hpi). The irregular shaped areas are necrotic tissues caused by the WT isolate, whereas broken circles indicate the gene-silenced transformant inoculation sites without visible necrosis. (d–f) Transient expression of *scr96* partially recovered the virulence of gene-silenced transformants on *N. benthamiana*. At 48 h after agroinfiltration, infiltration sites in each leaf expressing both *gfp* (left) and *scr96* (right) were challenged with the WT isolate and gene-silenced transformants [S10 (d) or S21 (e)]. Photographs were taken 48 h after pathogen inoculation. Leaves were stained by trypan blue and then destained by ethanol. The broken circles indicate the infiltration and inoculation sites. (f) The diameters of the lesions from (d) and (e) were measured and analysed. Error bars represent standard errors from technical replicates. There was no significant difference between left and right treatments (Student's *t*-test).

pathogen interactions is determined (Mott *et al.*, 2014). We found that *P. cactorum scr96*-silenced transformants lost their pathogenicity to *N. benthamiana*, indicating that this effector plays an important role in virulence during pathogen infection (Fig. 4). On detection of pathogen invasion through the recognition of PAMPs, one of the early reactions of plants is the production of toxic ROS. This production is classically associated with direct microbial killing (Peng and Kuc, 1992). To elucidate whether PcF/SCR effectors are involved in ROS responses, gene-silenced transformants were tested on Plich's medium supplemented with H_2O_2 . We found that *scr96*-silenced transformants were more sensitive to H_2O_2 than the control isolates (Fig. 5). The results indicated that the PcF/SCR effectors contribute positively to the virulence of the pathogens, and loss of pathogenicity of the transformants may be caused by a compromised ability to degrade H_2O_2 and/or reductions in other stress tolerance capabilities during infection. Therefore, SCR96 is probably a virulence determinant and involved in ROS responses. On the basis of these results, we assume that SCR proteins may

not act as toxins, or may be targeted by ROS. This speculation is partially supported by previous findings indicating that PcF shows structural similarity to Ole-e6, a major allergen from olive tree pollen, by nuclear magnetic resonance (Nicastro *et al.*, 2009), and PcF/SCR proteins share certain characters with fungal Avr proteins, such as being SCR secretory proteins. Nonetheless, the exact mode of action of PcF/SCR family proteins remains to be determined.

Studies have shown how the primary sequences of host-translocated effectors have been shaped by positive selection, and how genes undergo accelerated birth and death evolution (Cui *et al.*, 2012; Stassen and Van den Ackerveken, 2011; Win *et al.*, 2007). In this study, sequence polymorphism was not observed for SCR96. This is possibly because only a few *P. cactorum* isolates were investigated for sequence polymorphism. Another possible reason may be that this effector is conserved in *P. cactorum* with slow rates of evolution. This effector is seemingly specific to oomycetes because homologues exist only in oomycetes, but not in fungi (Fig. 3). In contrast, a previous study has shown that

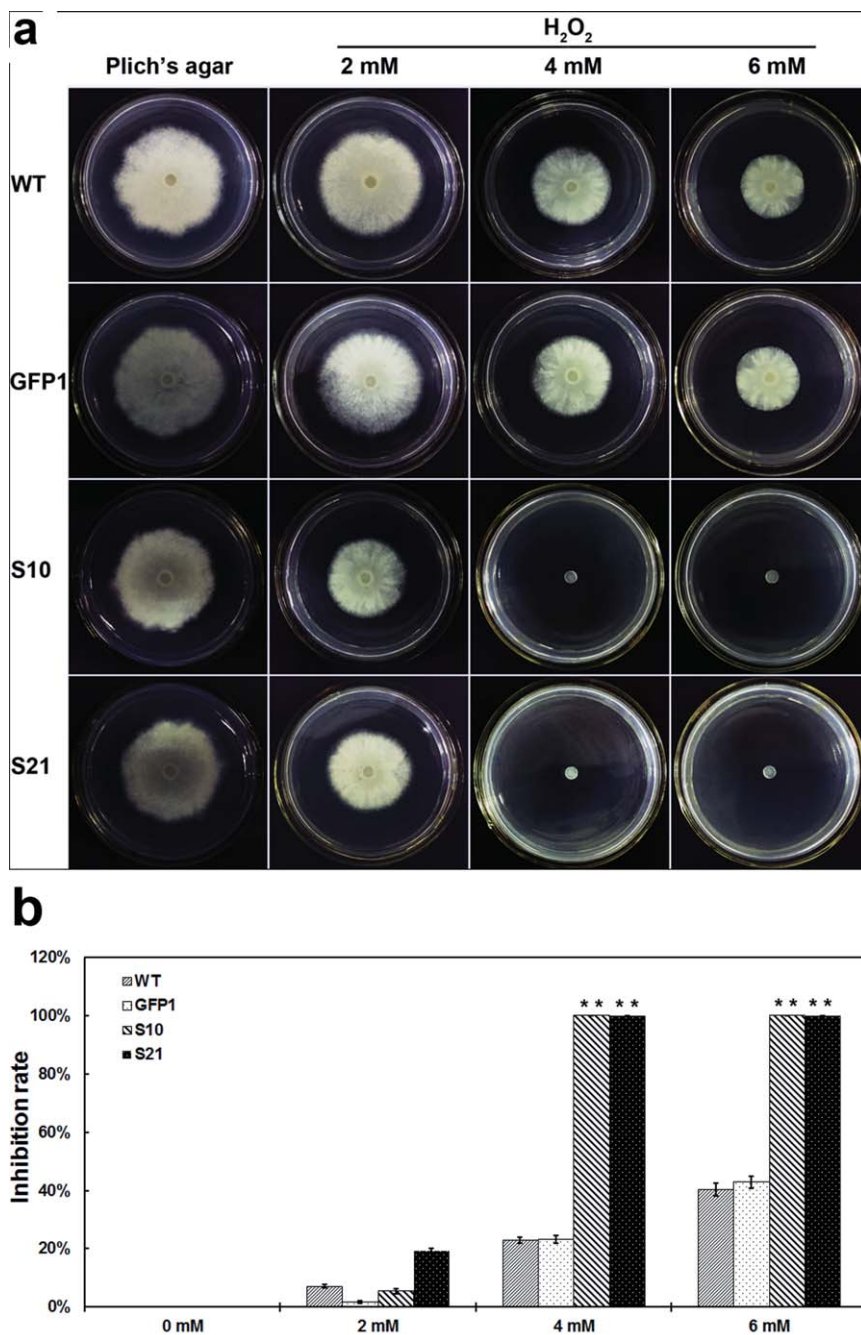


Fig. 5 Mycelial growth assay of *Phytophthora cactorum* *scr96*-silenced transformants under the oxidative stress condition. (a) Assay of mycelial growth of the wild-type (WT), transformation control (GFP1) and *scr96*-silenced transformants (S10 and S21 shown here) on Plich's agar medium only or supplemented with 2, 4 or 6 mM H_2O_2 . (b) Rates of growth inhibition were calculated for each treatment relative to growth on Plich's medium only. Colony diameters were measured in each independent biological experiment after 5 days of growth. Three independent biological experiments were performed with three replicates each time. Error bars represent the standard errors and asterisks indicate significant differences between the *scr96*-silenced transformants and the WT (** $P < 0.01$).

positive selection probably caused the extensive polymorphism observed within the SCR74 family of *P. infestans* (Liu *et al.*, 2005). This may suggest that distinct selective forces have shaped different PcF/SCR family members throughout the evolution of *Phytophthora* species.

This is the first study to report that PCD-triggering PcF/SCR effectors are important for pathogenicity and oxidative stress tolerance in *Phytophthora*. However, their molecular mechanisms remain to be elucidated. On the basis of an 'arms race' model, all PcF/SCR effectors have been tentatively classified as 'apoplasmic

effectors', interacting with plant surface receptors (Bos *et al.*, 2003; Liu *et al.*, 2005; Tyler *et al.*, 2006). Although PcF/SCR effectors have no known protein domains, they are rich in cysteine residues that can form disulfide bridges (Orsomando *et al.*, 2003, 2011). The intramolecular S-S bridge is presumed to be important for the protection of such proteins in the harsh acidic and protease-rich apoplast environment in which they are delivered during plant infection (Kamoun, 2006). In the near future, the nature of effectors and plant target proteins should be determined. Ultimately, the knowledge obtained from these studies will

help us to establish functional connections between pathogen effectors and host defence processes, and to provide insights into novel disease control strategies.

EXPERIMENTAL PROCEDURES

Growth of plant materials and *Phytophthora* strains

Nicotiana benthamiana, *N. tabacum* (cv. Samsun NN) and *S. lycopersicum* (cv. Maofen-802) were grown in Styrofoam cups containing sterile soil at 25 °C under a 16-h/8-h light/dark regime.

The strains (Table S2) of *P. cactorum* were routinely cultured on 10% V8 agar medium at 25 °C in the dark (Erwin and Ribeiro, 1996). To test for stress sensitivity, culture plugs (5 mm × 5 mm) were placed onto freshly prepared Plich's agar plates supplemented with H₂O₂ (2–8 mM) and cultured in the dark at 25 °C.

Nucleic acid isolation and mRNA sequencing

Genomic DNA was isolated from *Phytophthora* mycelia as described by Zelaya-Molina *et al.* (2011). Total RNA from different life stages of *P. cactorum* and from inoculated plant samples was prepared as described by Chen *et al.* (2014). Total RNA was isolated using RNAiso Plus reagents (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions.

Three *P. cactorum* RNA-Seq libraries (MY, SP and GC) were constructed according to the manufacturer's instructions using an Illumina Gene Expression Preparation Kit. DNA fragments were single-end sequenced on the Illumina Cluster Station and Illumina Genome Analyzer Ix sequencing platform. The clear reads were mapped to *P. cactorum* transcriptome reference sequences (Chen *et al.*, 2014) using the BLAST-Like Alignment Tool (BLAT) (Kent, 2002) with all default parameters, except $-\text{tileSize} = 9$ and $-\text{stepSize} = 6$. The datasets are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database through the project accession no. SRP059996.

RT-PCR and quantitative RT-PCR analysis

mRNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (RNase H Minus) and random hexamer primers (TaKaRa Biotechnology) following the manufacturer's protocol. PCRs were run on an LS-96G thermocycler (ThermoFisher, Dubuque, IA, USA) with the following program: 94 °C for 5 min, followed by 29 cycles (40 cycles for quantitative PCR) of 95 °C for 15 s, 46–60 °C for 30 s and 72 °C for 1 min, and, finally, melting curve analysis. For quantitative PCR, SYBR Green dye (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions. In each case, the housekeeping gene encoding ubiquitin-conjugating enzyme (*PcUbc*) (GenBank accession no. KM068098) was used as the internal control (Chen *et al.*, 2014). For quantitative RT-PCR, expression data were analysed by Student's *t*-test ($P < 0.01$). When gene expression was monitored in infected *N. benthamiana*, cDNA from mock-inoculated plants was used as an additional negative control for each primer pair to exclude the possibility that the amplification was caused by homologous plant genes. The primers

used in the reactions are listed in Table S3 (see Supporting Information). All RT-PCRs were performed three times using independently isolated RNA samples.

Molecular cloning and sequence analysis

For transient expression in solanaceous plants, full-length effector genes amplified from genomic DNA using high-fidelity DNA polymerase (PrimeSTAR[®] HS DNA Polymerase, TaKaRa Biotechnology) and primer pairs (Table S3) were cloned into the *Sma*I site of the binary PVX vector pGR107 or *Clal*-*Sall* sites of pGR106 (Jones *et al.*, 1999). For *Phytophthora* transformation, a full-length *scr96* gene fragment was amplified from genomic DNA using high-fidelity DNA polymerase and primer pairs (SCR96-*Clal*F/*Bsi*WIR; Table S3), digested with *Clal* and *Bsi*W1, and ligated with equally digested pTORMRFP4 (Whisson *et al.*, 2007) to yield the plasmid pTOR-*scr96*.

BLASTX ($E \leq 1e-5$) was used for homology searches of the FungiDB database (<http://fungidb.org>) and genome websites for several oomycetes. The homologues are arbitrarily defined here to have a predicted signal peptide, a mature length of ≤ 200 amino acid residues, at least four cysteines and a total cysteine content of $\geq 5\%$. Alignment of SCR96 and its homologues from other organisms was performed with BioEdit (v7.2.0). Phylogenetic trees were generated by the neighbour-joining algorithm implemented in MEGA (v5) with default parameters. Tree node support was evaluated by bootstrapping with 1000 pseudoreplicate datasets.

High-fidelity PCR amplification of polymorphism analysis for *scr96* was conducted as described by Chen *et al.* (2014). Briefly, the primer pair (SCR96snp-F/R; Table S3) was designed to amplify a 392-bp fragment containing the entire open reading frame (ORF) of the gene. The genomic DNA from each of eight *P. cactorum* isolates (Table S2) was used as PCR template. The fragments were cloned into a TA vector pMD-19T (TaKaRa Biotechnology) and 30 randomly selected clones for each fragment were sequenced using the universal M13 primers (Sangon Biotech, Shanghai, China).

Agroinfiltration assay

The culture of *A. tumefaciens* strain GV3101 carrying pGR107- or pGR106-derived constructs and infiltration into leaves of *N. benthamiana*, *N. tabacum* and *S. lycopersicum* were performed as described by Chen *et al.* (2014). Each assay consisted of at least five plants inoculated on four leaves (total of 20 leaves). Symptom development was monitored visually 3–15 days after infiltration. The experiments were repeated at least three times on independent occasions, producing similar results in each case.

Phytophthora transformation

Stable transformation of *P. cactorum* was performed using a PEG/CaCl₂-mediated protoplast transformation protocol (Mcleod *et al.*, 2008) with minor modifications. Briefly, isolate 10 300 was grown on nutrient pea broth (NPB) agar plates (Mcleod *et al.*, 2008) for 4 days before being transferred into NPB broth for 2–3 days of cultivation. Transformants were selected on pea broth-mannitol (PM) agar medium (Mcleod *et al.*, 2008) containing 17.5 µg/mL G418 (geneticin) (Sangon Biotech). The plasmid

pGFPN containing both the *gfp* gene driven by the promoter of the *Bremia lactucae* *Ham34* gene and the *nptII* gene driven by the promoter of the *B. lactucae* *Hsp70* gene (Ah-Fong and Judelson, 2011) was introduced into *P. cactorum* using the above transformation strategy, and green fluorescence of the different cell types was observed using an Olympus BX53 fluorescence microscope at excitation and emission wavelengths of 488 nm and 495–530 nm, respectively.

For gene silencing, the plasmid pTOR-*scr96* was introduced into *P. cactorum* using the same protoplast transformation protocol, and putative SCR gene-silenced transformants were screened by quantitative RT-PCR using gene-specific primer, with the *PcUbc* gene (Table S3) serving as a constitutively expressed endogenous control.

Pathogenicity test

Zoospores of the WT isolate, transformation control and SCR gene-silenced transformants of *P. cactorum* were used to inoculate plants. The abaxial sides of detached leaves of *N. benthamiana* plants were inoculated with 1000 zoospores of each strain. Inoculated leaves were kept in 80% humidity and darkness at 25 °C, and inspected every 2 h for disease symptoms.

Trypan blue staining

Trypan blue staining was performed to detect cell death in *N. benthamiana* leaves. Leaves were soaked in Farmer's solution (95% ethanol–chloroform–glacial acetic acid, 6 : 3 : 1) for 30 s and then submerged in a 0.05% trypan blue mixture (Sigma-Aldrich, St. Louis, MO, USA) for 8 h in the dark. The leaves were rinsed with deionized water and then destained using multiple changes of boiling 95% ethanol. The infected areas of the leaves were then compared with those of the uninoculated controls and photographed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Protein sequence alignment of SCR96, SCR99, SCR121 and PcF identified in this study. The gene sequences of *scr96*, *scr99*, *scr121* and *PcF* were cloned from *Phytophthora cactorum* isolate 10 300. The deduced protein sequences were aligned and shaded for consensus (50% threshold for shading) using BioEdit.

Fig. S2 The growth of *Phytophthora cactorum* wild-type isolate 10 300 on nutrient pea broth (NPB) medium (Mcleod *et al.*, 2008) supplemented with geneticin (G418) at different concentrations. Photographs were taken at 4 days post-inoculation (dpi).

Fig. S3 *Phytophthora cactorum* transformant GFP1 expressing green fluorescent protein. (a) Mycelia. (b) Sporangia. (c) A germinating cyst. (d) Oospores.

Table S1 Summary of Illumina reads mapping to *Phytophthora cactorum* reference transcriptome by BLAST-Like Alignment Tool (BLAT) analysis.

Table S2 *Phytophthora cactorum* isolates used in this study.

Table S3 List of primers used in this study.