



The essential effector SCRE1 in *Ustilagoidea virens* suppresses rice immunity via a small peptide region

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SUMMARY

The biotrophic fungal pathogen *Ustilagoidea virens* causes rice false smut, a newly emerging plant disease that has become epidemic worldwide in recent years. The *U. virens* genome encodes many putative effector proteins that, based on the study of other pathosystems, could play an essential role in fungal virulence. However, few studies have been reported on virulence functions of individual *U. virens* effectors. Here, we report our identification and characterization of the secreted cysteine-rich protein SCRE1, which is an essential virulence effector in *U. virens*. When SCRE1 was heterologously expressed in *Magnaporthe oryzae*, the protein was secreted and translocated into plant cells during infection. SCRE1 suppresses the immunity-associated hypersensitive response in the nonhost plant *Nicotiana benthamiana*. Induced expression of SCRE1 in rice also inhibits pattern-triggered immunity and enhances disease susceptibility to rice bacterial and fungal pathogens. The immunosuppressive activity is localized to a small peptide region that contains an important ‘cysteine-proline-alanine-arginine-serine’ motif. Furthermore, the *scre1* knockout mutant generated using the CRISPR/Cas9 system is attenuated in *U. virens* virulence to rice, which is greatly complemented by the full-length *SCRE1* gene. Collectively, this study indicates that the effector SCRE1 is able to inhibit host immunity and is required for full virulence of *U. virens*.

Keywords: fungal effector, immunosuppressive peptide, rice immunity, SCRE1, *Ustilagoidea virens*.

INTRODUCTION

Ustilagoidea virens (telemorph *Villosiclava virens*) is an important fungus that causes false smut of rice (*Oryza sativa*). The

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disease spreads rapidly throughout the world and poses a significant economic impact (Rush *et al.*, 2000). Particularly in China and in South Asia, false smut has become one of the most important rice diseases (Tang *et al.*, 2013). *U. virens* infects rice flowers through stamen filaments at the late booting stage and develops false smut balls on the panicles at the heading stage (Han *et al.*, 2015; Hu *et al.*, 2014; Zhang *et al.*, 2014). In the formation of false smut balls during infection, the pathogen produces two major types of mycotoxins: ustiloxins and ustilaginoidins (Koiso *et al.*, 1994; Koyama and Natori, 1988). These mycotoxins inhibit cell division and impose a health threat to humans and animals (Koiso *et al.*, 1994). Because false smut is a newly emerging disease, little is known about the virulence mechanisms of *U. virens*, which makes this fungal disease difficult to predict and manage (Fang *et al.*, 2016; Han *et al.*, 2015; Zhang *et al.*, 2014).

Phytopathogenic fungi and oomycetes secrete a large number of effector proteins into the extracellular matrix (Alfano and Collmer, 2004; Galán and Collmer, 1999; Lo Presti *et al.*, 2015). Some of these effectors remain and function in the apoplast and are called apoplastic effectors, while others, named cytoplasmic effectors, enter the host cells (Giraldo and Valent, 2013). During host–pathogen coevolution, some pathogen effectors are recognized by host immune receptors to trigger plant immunity in resistant plants. In the absence of such recognition (i.e. in susceptible plants), many effectors subvert plant immunity and other host cellular processes to promote infection (Cui *et al.*, 2015; Oliveira-Garcia and Valent, 2015). Studies have shown that fungal effectors play virulence functions using a variety of molecular mechanisms. Multiple apoplastic effectors, such as Avr4 and Ecp6 from *Cladosporium fulvum*, Slp1 from *Magnaporthe oryzae* and Mg3LysM from *Mycosphaerella graminicola*, block the elicitation of chitin-triggered immunity (van Esse *et al.*, 2007; de Jonge *et al.*, 2010; Marshall *et al.*, 2011; Mentlak *et al.*, 2012). Avr4, Mg1LysM and Mg3LysM, as well as the effector SnTox1 from the

necrotrophic fungus *Parastagonospora nodorum* bind to chitin in the fungal cell wall and protect them against hydrolysis by plant chitinases (van Esse *et al.*, 2007; Liu *et al.*, 2012; Marshall *et al.*, 2011; Mentlak *et al.*, 2012). On the other hand, Ecp6 and Slp1 sequester chitin oligosaccharides that are released from the cell walls of invading hyphae to prevent activation of chitin-induced immunity (Chen *et al.*, 2014; de Jonge *et al.*, 2010; Mentlak *et al.*, 2012). The cytoplasmic effector Cmu1 of *Ustilago maydis* counteracts salicylic acid-induced immunity by functioning as a chorismate mutase to reduce the level of chorismate, a precursor for salicylic acid synthesis (Djamei *et al.*, 2011). In addition, the fungal effector candidate PpEC23 from *Phakopsora pachyrhizi* suppresses plant immunity and interacts with the transcription factor GmSPL12I, a negative immune regulator in soybean (Qi *et al.*, 2016a). Fungal effectors can also target host proteins residing in subcellular organelles. For example, the small cysteine-rich effector SsSSVP1 from *Sclerotinia sclerotiorum* interacts with the host mitochondrial protein QCR8 and facilitates *S. sclerotiorum* infection, probably through disrupting the host cell respiratory chain (Lyu *et al.*, 2016).

In addition, pathogen effectors often suppress plant immunity through targeting various types of catalytic enzymes. The Pep1 effector from *U. maydis* interacts with the secreted maize peroxidase POX12 and inhibits its activity, thereby suppressing the reactive oxygen species (ROS) burst in plants (Hemetsberger *et al.*, 2012). Another *U. maydis* effector, Pit2, inhibits a set of maize apoplastic cysteine proteases whose activities promote salicylic acid-associated plant defences (Mueller *et al.*, 2013). The *C. fulvum* apoplastic effector Avr2 also inhibits several cysteine proteases that contribute to plant basal defences (Shabab *et al.*, 2008). The rust transferred protein 1, a cytoplasmic effector, represents yet another family of fungal effectors that exhibit inhibitory activity toward plant proteases (Pretsch *et al.*, 2013). In addition, several effectors have been identified to target the host ubiquitin-proteasome system and inhibit plant immunity. For example, AvrPiz-t in *M. oryzae* binds to the rice RING E3 ligase APIP6 and inhibits its ubiquitination activity (Park *et al.*, 2012). APIP6 is required for ROS generation and defence against *M. oryzae* (Park *et al.*, 2012). APIP10, another E3 ligase that functions as a positive regulator of plant immunity in the non-*Piz-t* background in rice, is also a target of AvrPiz-t (Park *et al.*, 2016). In addition, PEC6 from the wheat stripe rust pathogen *Puccinia striiformis* interacts with adenosine kinases and suppresses pattern-triggered immunity (Liu *et al.*, 2016a).

Comparative transcriptome analyses suggest that a set of *U. virens* secreted proteins could be putative effectors (Han *et al.*, 2015; Zhang *et al.*, 2014). In a large-scale screening, eight *U. virens* putative effectors were identified to induce the immunity-associated hypersensitive responses when transiently overexpressed in the nonhost plant *Nicotiana benthamiana* (Fang *et al.*, 2016). On the other hand, when delivered

by the type III secretion system of the bacterium *Burkholderia glumae*, many putative *U. virens* effectors were found to suppress *B. glumae*-induced hypersensitive cell death in *N. benthamiana* (Zhang *et al.*, 2014). The *U. virens* secreted cysteine-rich effector (SCRE) SCRE2 (UV_1261) has been recently demonstrated to be an essential virulence factor (Fan *et al.*, 2019; Fang *et al.*, 2019).

In this study, we demonstrate that the small SCRE candidate 1 (SCRE1) from *U. virens* not only inhibits hypersensitive cell death in the nonhost *N. benthamiana* but also suppresses pattern-triggered immunity and enhances disease susceptibility in the host rice. When ectopically expressed in *M. oryzae*, SCRE1 is secreted and accumulated in the biotrophic interfacial complexes during infection, suggesting that SCRE1 is a cytoplasmic effector. The study identifies SCRE1 as an essential virulence effector in *U. virens*.

RESULTS

SCRE1 is a secreted protein in *U. virens*

It was predicted that UV_2799 encodes a putative secreted cysteine-rich effector (named thereafter SCRE1) in *U. virens* (Zhang *et al.*, 2014). We first used a yeast secretion system to verify the functionality of the SCRE1 signal peptide (Fang *et al.*, 2016; Jacobs *et al.*, 1997). The predicted signal peptide-encoding sequence of SCRE1 was fused in frame with a truncated *SUC2* gene that encodes invertase but lacks its own signal peptide. When the fusion construct was transformed into the YTK12 strain, the yeast strain restored the ability to grow on YPRAA medium (Fig. 1A). The N-terminal sequences of Avr1b and Mg87 were used as positive and negative controls, respectively. The results indicate that the predicted SCRE1 signal peptide is functional in yeast and can guide the secretion of invertase. Furthermore, pCAMBIA1301-*RP27::SCRE1-HA* was transformed into the *U. virens* strain P1 to express SCRE1-HA under the fungal *RP27* promoter (Bruno *et al.*, 2004). HA-tagged β -tubulin (UV_1410-HA), which is a major component of the eukaryotic cytoskeleton, was also expressed in *U. virens*. The results from immunoblotting showed that SCRE1-HA was detected in total cell lysate and in the culture medium, while β -tubulin-HA was only detected in total cell lysate, but not in the culture medium (Fig. 1B). More convincingly, the native β -actin was also only detectable in total cell lysates, but not in the culture medium of the transformed strains, indicating that no cytoplasmic protein was leaking into the culture medium (Fig. 1B). These results confirmed that SCRE1 is a secreted protein in *U. virens*.

Ectopically expressed SCRE1 in *M. oryzae* is translocated into plant cells during infection

Generally, effector genes are transcriptionally regulated during pathogen infection. To investigate the expression pattern

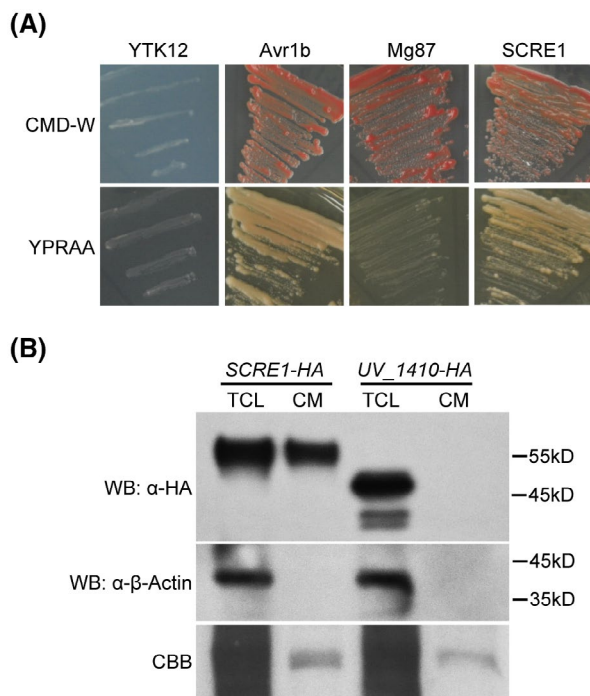


Fig. 1 SCRE1 is a secreted protein in *Ustilagoideae virens*. (A) The putative signal peptide of SCRE1 is functional to guide invertase secretion into culture medium (CM). YTK12 is an invertase secretion-deficient yeast strain. The secretion of invertase is indicated by the growth of YTK12 on YPRAA plates with raffinose as sole carbon source. The signal peptide of Avr1b and the first 25 amino acids of Mg87 were used for positive and negative controls, respectively. (B) SCRE1-HA was detected in CM and in the total cell lysate (TCL) via western blot (WB) analysis. The non-secreted β -tubulin protein UV_1410-HA and β -actin, as negative controls, were only detected in the TCL, but not in the CM. α -HA, anti-haemagglutinin antibody; α - β -actin, anti- β -actin antibody. CCB, Coomassie brilliant blue staining; SCRE1-HA, *U. virens* transformed with pCAMBIA1301-RP27::SCRE1-HA; UV_1410-HA, *U. virens* transformed with pCAMBIA1301-RP27::UV_1410-HA.

of *SCRE1*, the transcript level of *SCRE1* was quantified at different time points after inoculation via quantitative reverse transcription-PCR (RT-qPCR) analyses. *SCRE1* exhibited a strong up-regulated expression pattern during *U. virens* infection in the susceptible rice variety LYP9, with a peak of expression at 5 days after inoculation and a gradual decrease thereafter (Fig. 2A).

Next, living cell imaging was used to investigate whether SCRE1 is secreted and translocated into plant cells using a heterologous system. SCRE1 carrying a nuclear localization signal (NLS) from simian virus large T-antigen (Ai *et al.*, 2007) was ectopically expressed as a fusion with mCherry in *M. oryzae* driven by the *RP27* promoter. The conventional NLS is sufficient for nuclear localization of fusion proteins and thus facilitates visualization of mCherry fluorescence (Park *et al.*, 2012). The engineered *M. oryzae* strains were inoculated onto detached barley leaves. Red fluorescence was detectable inside the invasive hyphae of mCherry-labelled *M. oryzae* by microscopy at 30 h after

inoculation (Fig. 2B,C). The majority of barley epidermal cells infected by *SCRE1*-mCherry-NLS-transformed *M. oryzae* exhibited red fluorescence in the nuclei at 30 h after inoculation (Fig. 2B,C). However, red fluorescence was only observed in the hyphae of mCherry-NLS expressing *M. oryzae* during infection (Fig. 2D).

Cytoplasmic effectors in *M. oryzae* are generally accumulated in a novel structure called the biotrophic interfacial complex (BIC), where some effectors are translocated into rice cytoplasm during infection (Khang *et al.*, 2010). To further investigate SCRE1 translocation in *M. oryzae*, the Guy11 strain was transformed with pYF11-*RP27*::*SCRE1*-GFP and then inoculated into detached rice sheaths. At 30 h after inoculation of *M. oryzae*, green fluorescence from SCRE1-GFP was clearly observable in BICs by confocal microscopy (Fig. 2E). As a positive control, green fluorescence was visible in the BICs after the *M. oryzae* strain expressing AVR-Pia-GFP was inoculated into rice sheaths (Fig. 2E). Collectively, these data indicate that SCRE1, when expressed in *M. oryzae*, is secreted and translocated through BICs during infection, and thus is most likely a cytoplasmic effector.

SCRE1 is required for full virulence of *U. virens* to rice

To investigate whether *SCRE1* plays a role in *U. virens* virulence, we generated the *scre1* mutants using a modified CRISPR/Cas9 system. Five, six and five gene replacement mutants were preliminarily identified out of 32, 18 and 18 transformants via PCR screening using three single guide (sg) RNAs targeting different regions of *SCRE1*, respectively. Four independent mutants, *scre1-1-10*, *scre1-1-26*, *scre1-2-4* and *scre1-3-23*, were confirmed by Southern blot analysis (Fig. S1A). The *scre1-1-10*, *scre1-2-4* and *scre1-3-23* mutants generated from different sgRNAs were subsequently injected into rice panicles before heading for virulence assays. The disease incidence rate of panicles inoculated with the wild-type P1 strain was much higher than those inoculated with the three mutants. Consistently, the number of diseased grains per P1-inoculated panicle was significantly greater than that caused by these mutants (Figs 3 and S1B). Furthermore, the complementary strain was created by introducing the plasmid-borne *SCRE1* gene into the *scre1-10* mutant. Inoculation assays showed that the virulence of the complementary strain was partially restored (Fig. 3). These data indicate that *SCRE1* is required for the virulence of *U. virens*.

SCRE1 suppresses immunity-associated hypersensitive cell death in *N. benthamiana*

To determine the immunosuppressive ability of SCRE1, we demonstrated here that *B. glumae* carrying pEDV-*SCRE1*-HA caused a considerably reduced nonhost cell death response in *N. benthamiana* leaves compared with the wild-type *B. glumae* (Fig. S2A). Immunoblotting assays showed that SCRE1-HA was expressed in *B. glumae* and secreted into culture medium when bacteria

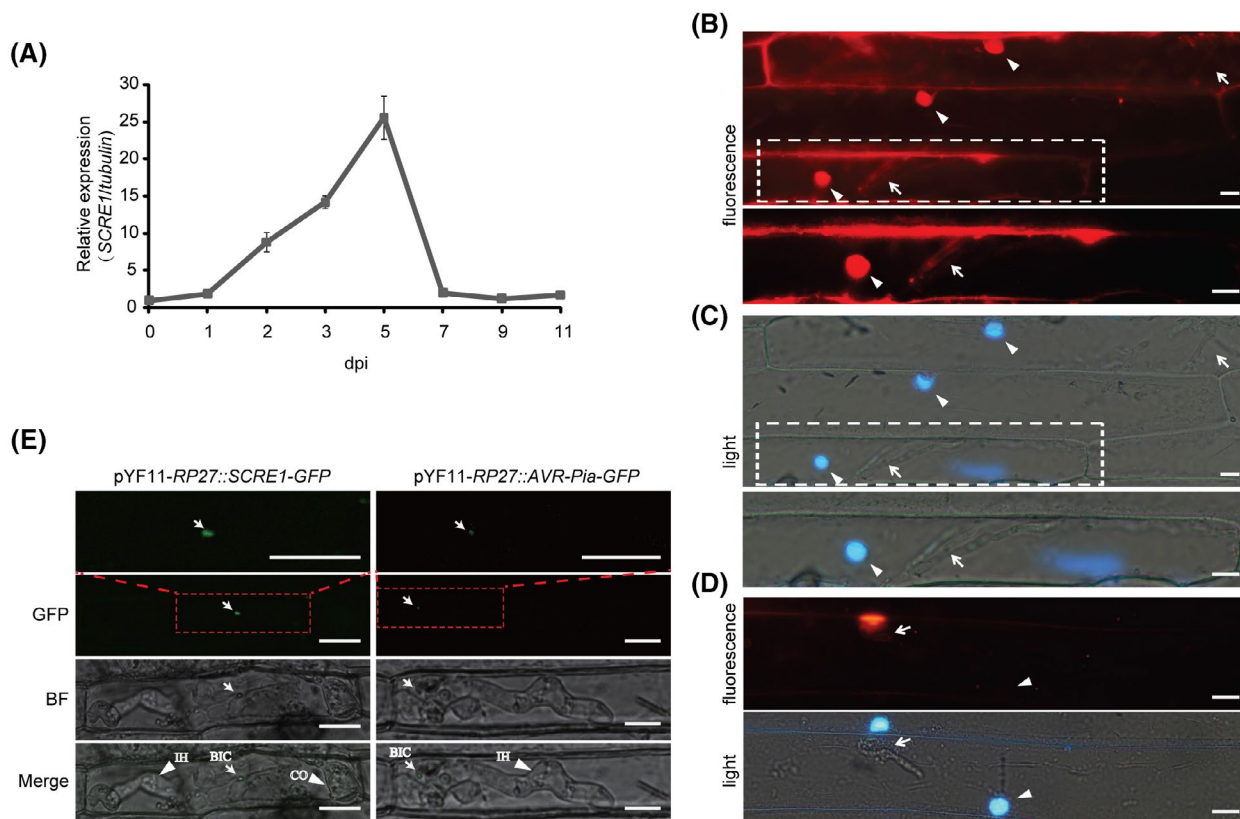


Fig. 2 SCRE1 is an effector in *Ustilagoideae virens* as revealed by expression and translocation assays. (A) SCRE1 expression was dramatically induced and peaked at 5 days post-inoculation (dpi) and gradually decreased thereafter during *U. virens* infection. SCRE1 expression was normalized to the internal reference *tubulin* gene. (B), (C) Red fluorescence was clearly observed in barley cell nuclei and invasive hyphae of *Magnaporthe oryzae*. The fluorescence (B) and light (C) microscopy images were taken 30 h after barley leaves were inoculated with *M. oryzae* carrying pKS-RP27::SCRE1-mCherry-NLS. The barley nuclei stained with DAPI are indicated by arrowheads, while *M. oryzae* invasive hyphae are indicated by arrows. The images in lower panels are enlarged from the regions in broken squares in upper panels. (D) Red fluorescence was only observed in *M. oryzae* hyphae, but not in barley nuclei. The fluorescence (upper panel) and light (lower panel) microscopy images were taken 30 h after barley leaves were inoculated with *M. oryzae* carrying pKS-RP27::mCherry-NLS. The nuclei stained with DAPI are indicated by arrowheads, while *M. oryzae* hyphae are indicated by arrows. (E) Green fluorescence was clearly observed in biotrophic interfacial complexes during the infection of *M. oryzae* strains transformed with pYF11-RP27::SCRE1-GFP and pYF11-RP27::AVR-Pia-GFP. The transformed *M. oryzae* Guy11 strains were inoculated into rice sheaths. The images were captured by confocal microscopy 30 h after inoculation. The images in the uppermost panels are enlarged from the regions in broken squares in the GFP panels. GFP, green fluorescent protein; BF, bright field; Merge, the overlay of GFP and BF images; IH, invasive hyphae; CO, conidia; BIC, biotrophic interfacial complex. Scale bar: 10 μ M.

were cultured in the type III secretion-inducing minimal medium (Fig. S2B). This result suggests that the delivery of SCRE1 via the bacterial type III secretion system into plant cells can suppress *B. glumae*-triggered hypersensitive responses (HR) in *N. benthamiana*. To elaborate this result, we demonstrated that transient expression of SCRE1 in *N. benthamiana* inhibited hypersensitive cell death triggered by the *Phytophthora infestans* elicitor INF1 (Fig. 4A) (Kamoun *et al.*, 1998). Similarly, SCRE1 also suppressed cell death in *N. benthamiana* induced by the mammalian protein BAX, which has been shown to cause HR in numerous plant species (Lam *et al.*, 2001; Oltval *et al.*, 1993) (Fig. 4A). Furthermore, co-expression of SCRE1 without signal peptide also suppressed INF1- and BAX-triggered HR in *N. benthamiana* (Fig. 4A). Electrolyte leakage in *N. benthamiana* triggered by INF1 and BAX was significantly

attenuated in the presence of SCRE1-FLAG compared with co-expression with FLAG-tagged green fluorescent protein (GFP-FLAG) (Fig. 4B). Western blot analysis showed that BAX-FLAG was well produced when co-expressed with SCRE1-FLAG and GFP-FLAG in *N. benthamiana* leaves (Fig. 4C). We further investigated subcellular localization of SCRE1/SCRE1-SP when mCherry-labelled proteins were transiently expressed in *N. benthamiana* cells. After plasmolysis, neither SCRE1-mCherry nor SCRE1-SP-mCherry was visible in periplasmic spaces (Fig. S3). As controls, SCRE2-mCherry was clearly observed in periplasmic spaces of plasmolysed cells, while SCRE2-SP-mCherry was not (Fig. S3; Fang *et al.*, 2019). Collectively, these data suggest that SCRE1, as a secreted protein, is taken up inside plant cells and functions as a suppressor of immunity-associated cell death in plants.

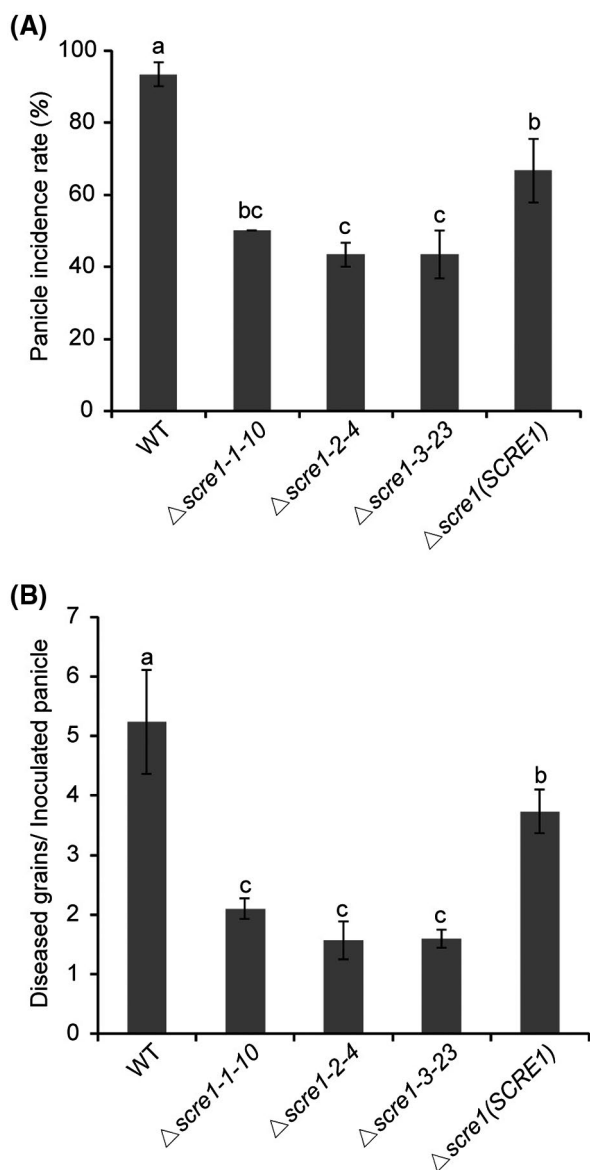


Fig. 3 *SCRE1* is required for full virulence of *Ustilaginoidea virens* to rice. The panicle incidence rate (%) (A) and average diseased grains per inoculated panicle (B) after injection inoculation of the wild-type, different *scre1* mutant and complementary strains in *U. virens*. The complementary strain was generated through transforming plasmid-borne full-length *SCRE1* gene into the *scre1-1-10* mutant. Different letters above error bars indicate statistically significant differences among the wild-type, *scre1* mutant and complementary strains of *U. virens*. Data shown are combined from three independent inoculation experiments. Error bars represent means \pm standard error (SE).

SCRE1 suppresses pattern-triggered immunity and promotes pathogen infection in rice

To investigate whether SCRE1 suppresses rice immunity, we generated 22 transgenic rice lines with dexamethasone (DEX)-inducible expression of SCRE1. Three transgenic lines, Z178, Z195 and Z196, with strong DEX-induced expression and no

detectable basal expression of SCRE1, were chosen for further studies (Fig. S4A). Chitin and flg22 triggered a strong and rapid generation of ROS in the wild-type, Z195 and Z196 transgenic rice plants without DEX induction. The induced oxidative burst was significantly suppressed in these transgenic plants after DEX treatment. In contrast, DEX treatment had no effect on flg22- and chitin-triggered ROS burst in the wild-type plants (Figs 5A,B and S4B–E), indicating that SCRE1 inhibits microbe-associated molecular pattern (MAMP)-triggered oxidative burst. Quantitative RT-PCR assays showed that expression of the defence marker genes *OsPR10a* and *OsPR1b* was strongly up-regulated by flg22 and chitin in the wild-type, Z178 and Z196 transgenic seedlings without DEX treatment. By contrast, flg22- and chitin-induced expression of these defence-related genes was significantly suppressed in the DEX-treated transgenic lines Z178 and Z196 (Figs 5C,D and S4F–G). Thus, transgenically expressed SCRE1 suppresses rice pattern-triggered immunity.

To investigate whether SCRE1 affects disease susceptibility in rice, the transgenic lines Z178, Z195 and Z196 were infected by *Xanthomonas oryzae* pv. *oryzae* PXO99 (a major bacterial pathogen of rice) or *M. oryzae* strain S5 (a major fungal pathogen of rice). The three transgenic lines after DEX treatment all exhibited significantly longer disease lesions on PXO99-inoculated leaves than the same transgenic lines without DEX treatment and the wild-type plants (Figs 5E and S4H). Similarly, *M. oryzae* inoculation caused significantly greater disease lesions on the leaves of DEX-treated transgenic lines than on the mock-treated wild-type and transgenic plant leaves (Figs 5F and S4I). These results demonstrated that transgenic expression of SCRE1 compromises rice basal defences against both bacterial and fungal pathogens.

SCRE1⁴⁵⁻⁷⁰ is an essential fragment for the virulence function of SCRE1

SCRE1 contains no known conserved functional domain but two types of tandem and almost identical repeats, each repeat containing two Cys residues (Fig. S5A). To delineate the region in SCRE1 that is essential for its virulence ability, a series of truncated SCRE1 variants were expressed in *N. benthamiana* (Fig. S5B). A small peptide that contains the 45–70th amino acid residues (SCRE1⁴⁵⁻⁷⁰) and the truncated SCRE1 proteins including this fragment had a similar ability as full-length SCRE1 to suppress INF1- or BAX-induced hypersensitive responses and/or electrolyte leakage in *N. benthamiana* (Figs 6A and S5B). In contrast, other small peptides that contain only part of this fragment, such as SCRE1⁵⁰⁻⁷⁰ and SCRE1⁴⁵⁻⁶⁵, largely lost the HR-suppressive ability in *N. benthamiana*. Furthermore, alanine-scanning mutagenesis was performed to generate five multiple-point mutations in the 45–70 amino acid region of SCRE1 (Fig. 6B). Transient expression of SCRE1^{45AAAAA49}

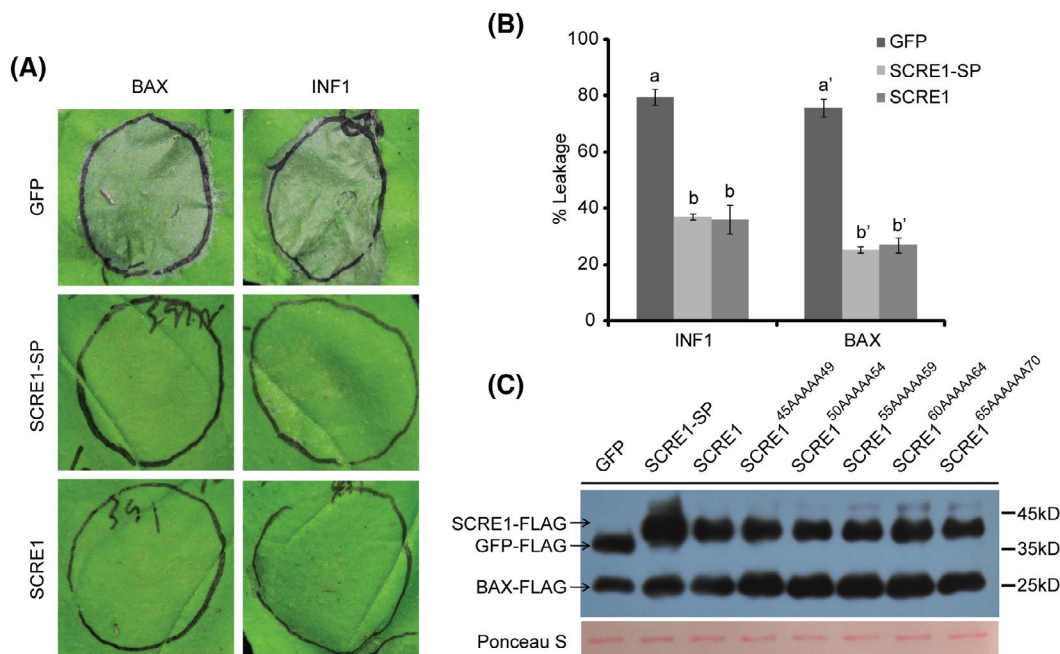


Fig. 4 SCRE1 suppresses different types of immunity-associated hypersensitive cell death in *Nicotiana benthamiana*. (A) Co-expression of SCRE1 or truncated SCRE1 without signal peptide (SCRE1-SP) suppressed BAX- (left panel) and INF1- (right panel) triggered hypersensitive cell death in *N. benthamiana*. (B) SCRE1/SCRE1-SP expression, in contrast to green fluorescent protein (GFP) expression, significantly suppressed BAX- and INF1-induced electrolyte leakage in *N. benthamiana*. Different letters over error bars indicate significant difference in BAX- and INF1-induced electrolyte leakage of *N. benthamiana* leaves between SCRE1/SCRE1-SP co-expression and GFP co-expression ($P < 0.05$). (C) SCRE1-FLAG and GFP-FLAG co-expression imposed no evident effect on BAX-FLAG expression in *N. benthamiana* leaves. Upper panel: the proteins isolated from the agroinfiltrated leaves were detected via immunoblotting with anti-FLAG antibody (α -FLAG). The bands of SCRE1-FLAG, GFP-FLAG and BAX-FLAG are indicated by arrows. Lower panel: protein loading is indicated by Ponceau S staining.

(the ⁴⁵CPARS⁴⁹ motif replaced by AAAAA) lost the ability to suppress BAX- and INF1-induced ion leakage, while the other four mutant proteins retained the immunosuppressive ability in *N. benthamiana* (Fig. 6C,D). These mutant proteins were well expressed in *N. benthamiana* (Fig. 4C). To further confirm these findings, we purified the truncated SCRE1¹⁻¹⁷⁹ from *Escherichia coli* (full-length SCRE1 was not well expressed in *E. coli*). Infiltration of purified SCRE1¹⁻¹⁷⁹ into *N. benthamiana* leaves was able to suppress BAX- or INF1-triggered cell death, while purified SCRE1^{1-179(45AAAAA49)} did not (Fig. 6E). Consistently, SCRE1¹⁻¹⁷⁹ infiltration significantly alleviated BAX- or INF1-triggered electrolyte leakage from *N. benthamiana* leaves, while SCRE1^{1-179(45AAAAA49)} did not (Fig. 6F). Taken together, these data define the 45–70 amino acid residues of SCRE1, particularly the ⁴⁵CPARS⁴⁹ motif, as the essential domain for the virulence function of SCRE1.

To investigate whether purified SCRE1 has the ability to suppress rice immunity, flg22- and chitin-triggered immunity was examined in SCRE1¹⁻¹⁷⁹-pretreated rice seedlings. As expected, expression of *OsPR10a* and *OsPR1b* was induced by flg22 and chitin in mock-pretreated rice seedlings. In contrast, flg22- and chitin-triggered expression of *OsPR10a* and *OsPR1b* was significantly attenuated in SCRE1¹⁻¹⁷⁹-pretreated seedlings, while SCRE1^{1-179(45AAAAA49)} pretreatment had no effect on flg22- or

chitin-induced expression of *OsPR10a* and *OsPR1b* (Fig. 7A,B). Furthermore, flg22- and chitin-triggered MAPK activation in rice seedlings was also greatly dampened with SCRE1¹⁻¹⁷⁹ pretreatment, but not with SCRE1^{1-179(45AAAAA49)} (Fig. 7C). Collectively, these results showed that purified SCRE1¹⁻¹⁷⁹ suppresses pattern-triggered rice immunity and that the ⁴⁵CPARS⁴⁹ motif is critical for its suppression activity.

DISCUSSION

Rice false smut is an emerging global disease and poses significant threats to rice production and human health. Currently, little is known about virulence functions of *U. virens* effectors (Rush *et al.*, 2000; Tang *et al.*, 2013; Zhang *et al.*, 2014). Effectors play essential roles in fungal virulence (Lo Presti *et al.*, 2015; Oliveira-Garcia and Valent, 2015). However, despite genomics-based predictions, few effectors have been functionally characterized in *U. virens* (Fang *et al.*, 2016). In this study, we identified an essential virulence effector, SCRE1, in *U. virens* and provided evidence that this effector suppresses rice immunity.

In *U. virens*, 193 secreted proteins have been predicted to be potential effectors based on sequence and structural features (Zhang *et al.*, 2014). Through a large-scale high-throughput

screen, many putative *U. virens* effectors, including SCRE1, were previously shown to suppress *B. glumae*-induced HR-like cell death in the nonhost plant *N. benthamiana* (Zhang *et al.*, 2014). In this study, we showed that SCRE1 also has the ability to inhibit HR-like cell death induced by BAX and INF1 in *N. benthamiana* (Fig. 4). Furthermore, transgenically expressed SCRE1 compromises the expression of defence-marker genes and the oxidative burst triggered by flg22 and chitin in rice plants (Fig. 5A–D) and the SCRE1 transgenic plants were more

susceptible to both bacterial and fungal pathogens (Fig. 5E,F). These results are consistent with the hypothesis that SCRE1 is a fungal effector that is involved in immunosuppression. SCRE1 has other features that are characteristic of a genuine fungal effector protein: it is transcriptionally induced during infection and secreted by *U. virens* (Figs 1 and 2). In addition, red fluorescence from SCRE1-mCherry-NLS was clearly visible in barley nuclei after infection by *M. oryzae* with heterologous expression of SCRE1-mCherry-NLS, indicating that SCRE1 is translocated

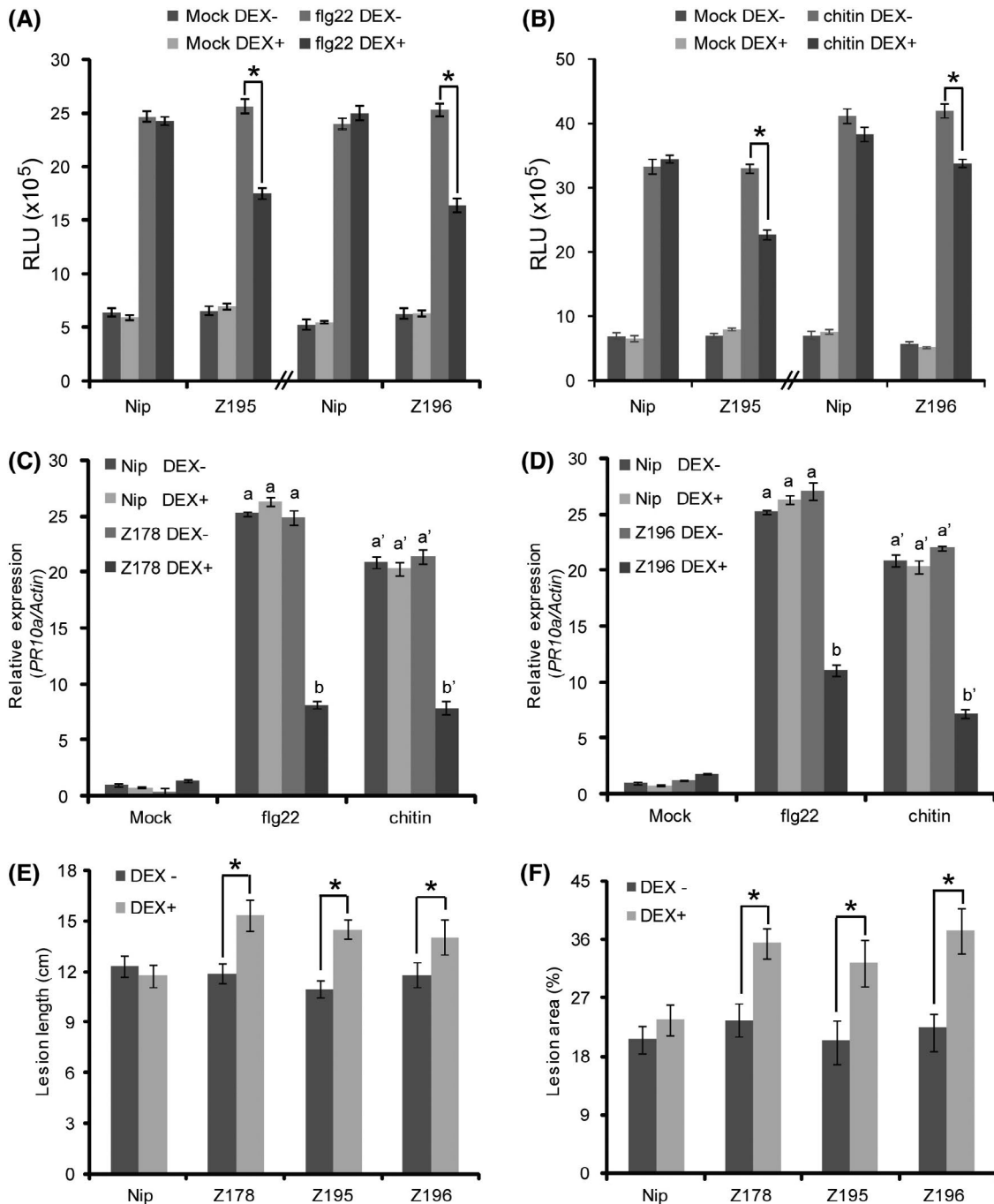


Fig. 5 Ectopic expression of SCRE1 suppresses pattern-triggered immunity and promotes pathogen infection in rice. (A), (B) Dexamethasone (DEX)-induced expression of SCRE1 inhibited the oxidative burst triggered by flg22 (A) and chitin (B) in the Z195 and Z196 transgenic rice plants. Leaf disks collected from the wild-type, Z195 and Z196 transgenic plants were immersed in DEX (10 μ M) and mock solution for 16 h followed by treatment of flg22 (10 μ M) and hexa-*N*-acetyl-chitohexaose (8 μ M). The area under the curve for 30-min oxidative burst (relative luminescence units \times time, see Fig. S4B–E) was determined for the wild-type and transgenic plants after different treatments. Asterisks indicate statistical significance in oxidative burst from flg22- and chitin-treated transgenic rice leaves with and without DEX treatment ($P < 0.05$). Histogram shows means \pm SE for nine leaf disks from different plants. RLU, relative luminescence unit. (C), (D) DEX-induced expression of SCRE1 significantly inhibited *OsPR10a* expression triggered by flg22 and chitin in the Z178 (C) and Z196 (D) transgenic lines. The wild-type and transgenic seedlings were treated with DEX (10 μ M) and mock solution for 24 h followed by flg22 (1 μ M) and chitin (10 μ g/mL). *OsPR10a* expression was detected by RT-qPCR and normalized to the reference gene *OsActin*. Different letters above error bars indicate statistically significant differences in the *OsPR10a* transcript levels in the wild-type and transgenic lines under different treatments ($P < 0.05$). (E), (F) SCRE1 expression in the transgenic lines significantly promoted pathogen infection after inoculation of rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (E) and rice blast fungus *Magnaporthe oryzae* (F). The wild-type and transgenic plants were challenged with pathogens at 24 h after spraying with DEX (10 μ M) and mock solution. At least 12 leaves were measured for lesion lengths at 12 days after bacterial inoculation. Lesion areas on 20 inoculated leaves were photographed at 6 days after fungal inoculation and calculated using Adobe Photoshop. Asterisks indicate statistically significant differences in disease lesions on the inoculated leaves with and without DEX treatment. Error bars represent means \pm SE. Nip, Nipponbare; Z178, Z195 and Z196, three independent transgenic lines; DEX–, no dexamethasone treatment; DEX+, with dexamethasone treatment.



into plant cells after secretion (Fig. 2B,C). Many cytoplasmic effectors in *M. oryzae*, such as BAS1, PWL2 and AVR-Pia, have been demonstrated to be secreted into the BIC structure and translocated to rice cytoplasm during *M. oryzae* infection (Khang *et al.*, 2010; Mosquera *et al.*, 2009; Sornkom *et al.*, 2017; Yoshida *et al.*, 2009). Here, we showed that ectopically expressed SCRE1-GFP in *M. oryzae* was accumulated in the BIC structure during infection of rice cells (Fig. 2E), indicating that SCRE1 is a cytoplasmic effector. In oomycetes, the RxLR-dEER motifs are sufficient for transit of cytoplasmic effectors into host cells (Dou *et al.*, 2008). However, autonomous translocation motifs for fungal effectors are still mysterious, although some cytoplasmic fungal effectors have been demonstrated to be transported into plant cells (Kale *et al.*, 2010; Manning and Ciuffetti, 2005; Rafiqi *et al.*, 2010). Furthermore, multiple independent *scre1* mutants of *U. virens* all exhibited a significant reduced virulence to rice and the plasmid-borne full-length *SCRE1* gene significantly restored the virulence of the mutant (Fig. 3), indicating that *SCRE1* is required for the virulence of *U. virens*. Taken together, we conclude that SCRE1 is an important cytoplasmic effector with virulence functions in *U. virens*.

Interestingly, we revealed that the truncated SCRE1 without its signal peptide retained HR-suppressive ability (Fig. 4A). In contrast, our previous study demonstrated that signal peptides of the *U. virens* secreted proteins that trigger cell death were all required for their HR-inducing ability. The difference is probably because the HR-inducing and immunosuppressive effectors might function in different locations. Similar to the HR-inducing effectors in *M. oryzae* (Chen *et al.*, 2013), HR-inducing effectors in *U. virens* likely function in apoplastic spaces while cytoplasmic effectors including SCRE1 might be transported inside host cells after they are secreted into periplasmic spaces by fungi. This speculation is supported by the subcellular localization assay of SCRE1-mCherry, demonstrating that red fluorescence from SCRE1-mCherry was localized predominantly inside the cells and was hardly visible in periplasmic spaces of plasmolysed *N. benthamiana* cells (Fig. S3).

The purified SCRE1¹⁻¹⁷⁹ had the ability to inhibit BAX- and INF1-triggered HR-like symptoms in nonhost *N. benthamiana* and to compromise pattern-triggered immunity in host rice seedlings (Figs 6E,F and 7). This finding indicates that SCRE1 in the extracellular matrix might be translocated inside the cells through an unknown mechanism. However, we cannot completely rule out the possibility of SCRE1 functioning as an apoplastic effector. Although no known conserved functional domain or motif was found in SCRE1, we discovered that SCRE1 possesses two types of Cys-containing tandem repeats (Fig. S5A). Through a series of truncations of SCRE1, we were able to define the key immunosuppressive region of SCRE1. Remarkably, a small 26 amino acid peptide containing amino acid residues 45–70, SCRE1⁴⁵⁻⁷⁰, was found to be sufficient for the HR-inhibitory ability in *N. benthamiana* (Figs 6 and S5). It is notable that transient expression of the small peptide SCRE1⁴⁵⁻⁷⁰ had an even stronger inhibition effect on INF1- and BAX-induced cell death than full-length SCRE1 (Fig. 6A). The flg22 peptide functions more effectively as an elicitor of defence responses than flagellin (Felix *et al.*, 1999). We propose two explanations for this phenomenon: (i) small peptides are more easily accessible to plant cells, and (ii) functional peptides are somewhat covered by other fragments of full-length proteins. It is well known that small peptides can induce plant immunity (Yamaguchi and Huffaker, 2011). However, to our knowledge, few studies have reported that a small peptide is able to disarm plant immunity. Furthermore, using site-directed mutagenesis, we identified a 5-amino-acid motif, ⁴⁵CPARS⁴⁹, that is critical for the immunosuppressive function of SCRE1 (Fig. 6). Interestingly, the HR-suppressive region (i.e. 45–70th amino acids) of SCRE1 encompasses mostly the sequence between the first tandem repeats (Fig. S5A). The finding suggests that the tandem repeats themselves in SCRE1 are not directly associated with its immunosuppressive activity. The role of these repeats, if any (e.g. in stabilizing SCRE1 protein *in vivo*), needs to be further investigated. Most recently, the conserved 14-amino-acid motif (PID14) in *U. maydis* Pit2 has been shown to function as an inhibitor of papain-like cysteine proteases to suppress plant immunity (Villamil *et al.*, 2019). In future studies,

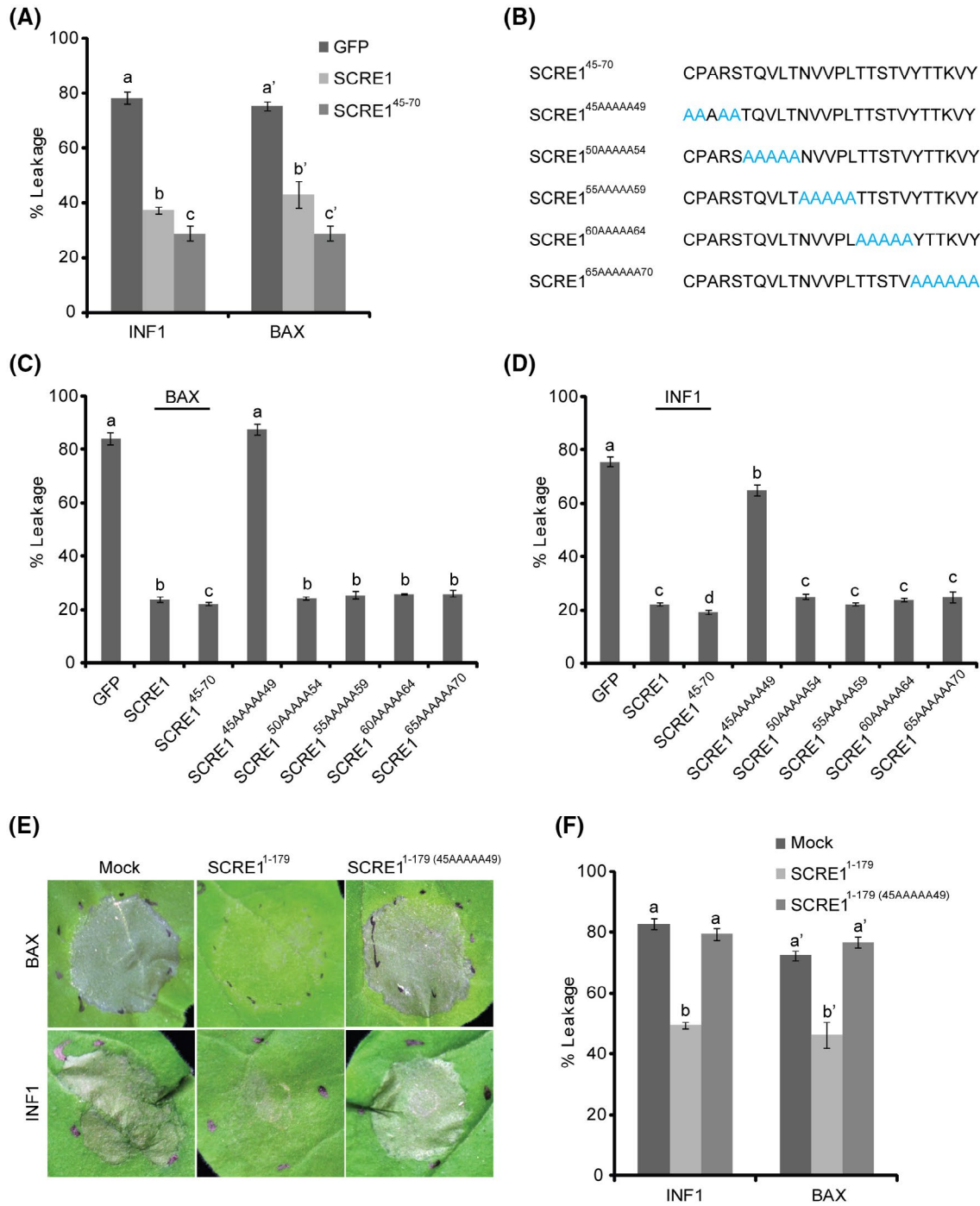


Fig. 6 SCRE1⁴⁵⁻⁷⁰ is an essential region that suppresses BAX- and INF1-triggered cell death in *Nicotiana benthamiana*. (A) Transient expression of SCRE1⁴⁵⁻⁷⁰ had an even stronger ability than full-length SCRE1 to inhibit BAX- and INF1-triggered electrolyte leakage in *N. benthamiana*. BAX and INF1 were transiently expressed at 6 h after expression of green fluorescent protein (GFP), SCRE1 and SCRE1⁴⁵⁻⁷⁰ in *N. benthamiana*. Letters above error bars indicate statistically significant differences in BAX- and INF1-triggered electrolyte leakage when co-expressing with different proteins in *N. benthamiana*. (B) Diagrams of alanine-scanning mutagenesis in the 45–70th residues of full-length SCRE1. (C), (D) SCRE1^{45AAAAA49}, in which the 45–49th residues of SCRE1 were all replaced with Ala, significantly lost the ability to suppress BAX- (C) and INF1- (D) induced ion leakage in *N. benthamiana*. Different letters (a–d) indicate statistically significant differences in BAX- and INF1-triggered electrolyte leakage in *N. benthamiana*. BAX and INF1 were expressed at 6 h after expression of the indicated proteins. (E), (F) The Ala replacement in the 45–49th residues of SCRE1¹⁻¹⁷⁹ greatly disrupted the ability of *in vitro*-purified SCRE1¹⁻¹⁷⁹ to suppress BAX- and INF1-induced hypersensitive response symptoms (E) and ion leakage (F). *In vitro*-purified proteins (1 µM) were co-infiltrated with *Agrobacterium* strains carrying pGR107-BAX and pGR107-INF1 constructs into *N. benthamiana* leaves. Hypersensitive response-like symptoms and electrolyte leakage in the infiltrated leaves were investigated at 5 days post-infiltration.

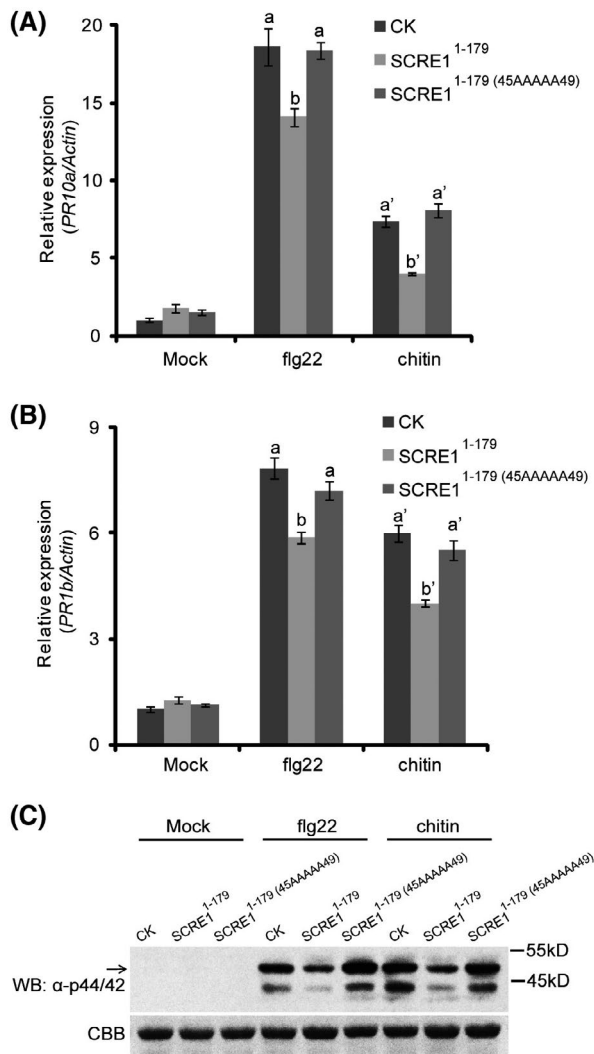


Fig. 7 SCRE1 compromises pattern-triggered immunity in rice. (A), (B) The flg22- and chitin-induced expression of *OsPR10a* (A) and *OsPR1b* (B) was significantly attenuated in SCRE1¹⁻¹⁷⁹-pretreated rice seedlings, but not in mock- and SCRE1^{1-179(45A444A49)}-pretreated seedlings. Rice seedlings were incubated with 1 μ M of SCRE1¹⁻¹⁷⁹, SCRE1^{1-179(45A444A49)} or mock solution for c. 12 h followed by treatment with flg22 (1 μ M) or chitin (10 μ g/mL) for 6 h. The expression level of *OsPR10a* and *OsPR1b* was detected by quantitative RT-PCR and normalized to that of the reference gene *OsActin*. Different letters above error bars indicate significant differences in the transcript levels of *PR* genes among SCRE1¹⁻¹⁷⁹, SCRE1^{1-179(45A444A49)} and mock treatments. (C) The flg22- and chitin-induced MAPK phosphorylation was greatly inhibited in the SCRE1¹⁻¹⁷⁹-pretreated rice seedlings, but not in the SCRE1^{1-179(45A444A49)}-pretreated seedlings. The SCRE1¹⁻¹⁷⁹-pretreated seedlings were treated by flg22 and chitin for 15 min and were then collected for protein extraction. MAPK activation was detected through immunoblotting with anti-Phospho-p44/42 MAPK antibody (α -p44/42). Lower panel: protein loading is indicated by Coomassie brilliant blue (CBB) staining.

it would be worthwhile identifying host targets of SCRE1 and clarifying whether the immunosuppressive peptide in SCRE1 functions in a similar way to PID14 as an enzyme inhibitor.

EXPERIMENTAL PROCEDURES

Plant materials, bacterial and fungal strains

Oryza sativa subsp. *japonica* 'Nipponbare', *N. benthamiana* and *Hordeum sativa* plants were grown in the greenhouse or in the growth chamber. Bacterial and fungal strains are listed in Table S1. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) medium. The other bacteria and fungi used were grown at 28 °C. *B. glumae* and *A. tumefaciens* strains were cultured in LB medium, *X. oryzae* pv. *oryzae* PXO99 in nutrient broth, *M. oryzae* in oatmeal-tomato paste medium, *U. virens* isolates P1 in potato sucrose medium, and yeast strains in yeast extract-peptone-dextrose medium, unless specifically noted. The PCR primers used in this study are listed in Table S2.

Agrobacterium-mediated transient assays in *N. benthamiana*

SCRE1 fragments were amplified using the primers in Table S2. The pGR107 constructs (Jones *et al.*, 1999) were transformed into *A. tumefaciens* strains using the freeze-thaw method (An *et al.*, 1988). Overnight-cultured *A. tumefaciens* cells were collected and resuspended in the infiltration buffer (10 mM MES, pH 5.7, 10 mM MgCl₂, 150 μ M acetosyringone). After being incubated at room temperature for 2 h, *A. tumefaciens* cells were pressure-infiltrated into the leaves of 4–6-week-old *N. benthamiana* plants at an OD₆₀₀ of 0.2 for INF1 and BAX expression or OD₆₀₀ = 0.5 for other protein expression. *A. tumefaciens* strains carrying pGR107-INF1 or pGR107-BAX were infiltrated together with different truncated constructs of SCRE1 or at 6 h after infiltration of different SCRE1 constructs. Subcellular localization of SCRE1-mCherry and SCRE1-SP-mCherry was performed as described previously (Fang *et al.*, 2019).

Electrolyte leakage measurement

Electrolyte leakage in the inoculated *N. benthamiana* leaves was measured as described previously (Fang *et al.*, 2016).

The yeast secretion assay

The yeast invertase secretion assay was performed using the SP trap vector pSUC2 as previously described (Jacobs *et al.*, 1997). Briefly, the signal sequence of SCRE1 was PCR-amplified and subcloned into pSUC2. The pSUC2-SCRE1(SP) construct was transformed into the invertase-deficient yeast strain YTK12 (SUC2⁻) as described previously (Jacobs *et al.*, 1997). The transformants were cultured on CMD-W (0.67% yeast N base, 0.075% tryptophan drop-out supplement, 2% sucrose, 0.1% glucose, 2% agar) or YPRAA (1% yeast extract, 2% peptone, 2% raffinose, 2 μ g/L antimycin A, 2% agar) plates for invertase secretion assays.

Agrobacterium-mediated transformation of *U. virens*

The fungal promoter *RP27* (Bruno *et al.*, 2004) was PCR-amplified and cloned into pCAMBIA1301. The $3 \times HA$ coding sequence was amplified from pGWB14 and cloned into pCAMBIA1301-*RP27*. The *SCRE1* coding sequence was amplified and ligated into pCAMBIA1301-*RP27*- $3 \times HA$. The construct was then transformed into *A. tumefaciens* strain AGL1. *Agrobacterium*-mediated *U. virens* transformation was performed as described with some modifications (Mullins *et al.*, 2001). Briefly, the *U. virens* P1 culture was filtered through lens paper. The conidia were collected from the filtrate by centrifugation. *A. tumefaciens* was cultured in LB medium at 28 °C for 12 h with shaking. *A. tumefaciens* cells were collected and resuspended in co-cultivation medium to a cell density of $OD_{600} \approx 0.15$, and were further cultured for 6 h. The culture was mixed with equal volume of P1 conidial suspension (10^6 spores/mL) and spread onto nitrocellulose membrane (Whatman, GE Healthcare, Amersham, UK) placed on co-cultivation medium plates. After the plates had been incubated for 3 days, the nitrocellulose membrane was transferred to TB3 (3 g/L yeast extract, 3 g/L casamino acids, 2% sucrose, 1.2% agar) plates containing 100 µg/mL of hygromycin B for transformant selection. Individual colonies appearing after 5–6 days of incubation were transferred to TB3 for further identification.

SCRE1 secretion assays

B. glumae transformed with pEDV-*SCRE1*-*HA* was cultured in the *hrp*-inducing SMMXC medium (Sun *et al.*, 2011). The wild-type *U. virens* and transformants carrying pCAMBIA1301-*RP27*::*SCRE1*-*HA* and pCAMBIA1301-*RP27*::*UV_1410*-*HA* were cultured in potato-sucrose medium for 5–7 days with shaking. The culture medium were collected by consecutive filtrations through filter paper and a 0.45 µm filter. Proteins in the medium were precipitated by 10% trichloroacetic acid on ice overnight and then spun down (13,800 g, 30 min). The pellet was washed with acetone and air-dried. The protein precipitants were dissolved in 1 × loading buffer and separated on SDS-PAGE gels (12%). The proteins were electrophoretically blotted on Immobolin-P membranes (Merck Millipore, Darmstadt, Germany), which were then probed with anti-*HA* (Roche Diagnostics, Basel, Switzerland) and anti-β-actin (CWBio, Beijing, China) antibodies.

Generation of gene knockout and complementary strains in *U. virens*

To create gene-replacement mutants in *U. virens*, polyethylene glycol (PEG)-mediated protoplast transformation was performed in combination with a modified CRISPR/Cas9 system (Zheng *et al.*, 2016). Briefly, the protoplasts were prepared from the mycelia after being incubated in lysis buffer (25 g/L driselase, 10 g/L lysing enzymes and 0.05 g/L lyticase in 1.2 M KCl).

The protoplasts were rinsed and then resuspended in STC buffer (20% sucrose, 50 mM Tris-Cl, pH 8.0, 50 mM CaCl₂) to 10^8 protoplasts/mL. The upstream and downstream sequences (c. 1 kb) of *SCRE1* were PCR-amplified and were then fused to the 5' and 3' termini of hygromycin resistance gene by fusion PCR, respectively. Three pairs of *SCRE1*-specific spacer primers for different sgRNA target sequences (Table S2) were designed following the instructions (<http://www.rgenome.net/>). These primers were annealed and inserted into the *BsmBI*-digested entry vector pmCAS9-tRp-gRNA. Individual plasmid constructs and PCR products (5 µg each) were transformed into *U. virens* protoplasts as described (Li *et al.*, 2019). The protoplasts were cultured for 12–14 days to screen transformants. In addition, the full-length *SCRE1* gene with the native promoter was amplified and cloned into pYP102 vector (Zhou *et al.*, 2018). The vector was transformed into the *scre1-1-10* mutant to construct the complementary strain.

Southern blot analysis

Southern blot analysis was performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) following the manufacturer's instructions (Li *et al.*, 2019).

The translocation assay

The pYF11-*RP27*::*SCRE1*-*GFP* construct was generated through homologous recombination in *Saccharomyces cerevisiae* (Qi *et al.*, 2016b). The recombinant plasmid was isolated using a yeast plasmid extraction kit (Zomanbio, Beijing, China) and was then transformed into *E. coli* DH5α. After being confirmed by sequencing, the plasmid was transformed into *M. oryzae* Guy11 via PEG-mediated protoplast transformation as described (Yang *et al.*, 2010). *M. oryzae* conidial suspension ($100 \mu\text{L}$, 10^5 spores/mL) was inoculated on the inner leaf sheath cuticle cells. After 30–36 h incubation under humid conditions at 28 °C, the leaf sheaths were observed under confocal microscopy.

Plant transformation

For conditional expression of *SCRE1*, the *SCRE1* open reading frame was amplified and cloned into pUC19-RBS- $3 \times FLAG$. The open reading frame was released together with the $3 \times FLAG$ coding sequence by *XhoI* and *SpeI* digestion, and was then ligated into pTA7001 (Aoyama and Chua, 1997). These constructs were transformed into *A. tumefaciens* EHA105. *Agrobacterium*-mediated rice transformation was performed as described previously (Li *et al.*, 2015; Lu *et al.*, 2015).

The oxidative burst assay

The oxidative burst after MAMP treatment was detected in the leaf disks using luminol chemiluminescence assay with minor

modifications (Schwacke and Hager, 1992; Park *et al.*, 2012). Briefly, leaf disks were punched from 4-week-old wild-type and transgenic plants and were then pre-incubated in 10 μ M DEX or mock solution for 16 h. To detect ROS generation, three leaf discs were soaked in 100 μ L solution with luminol substrate, peroxidase and different MAMPs [10 μ M flg22, 8 μ M hexa-*N*-acetyl-chitohexaose (Toronto Research Chemicals H290750), 10 μ g/mL chitin (Sigma C9752), or sterile H₂O as control]. Luminescence was recorded continuously at 1-min intervals for 30 min with a GloMax 20/20 luminometer (Promega).

Rice defence response assays

Seven-day-old rice seedlings were pretreated with 10 μ M DEX and mock solution overnight, and were then treated with 1 μ M flg22, 10 μ g/mL chitin or sterile H₂O plus 0.01% Silwet L-77 (GE Healthcare, Amersham, UK). The seedlings were collected at 6 h after treatment for detection of defence-marker gene expression and at 15 min for MAPK activation assay. Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the leaves of treated seedlings following the manufacturer's instructions. The synthesis of cDNA was performed with the Reverse Transcription System (Takara, Dalian, China) using oligo-d(T)₁₈ as primers. The transcript levels of defence-marker genes were analysed using the SYBR PrimeScript RT-PCR kit (Takara) on an ABI PRISM 7500 system and normalized to the reference gene *OsActin*. The proteins were isolated from rice seedlings and MAPK activation was detected as described (Wang *et al.*, 2015). Phospho-serine/-threonine was detected using anti-Phospho-p44/42 MAPK antibody (1:2000 dilution) (Cell Signaling Technology, Danvers, MA, USA) in 4 °C overnight, followed by anti-rabbit-HRP conjugated secondary antibody (CWBio) at 1:5000 dilution.

Bacterial inoculation assays

Resistance to bacterial blight was evaluated on 6-week-old rice plants using the leaf clipping method (Kauffman *et al.*, 1973; Wang *et al.*, 2018). The wild-type and transgenic plants were sprayed with 10 μ M DEX at 24 h before inoculation. The *X. oryzae* pv. *oryzae* strain PX099A was cultured overnight and then resuspended with 10 mM MgCl₂ to an OD₆₀₀ of 0.8 for inoculation. Lesion lengths of the inoculated leaves (≥ 12 leaves, each line) were measured at 12–14 days after inoculation.

Fungal inoculation assays

The *U. virens* isolate P1 and mutant strains were inoculated into rice panicles of the false smut susceptible cv. LYP9 at the booting stage by injection (Han *et al.*, 2015; Wang *et al.*, 2008). *M. oryzae* S5 inoculation was performed as described previously (Lu *et al.*, 2015; Peng and Shishiyama, 1988). At least 20 inoculated leaves were photographed at 5–6 days post-inoculation and disease

severity was scored by estimating lesion areas using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Recombinant protein expression and purification

SCRE1¹⁻¹⁷⁹ and its variants were generated via PCR using the primers in Table S2 and were subcloned into pET32b (His₆-tag). *E. coli* BL21 (DE3) cells (OD₆₀₀ \approx 0.6) transformed with individual constructs were incubated with 100 μ M isopropyl β -D-thiogalactopyranoside for 3–5 h at 25 °C to induce protein expression. The His₆-tagged proteins were purified using Ni-NTA beads (Novagen, Billerica, MA, USA) according to the manufacturer's instructions. The protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo, Rockford, IL, USA) and was also confirmed by SDS-PAGE gels after staining with Coomassie brilliant blue.

Protein extraction and immunoblotting

Total proteins were isolated from the inoculated *N. benthamiana* leaves and the transfected protoplasts following the described procedure (Liu *et al.*, 2016b; Sun *et al.*, 2006). Briefly, the ground powder and the collected protoplasts were incubated in 1 mL of extraction buffer [50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 10% glycerol, 0.1% Triton X-100, 20 mM dithiothreitol and 1% protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA)] on ice for 1 h. The extracts were separated in a 12% polyacrylamide gel and then electrophoretically transferred onto Immobilon-P membrane (Millipore, Bedford, MA, USA) as described (Wang *et al.*, 2015). The membranes were subjected to immunoblotting with different antibodies.

Statistical analysis

All experiments were independently repeated at least three times with similar results unless specifically noted. Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test using SPSS software. Pairwise comparisons were performed by Student's *t*-test using Microsoft Excel.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 Virulence assay to rice of the wild-type, *scre1* mutant and complementary strains. (A) The *scre1* mutants were confirmed via Southern blot analyses. Genomic DNA isolated from the wild-type (WT) strain, *scre1-3-23*, *scre1-2-4*, *scre1-1-26* and *scre1-1-10* mutant candidates was digested with *SphI*. After separation by gel electrophoresis, the digested DNA was blotted on Hybond-N membrane. The membrane was hybridized with the probe that was PCR-amplified and DIG-labelled. The molecular markers are shown in bp on the left. (B) Disease symptoms on rice panicles after injection inoculation of the wild-type P1, *scre1-1-10* mutant and *scre1(SCRE1)* complementary strains. The images were taken at 4 weeks after inoculation.

Fig. S2 SCRE1 expressed and secreted from *Burkholderia glumae* suppresses nonhost cell death in *Nicotiana benthamiana*. (A) Infiltration of the rice bacterial pathogen *B. glumae* caused non-host cell death in *N. benthamiana* leaves while the engineered *B. glumae* transformed with pEDV-SCRE1-HA did not induce cell death. *B.g.*, *B. glumae*; *B.g. (SCRE1)*, *B. glumae* transformed with pEDV-SCRE1-HA. (B) SCRE1-HA was secreted by *B. glumae* in the SMMXC *hrp*-inducing minimal medium under different pH conditions. Upper panel: SCRE1-HA was detected in the cell pellet and culture medium by western blotting. SCRE1-HA secretion was dramatically induced at pH 6.5 in the SMMXC medium. Lower panel: protein loading is indicated by Ponceau S staining. CM, culture medium; TCL, total cell lysate; WB, western blot; α -HA, anti-haemagglutinin antibody.

Fig. S3 Subcellular localization of SCRE1 and SCRE1-SP in *Nicotiana benthamiana* cells. Red fluorescence was observed in the plasmolysed *N. benthamiana* cells transiently expressing mCherry, SCRE1/2-mCherry and SCRE1/2-SP-mCherry via laser scanning confocal microscopy. Neither SCRE1-mCherry nor SCRE1-SP-mCherry was visible in periplasmic spaces of plasmolysed *N. benthamiana* cells. In contrast, SCRE2-mCherry was clearly observed in periplasmic spaces, while SCRE2-SP-mCherry was not. SCRE1/2-SP, SCRE1/2 without signal peptides; mCherry panels, red fluorescence; zoom-in panels, enlarged images from broken square areas in mCherry panels. Arrows indicate the periplasmic spaces after *N. benthamiana* cells were plasmolysed. Scale bar: 75 μ m.

Fig. S4 Induced expression of SCRE1 suppresses microbe-associated molecular pattern (MAMP)-triggered immunity and promotes pathogen infection in transgenic rice plants. (A) SCRE1-FLAG was conditionally expressed after dexamethasone (DEX) induction in three independent transgenic lines, Z178, Z195 and Z196, as detected by immunoblotting with anti-FLAG antibody (α -FLAG). Lower panel: protein loading is indicated by Coomassie brilliant blue (CBB) staining. Nip, Nipponbare; DEX, dexamethasone; –, no DEX treatment; +, with DEX treatment. (B–E) SCRE1 expression induced by DEX significantly suppressed reactive oxygen species (ROS) burst triggered by flg22 (B, D) and chitin (C, E) in the Z195 (B, C) and Z196 (D, E) transgenic lines. Leaf discs were collected from the wild-type and transgenic lines at 24 h after spraying with 10 μ M DEX and mock solution, and were treated with 10 μ M flg22 and 8 μ M hexa-*N*-acetyl-chitohexaose. (F, G) DEX-induced expression of SCRE1 significantly inhibited *OsPR1b* expression triggered by flg22 and chitin in the Z178 (F) and Z196 (G) transgenic lines. The wild-type and transgenic lines were treated with 10 μ M DEX and mock solution for 24 h followed by 1 μ M flg22 and 10 μ g/mL chitin. The expression of *OsPR1b* was detected by quantitative RT-PCR and normalized to the reference gene *OsActin*. Different letters above error bars indicate significant differences in the transcript levels of *OsPR1b* in the wild-type and transgenic lines under different treatments ($P < 0.05$). Error bars represent means \pm SE. (H, I) Disease symptoms on the leaves of the wild-type, Z178, Z195 and Z196 transgenic rice lines with and without DEX treatment after *Xanthomonas oryzae* pv. *oryzae* PXO99 inoculation (H) and after *Magnaporthe oryzae* inoculation (I). The images in H and I were photographed at 12 and 6 days after inoculation, respectively.

Fig. S5 The SCRE1 fragment including the 45–70th amino acid residues retains the ability to suppress BAX-triggered cell death. (A) The derived protein sequences and structural features of SCRE1. The predicted signal peptide is highlighted in orange. The two types of tandem and almost identical repeats are shown in yellow and purple, respectively. The Cys residues are in bold. The 45–70th amino acid residues are underlined and highlighted in blue. (B) Different truncated SCRE1 proteins have distinct abilities to suppress BAX1-triggered hypersensitive responses in *Nicotiana benthamiana*. The truncated proteins including the 45–70th residues all have hypersensitive response (HR)-suppressive ability similar to the full-length SCRE1. +, having the ability to suppress BAX-induced cell death; –, having no HR suppressive ability. BAX was transiently expressed at 6 h after expression of GFP, SCRE1 and different truncated SCRE1 proteins in *N. benthamiana*. HR symptoms in the infiltrated leaves were observed and photographed at 5 days post-infiltration.

Table S1 The strains used in this study

Table S2 The primers used in this study