A Small Secreted Virulence-Related Protein Is Essential for the Necrotrophic Interactions of Sclerotinia sclerotiorum with Its Host Plants

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Abstract

Small, secreted proteins have been found to play crucial roles in interactions between biotrophic/hemi-biotrophic pathogens and plants. However, little is known about the roles of these proteins produced by broad host-range necrotrophic phytopathogens during infection. Here, we report that a cysteine-rich, small protein SsSSVP1 in the necrotrophic phytopathogen Sclerotinia sclerotiorum was experimentally confirmed to be a secreted protein, and the secretion of SsSSVP1 from hyphae was followed by internalization and cell-to-cell movement independent of a pathogen in host cells. SsSSVP1ΔSP could induce significant plant cell death and targeted silencing of SsSSVP1 resulted in a significant reduction in virulence. Through yeast two-hybrid (Y2H), coimmunoprecipitation (co-IP) and bimolecular fluorescence complementation (BiFC) assays, we demonstrated that SsSSVP1ΔSP interacted with QCR8, a subunit of the cytochrome b-c1 complex of mitochondrial respiratory chain in plants. Double site-directed mutagenesis of two cysteine residues (C38 and C44) in SsSSVP1ΔSP had significant effects on its homo-dimer formation, SsSSVP1ΔSP-QCR8 interaction and plant cell death induction, indicating that partial cysteine residues surely play crucial roles in maintaining the structure and function of SsSSVP1. Co-localization and BiFC assays showed that SsSSVP1ΔSP might hijack QCR8 to cytoplasm before QCR8 targeting into mitochondria, thereby disturbing its subcellular localization in plant cells. Furthermore, virus induced gene silencing (VIGS) of QCR8 in tobacco caused plant abnormal development and cell death, indicating the cell death induced by SsSSVP1ΔSP might be caused by the SsSSVP1ΔSP-QCR8 interaction, which had disturbed the QCR8 subcellular localization and hence disabled its biological functions. These results suggest that SsSSVP1 is a potential effector which may manipulate plant energy metabolism to facilitate the infection of S. sclerotiorum. Our findings indicate novel roles of small secreted proteins in the interactions between host-non-specific necrotrophic fungi and plants, and highlight the significance to illuminate the pathogenic mechanisms of this type of interaction.
Author Summary

To resist biotrophic and hemibiotrophic phytopathogens, plants utilize an innate immune system, mediated through nucleotide binding (NB)-leucine rich repeat (LRR) proteins, to respond to effectors, most of which are small secreted proteins. Hypersensitive responses (HRs) resulting from this type of interaction can effectively restrain the expansion of biotrophic or hemibiotrophic phytopathogens in plant tissues. However, it is not effective against typical necrotrophs with remarkably broad host range, such as *S. sclerotiorum*, because these necrotrophs have long been thought to just simply kill hosts and complete their life cycles using nutrients derived mostly from dead plant tissues. This type of phytopathogen-plant interaction obviously does not comply with the gene-for-gene or inversed gene-for-gene relationship. The results in present study show that SsSSVP1 of *S. sclerotiorum* functions as an effector in pathogen-plant interactions. SsSSVP1 is dramatically induced during infection, and required for the full virulence of *S. sclerotiorum*. SsSSVP1 can be internalized by plant cells after being secreted from fungal cells in the absence of a pathogen during infection. Furthermore, SsSSVP1ΔSP interacts with QCR8, a subunit of cytochrome b-c1 complex, and disturbs the localization of QCR8 in mitochondria, which may disable its biological function. The nonfunctionalization of QCR8 caused significant plant cell death. Hence, SsSSVP1 acts as an effector to manipulate the host cell physiology to facilitate the colonization of *S. sclerotiorum*. Obviously, this is a completely different interaction model from the gene-for-gene or inversed gene-for-gene paradigm. These findings suggest that the pathogenesis of *S. sclerotiorum* is more subtle and complex than previously appreciated and highlight the significance to investigate the interaction models between the host-non-specific necrotrophs and their hosts.

Introduction

*Sclerotinia sclerotiorum* (Lib.) de Bary is an exemplary necrotrophic phytopathogenic fungus with a broad host range. At least 408 species of plants are susceptible to this white mold fungus, most of them are from Dicotyledonae but a few are from Monocotyledonae such as onion and garlic [1]. *S. sclerotiorum* is also a cosmopolitan pathogen of many economically important crops, including oilseed rape (*Brassica* spp.), sunflowers, soybeans, peanuts and lentils, and its infection often leads to a significant loss of crop production.

Plant pathogens have been categorized as biotrophic, hemibiotrophic and necrotrophic pathogens based on the lifestyles of these agents, and the pathogenic mechanisms are obviously different among the different types of pathogens. Biotrophic pathogens must manipulate host physiology and derive nutrients from living host cells and tissues, whereas hemibiotrophic pathogens absorb nutrients from living cells during the early biotrophic stage of infection and subsequently kill host cells during the later necrotrophic stage of infection. The nutrient acquisition of necrotrophic pathogens is based on host cell killing [2]. Often, biotrophic and hemibiotrophic fungi secrete effectors that manipulate host cell structure and function to obtain nutrients and suppress plant defenses, thereby facilitating infection [3]. The secretion and transfer of effectors into plant host cells are also essential for the pathogenesis of many biotrophic and hemibiotrophic fungi [4–7]. Plant cell death triggered through hypersensitive responses (HRRs) is a major obstacle for the further expansion of biotrophic and hemibiotrophic fungi during the initial stage of infection. However, for necrotrophic fungi, host cell death might be beneficial rather than detrimental for pathogenesis; thus, the canonical necrotrophic fungus *S. sclerotiorum* secretes a wide array of cell-wall-degrading enzymes (CWDEs) to
facilitate host cell wall degrading and ultimately promote infection [8]. As a non-selective phytotoxin, oxalic acid (OA) produced by *S. sclerotiorum* can also contribute to pathogenesis in a number of ways (e.g. acidification, chelation of Ca^{2+}, low pH activation of degradative enzymes etc.) that augment fungal colonization of host plants [9]. In addition, OA plays a subtle role in the interaction between *S. sclerotiorum* and its hosts. For example, OA can suppress the oxidative burst of the host plant [10] and suppress host defenses by manipulating the host redox environment [11]. It also induces apoptotic cell death [12] and plays a crucial role in the control of the interplay of host cell apoptosis and autophagy during infection [13].

Necrotrophic fungi have long been considered as host killers. Previous studies have shown that host-specific necrotrophic fungal pathogens may utilize plant resistance signaling pathways to subvert PCD and enable pathogen growth [14,15]. To date, many interactions between host-specific necrotrophic fungal pathogen effector molecules and their host targets have been reported, including the victorin of *Cochliobolus victoriae* and TRX-h5 as well as LOV1 of *Arabidopsis thaliana* [16], the PC toxin of *Periconia circinata* and Pc locus of sorghum [17], the Ptr ToxA of *Pyrenophora tritici-repentis* and Tsn1 of wheat [14] as well as the SnTox1-Snn1 [18], SnToxA-Tsn1 [19,20], SnTox2-Snn2 [21], SnTox3-Snn3-B1 [22], SnTox4-Snn4 [23], and SnTox3-Snn3-D1 [24] in *Stagonospora nodorum*-wheat pathosystem. These interactions induce a resistance-like response that confers disease susceptibility in an inverse gene-for-gene manner. However, for host-non-specific fungi with remarkably broad host range such as *S. sclerotiorum* and *Botrytis cinerea*, emerging evidence suggests that they have more sophisticated and comprehensive strategies for infecting hosts than previously considered. They can manipulate the antagonistic effects between immune pathways to promote disease development in tomato [25]. Actually, even for these kinds of fungi, there is a transition from a biotrophic to necrotrophic lifestyle and the hemi-biotrophic lifestyle may be more temporally and spatially complex than currently depicted [26]. In addition to CWDEs and OA related pathogenic factors, some potential secreted proteinaceous effectors also play crucial roles in the pathogenesis of host-non-specific necrotrophic fungi. For example, we previously reported that a secreted integrin-like protein SSITL of *S. sclerotiorum* promotes virulence and directly or indirectly suppresses host resistance during the early stages of infection [27]. Another small secreted protein, Ss-Caf1, functions as a pathogenicity factor to trigger host cell death during the early stages of *S. sclerotiorum* infection [28]. Kabbage *et al.* also identified an effector-like protein in *S. sclerotiorum* (SsCm1) [13]. The xylanase Xyn11A can induce necrosis independently of the catalytic activity of this enzyme during *B. cinerea* infection [29]. However, until recently, there has been little experimental evidence for the existence of the interactions between proteinaceous effectors and host targets for typical necrotrophic phytopathogens, such as *S. sclerotiorum* and *B. cinerea*. The molecular mechanisms of the interactions between host-non-specific necrotrophic fungal effectors and their host targets is still poorly understood. The identification and characterization of this type of the necrotrophic interactions are difficult because they obviously do not act in the gene-for-gene manner or follow the inverse gene-for-gene scenario.

A recent study reported that the *S. sclerotiorum* genome encodes many predicted secreted proteins that might be involved in the interaction between this fungus and its hosts [30]. Notably, in plant-pathogen interactions, most of effectors are small secreted proteins [31–34] except for some non-proteinaceous toxins and secondary metabolites. However, the biological functions of small secreted proteins from many eukaryotic pathogens remain largely unknown. In the present study, we aim at identifying and characterizing proteinaceous effectors which play crucial roles in the interaction between *S. sclerotiorum* and its hosts. Digital gene expression profiles (DGE; Solexa/Illumina) and bioinformatics approaches were combined to screen for proteinaceous effector candidates in *S. sclerotiorum*. A cysteine-rich, small, secreted protein
SsSSVP1 was experimentally confirmed to interact with a component of plant cytochrome b-c1 complex in mitochondrial respiratory chain, which play a crucial role during *S. sclerotiorum*-hosts interaction. Our result demonstrated that the necrotrophic fungus *S. sclerotiorum* also secretes proteinaceous effectors that has targets in plants and the interaction between these effectors and their targets may seriously disturb the physiological processes of its hosts.

Results

**SsSSVP1 is a Sclerotinia- and Botryotinia-specific, cysteine-rich, small, secreted protein**

In our previous study, the DGE based on deep sequencing technology was used to illuminate the wide range of transcriptional responses associated with six different developmental stages of a virulent wild-type strain, Ep-1PNA367 [35]. In this study, the DGE data was used to identify the differentially expressed genes encoding putative secreted proteins during the vegetative growth stage on PDA and the infection stage on *A. thaliana* leaves. There were 314 genes encoding predicted secreted proteins that were identified to be significantly up-regulated during infection (S1 Table). We focused our study on those genes which encode cysteine-rich small proteins. RNAi technique was used to study the biological functions of *S. sclerotiorum* genes because of the multinucleated cells of this fungus. Our results showed that silencing SS1G_02068 significantly reduced the virulence of *S. sclerotiorum* and SS1G_02068 (GenBank accession: XM_001597822) could induce significant plant cell death when constitutively expressed in host cells. Thus, we named this protein “SsSSVP1”, as this is the first report that a small secreted virulence-related protein in *S. sclerotiorum* that has a target in plant cells.

SsSSVP1 is a protein without any known domains which may be specific to *Sclerotinia* and *Botryotinia*, as the homologs of SsSSVP1 have only been identified in *Sclerotinia* and *Botryotinia* in the non-redundant protein sequence database at NCBI to date. SsSSVP1 contains 163 amino acid residues including eight cysteine residues, which account for over 4% (Fig 1A). Multiple sequence alignment indicated that all the cysteine residues in SsSSVP1 are well conserved in its homologues (Fig 1B), indicating these cysteine residues may play an important role in the structure and function of SsSSVP1. Bioinformatics analysis revealed that SsSSVP1 has a predicted N-terminal signal peptide (SP, 1–17 aa), suggesting that it may be a secreted protein (Fig 1A). To test this hypothesis, the FLAG-tagged SsSSVP1 engineered strains were constructed and inoculated in liquid CM medium for shake culture. Western blot result showed that SsSSVP1-FLAG could be detected in the liquid culture medium (Fig 1C), indicating SsSSVP1 is indeed a secreted protein.

**SsSSVP1ΔSP** can induce significant plant cell death

To characterize the influence of SsSSVP1 over host cells after being secreted, considering a SP is cut off when a secreted protein is secreted from hyphae into plant cells, SsSSVP1ΔSP without its SP was constitutively expressed in *Nicotiana benthamiana* using Agrobacterium tumefaciens-mediated transformation method. Agrobacterium strains carrying the pTRV2-SsSSVP1ΔSP virus vector and the pTRV1 vector, the latter of which facilitates the movement of the recombinant virus, were mixed and co-infiltrated into *N. benthamiana* leaves. Our result showed that SsSSVP1ΔSP could induce significant cell death in leaves, stems and the whole plant (Fig 2A). However, the GFP alone for control did not induce plant cell death, suggesting that plant cell death was specifically induced by SsSSVP1ΔSP (Fig 2A). This result indicates SsSSVP1ΔSP is toxic to plant cells.
SsSSVP1 can be internalized into plant cells independently and translocated from cell to cell

A previous report in our lab showed that a small, secreted protein, Ss-Caf1 of *S. sclerotiorum* without its SP could induce significant plant cell death, however, full Ss-Caf1 with its SP could not induce plant cell death [28], suggesting that plant cells can recognize SPs from fungi and direct the secretion of fungal proteins expressed in plant cells. Interestingly, we found that full SsSSVP1 with its SP still could induce plant cell death similar to SsSSVP1ΔSP (Fig 3A and 3B). So, we postulated that SsSSVP1 could be internalized by plant cells in the absence of a pathogen. If this hypothesis is true, we should still be able to detect SsSSVP1 in plant cells after its secretion. To test this hypothesis, we first examined the subcellular localization of SsSSVP1ΔSP in host plant cells. The pTRV2-SsSSVP1ΔSP-GFP virus vector was constructed and transformed into an *Agrobacterium* strain to conduct infiltration assay on tobacco leaves. Confocal images showed that SsSSVP1ΔSP mainly distributed throughout the cytoplasm, particularly concentrated at the periphery of cell membrane (Fig 2B). In addition, SsSSVP1ΔSP occasionally localized in nuclei under unknown conditions, and sometimes it scattered in cytoplasm in a particle-like form (S1A Fig). Afterwards, we examined the subcellular localization of SsSSVP1 with its SP and SP-GFP (used for control) in tobacco leaf cells using the same protein expression system. Results showed that both SsSSVP1-GFP and SP-GFP localized in endoplasmic reticulum (ER)-like structure (S2 Fig), however, only SsSSVP1-GFP could be observed to localize in cytoplasmic compartments in a particle-like form, no particle-like form of SP-GFP was observed in cytoplasm, indicating the specificity of the fluorescence signal (Fig 3C).
results indicated the SsSSVP1 could be secreted by plant cells and had plant cell re-entry activity which may result from the internalization of SsSSVP1.

In order to further confirm that SsSSVP1 can be internalized into plant cells independently, nuclear targeting assay was used to facilitate visualization of the translocation of SsSSVP1 according to Khang et al. [4]. A small nuclear localization signal (NLS) from simian virus large T-antigen [36] was added at the C terminus of the SsSSVP1-mCherry fusion (SsSSVP1-mCherry-NLS) and SP-mCherry fusion (SP-mCherry-NLS, used for control). It is difficult to obtain pure transgenic lines because of the multi-nucleated trait of S. sclerotiorum and the hyper-virulence of S. sclerotiorum is not conducive to observe effector translocation, the constructs described above were transformed into B. cinerea (which is phylogenetically close to S. sclerotiorum) to more easily visualize faint fluorescence. The result showed that the SP-mCherry-NLS fluorescence was only observed in the nuclei of infected host cells but not the neighboring host cells, however, the SsSSVP1-mCherry-NLS fluorescence was observed in the nuclei of infected host cells and intact surrounding host cells (Fig 4). All these intact surrounding host cells were checked in different layers using z-axis scanning of a confocal laser microscope to ensure there were no hyphae in these cells (S3 Fig). These results further
indicated SsSSVP1 can be internalized into plant cells independently and move from cell to cell like effectors in other hemibiotrophic fungi [4].

**SsSSVP1 plays a crucial role in virulence**

Quantitative reverse transcription PCR (qRT-PCR) analysis showed that when pure actively growing hyphal fragments of *S. sclerotiorum* without culture medium were inoculated onto the leaves of *A. thaliana* (Col-0), the transcript levels of *SsSSVP1* rapidly increased by more than 50-fold at 3 hours post inoculation (hpi) and then gradually increased during the later infection stages (6–12 hpi, Fig 5). This result is consistent with the DGE data and suggests that *SsSSVP1* may be involved in infection of *S. sclerotiorum*. In order to explore the roles of *SsSSVP1* in virulence of *S. sclerotiorum*, RNAi technology was used because of the multi-nucleated cells. QRT-PCR was used to examine the transcript accumulation in *SsSSVP1*-silenced transformants. Three transformants (SsSSVP1-136, SsSSVP1-37 and SsSSVP1-70) showing dramatically reduced *SsSSVP1* expression and one transformant (SsSSVP1-2) with a slightly reduced *SsSSVP1* expression (Fig 6C) were selected for further study. The colony morphology, virulence and growth rate of these transformants were compared to the wild-type strain Ep-1PNA367 (Fig 6A, 6B, 6D and 6E). The virulence of *SsSSVP1*-silenced mutants was significantly reduced,
and only small lesions were developed on the detached *Brassica napus* leaves at 2 days post inoculation (dpi). For example, on average of three independent experiments, lesions induced

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**Fig 4. The nuclear targeting-based translocation assay of SsSSVP1-mCherry.** *B. cinerea* wild-type strain B05.10 and transformants of SP-mCherry-NLS and SsSSVP1-mCherry-NLS constructs were used to perform nuclear targeting assay, and the former two were used as controls. SsSSVP1-mCherry-NLS fluorescence occurred in the nuclei of invaded onion bulb lower epidermal cells and surrounding cells while mCherry-NLS fluorescence occurred only in the nuclei of invaded cells. No fluorescence was observed in the onion tissues infected by B05.10. The same imaging conditions were used in the three channels. Images were taken at 48 hpi using confocal laser scanning microscopy. Different layers of the intact surrounding cells were observed independently to ensure there were no hyphae in these cells. See S3 Fig for an example. The images show maximum projections of 4 confocal images captured along the z-axis. Areas within yellow dotted line indicate hyphal invaded onion epidermal cells.

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by SsSSVP1-70 were approximately 0.9 cm in diameter, while lesions induced by the wild-type strain were approximately 2.6 cm in diameter. Furthermore, the decreases in virulence were positively correlated with the silencing efficiency (Fig 6C and 6D), indicating the virulence reduction of the silenced transformants was caused by the silencing of SsSSVP1. In vivo inoculation assay showed the virulence of SsSSVP1-silenced mutants was also dramatically reduced on A. thaliana leaves compared to that of the wild-type strain (S4A and S4B Fig), indicating the virulence reduction of SsSSVP1-silenced mutants is not host-specific. Although the growth rate of SsSSVP1-silenced transformants was slightly reduced compared to that of wild-type strain (Fig 6E), statistical analysis indicated the expression reduction of SsSSVP1 had more effect on virulence than growth rate, which means the virulence reduction is not intimately associated with growth rate.

In order to further investigate the biological functions of SsSSVP1, S. sclerotiorum transformants over-expressing SsSSVP1-FLAG were used to perform virulence assay. QRT-PCR results showed that increase in the expression of SsSSVP1 varied in different transformants (S5C Fig). Western blot analysis showed that the SsSSVP1-FLAG could be detected in total protein extracts from the mycelia of these over-expression transformants, of which the OESsSSVP1-3 was used as an example (S5D Fig). However, there was no obvious difference between colonial morphology, virulence and growth rate of the over-expression transformants and the wild-type strain (S5A, S5B, S5E and S5F Fig). The rapid increase of SsSSVP1 expression level in the wild-type strain during infection could possibly explain the lack of difference in virulence between SsSSVP1-overexpression strains and the wild-type strain.

SsSSVP1ΔSP forms homo-dimer and interacts with QCR8

To further understand how SsSSVP1 affects the virulence of S. sclerotiorum, yeast two-hybrid (Y2H) technique was used to screen an A. thaliana cDNA library to identify the targets that interact with SsSSVP1ΔSP in plants. Our Y2H assay showed that SsSSVP1ΔSP interacted with itself (Fig 7A), indicating SsSSVP1ΔSP may function in plant cells in the form of homo-dimer. Meanwhile, our results demonstrated that SsSSVP1ΔSP could interact with QCR8.
(AT3G10860), the subunit 8 of cytochrome b-c1 complex which is the component of mitochondrial respiratory chain (Fig 7A). The QCR8 gene is well conserved in plants, and our Y2H
assay further showed that SsSSVP1ΔSP could interact with all the homologs of QCR8 in *A. thaliana* and *N. benthamiana* (Fig 7A and S6 Fig), indicating the possible universal existence of this necrotrophic interaction during the infection of *S. sclerotiorum* on many hosts. To determine if SsSSVP1ΔSP interacts with QCR8 in plant tissues, we co-expressed the GFP-tagged SsSSVP1ΔSP and 3×FLAG-tagged QCR8 in *N. benthamiana* leaves by *A. tumefaciens* infiltration method, our co-immunoprecipitation (co-IP) assay also supported that SsSSVP1ΔSP interacted with
QCR8 (Fig 7B). Furthermore, this result was further confirmed in planta using the bimolecular fluorescence complementation (BiFC) technique. SsSSVP1ΔSP-nYFP (N-terminal yellow fluorescent protein fragment) and QCR8-cYFP (C-terminal yellow fluorescent protein fragment) were transiently co-expressed in N. benthamiana leaves. Yellow fluorescence was detected in cytoplasm, especially at the periphery of cell membrane (Fig 7C), suggesting that SsSSVP1ΔSP interacts with QCR8 in plant cell cytoplasm.

C³⁸ and C⁴⁴ are essential for function of SsSSVP1ΔSP

As described above, many effectors are cysteine-rich proteins. Additionally, the eight cysteine residues are well conserved in the homologs of SsSSVP1. In order to examine if these cysteine residues play crucial roles in the function of SsSSVP1, single site-directed mutagenesis of the eight cysteine residues was conducted in SsSSVP1ΔSP. Our results showed that all the single-point mutations had little effects on the dimer formation of SsSSVP1ΔSP (Fig 8A), and the interaction between SsSSVP1ΔSP and QCR8 (Fig 8B). In addition, the expression of SsSSVP1ΔSP with all single-point mutations still could induce plant cell death (Fig 8C). However, our Y2H and single site-directed mutagenesis combined assays showed that SsSSVP1ΔSP-C³⁸A could not interact with SsSSVP1ΔSP-C⁴⁴A (S7 Fig). Furthermore, double-point mutation at residues 38 (C to A) and 44 (C to A) made SsSSVP1ΔSP lose the ability to interact with itself and with QCR8 (Fig 8A and 8B). These results further indicated the specificity of homo-dimer formation of SsSSVP1ΔSP and the interaction between SsSSVP1ΔSP and QCR8. Meanwhile, SsSSVP1ΔSP-C³⁸A-C⁴⁴A also could not induce plant cell death any more (Fig 8C), although it could express well and was stability in plant (Fig 8D). These results indicated C³⁸ and C⁴⁴ play a crucial role in maintaining the structure and biological functions of SsSSVP1.

Interaction between SsSSVP1ΔSP and QCR8 disturbs the subcellular localization of QCR8 in mitochondria

Our BiFC result showed that SsSSVP1ΔSP interacted with QCR8 in cytoplasm, especially at the periphery of cell membrane. However, QCR8 is one subunit of cytochrome b-c₁ complex, which localizes in mitochondria [37], so we hypothesize the interaction between SsSSVP1ΔSP and QCR8 might change the native subcellular localization of QCR8, and SsSSVP1ΔSP could hijack QCR8 to cytoplasm. To test this hypothesis, SsSSVP1ΔSP-mCherry and QCR8-GFP were co-expressed in N. benthamiana leaves using Agrobacterium infiltration method for the observation of their co-localization. As expected, QCR8 alone localized in mitochondria (Fig 9A), because it co-localized with the mitochondria-mcherry marker [38] (Fig 9B). However, SsSSVP1ΔSP and QCR8 co-localized in cytoplasm (Fig 9B), which is in accordance with the BiFC results. Additionally, QCR8 still localized in mitochondria when it was co-expressed with the double site-directed mutant SsSSVP1ΔSP-C³⁸A-C⁴⁴A losing the ability to interact with QCR8 (Fig 9B), indicating the specificity of fluorescence distribution of the SsSSVP1ΔSP and QCR8 co-localization. Occasionally, the co-localization of SsSSVP1ΔSP and QCR8 in nuclei or in cytoplasmic compartments of plant cells could also be observed (S1B Fig). QCR8 is encoded by nuclear genome and translated in cytoplasm. Our results indicated that the interaction between SsSSVP1ΔSP and QCR8 could disturb the native localization of QCR8, and SsSSVP1ΔSP might hijack QCR8 to cytoplasm before QCR8 was translocated into mitochondria.

Silencing of QCR8 led to plant abnormal development and cell death

Our co-localization and BiFC assays showed the SsSSVP1ΔSP-QCR8 interaction disturbed the subcellular localization of QCR8, which might disable the biological functions of QCR8. To test this hypothesis, a tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS) system
[39] was used to knock-down the three homologs of QCR8 encoding genes in N. benthamiana. The endogenous tobacco phytoene desaturase gene (PDS) was used to examine the effectiveness of the TRV-VIGS system (S8 Fig). QRT-PCR results showed that the transcript abundance of the three QCR8 encoding genes was reduced in both upper leaves and middle leaves of the silenced
Targeted silencing of QCR8 resulted in stunted development of stem apex which caused most of the QCR8-silenced plants to exhibit dwarf phenotype (Fig 10B). More importantly, approximately 78.9% (45/57) of the silenced lines showed plant cell death phenotype on the leaves with and without infiltration sites. No control lines exhibited these phenotypes. Together with the data that the double site-directed mutant SsSSVP1ΔSP-C38A-C44A cannot interact with QCR8 and also lost the capability to induce plant cell death, these results indicated the plant cell death induced by SsSSVP1ΔSP might be caused by the SsSSVP1ΔSP-QCR8 interaction, which disturbed the subcellular localization of QCR8 and hence made the QCR8 lose its biological function.

**Discussion**

*S. sclerotiorum* is a typical necrotrophic fungal pathogen that produces oxalic acid and CWDEs to kill plant cells and subsequently feeds on the dead tissues. However, increasing evidence
suggests the pathogenesis of *S. sclerotiorum* is more complex than originally considered. In this study, a *Sclerotinia*- and *Botrytis*-specific, small, secreted protein SsSSVP1 was identified, and its biological functions in the interactions between *S. sclerotiorum* and its hosts were explored. SsSSVP1 is a cysteine-rich protein which is predicted to form disulfide bonds intramolecularly. The cysteine residues are essential for the formation of disulfide bonds, which may facilitate the formation of stable homodimers, heterodimers, homopolymers, or heteropolymers, suggesting the important roles for these residues in protein folding and in maintaining the structural stability of some secreted proteins [40,41], particularly those that are secreted into the

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**Fig 10. Silencing of QCR8 leads to plant abnormal development and cell death.** (A) The relative expression levels of three QCR8 genes (QCR8-1, QCR8-2 and QCR8-3) in silenced *N. benthamiana* lines were determined through qRT-PCR. The expression levels of the actin gene in *N. benthamiana* were used to normalize the expression levels of QCR8. The QCR8 expression level in the control lines was set as 1.0. This qRT-PCR assay was performed one month after *A. tumefaciens* infiltration. “Up” and “M” indicated these samples were from the upper leaves and middle leaves, respectively. “CK” and “RNAi” indicated these samples were from the control lines and the silenced lines which were infiltrated with *A. tumefaciens* containing empty vectors and silencing vectors, respectively. (B) The phenotype of QCR8-silenced *N. benthamiana* lines (RNAi-QCR8) using TRV-based VIGS system. The lines transformed using *A. tumefaciens* containing VIGS-pTRV2 empty vector were used as control. Silencing of the QCR8 in *N. benthamiana* plants caused growth retardation and cell death. No obvious phenotype was observed in the control. Photos were taken 10 days post *A. tumefaciens* infiltration.

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oxidizing environment of extracellular medium [40]. The single site-directed mutagenesis of the eight cysteine residues in SsSSVP1^ΔSP had little effects on its structure and function because the mutants still can form homo-dimer, interact with QCR8 and induce plant cell death. Although it seemed that the degree of cell death induced by different single-point mutants of SsSSVP1^ΔSP varied at the early stage (10 dpi) after *A. tumefaciens* infiltration, the plant cells eventually died at the late stage (30 dpi). The difference of the degree of cell death at the early stage may be due to different plant growth status and different transmission speed of the virus. However, the double-point mutant SsSSVP1^ΔSP-C38A-C44A could not induce plant cell death no matter at the early stage or at the late stage after *A. tumefaciens* infiltration. Meanwhile, SsSSVP1^ΔSP-C38A-C44A also could not form homo-dimer or interact with QCR8. The dimer formation may be very important for SsSSVP1 when it is exposed to plant intercellular space during infection. This molecular mechanism has significant meaning in many cysteine-rich proteinase inhibitors, where even cleavage of the reactive site peptide bond does not change its overall conformation and such “modified” inhibitor still possesses antiproteinase activity [42]. QCR8 does not have any cysteine residues, indicating the interaction between SsSSVP1^ΔSP and QCR8 is not maintained by intermolecular disulfide bonds but by their respective tertiary structure. In conclusion, these results indicated there might be at least two disulfide bonds maintaining the tertiary structure of SsSSVP1 intramolecularly, affecting the stability and rigidity of this small secreted protein. Our results suggested C38 and C44 were essential to maintain the structure and function of SsSSVP1^ΔSP, however, we do not rule out the case that the other cysteine residues also play important roles.

A primary role of effectors is to inhibit host defense mechanisms [43–45]. However, the roles of effectors in biotrophic and necrotrophic fungi might be different, as the former require live host tissues, while the latter prefer dead plant tissues. Most effectors in biotrophic fungi suppress programmed cell death [46] while many effectors in necrotrophic fungi induce plant cell death. Our results also indicated that SsSSVP1^ΔSP induced significant plant cell death. In different repeated tests, SsSSVP1^ΔSP-GFP always mainly localized in the plant cytoplasm, occasionally localized in cytoplasmic compartments in a particle-like form or in nuclei in different areas even in the same infiltrated tobacco leaf. The difference in the fluorescence distribution in the plant cells expressing SsSSVP1^ΔSP might be due to the fact that the cells are at different stages of apoptosis. The mechanisms of the translocation of RXLR effectors in oomycetes or RXLR-like variants in fungi into plant cells has been documented and discussed [47–57], however, the mechanisms underlying the delivery in host cells of fungal effectors without RXLR motif are poorly understood, although the phenomena of the internalization and cell-to-cell movement of some fungal effectors were observed previously [4,58]. Previous research showed Ptr ToxA produced by *P. tritici-repentis* may be internalized via receptor-mediated endocytosis (RME) by sensitive wheat mesophyll cells and the endocytic vesicle-like structure was observed near plasma membrane [58]. Ptr ToxA is compartmentalized after internalization and forms particle-like structures in plant cells [58]. Interestingly, similar situations were observed in SsSSVP1 (Fig 3C). Hence, we infer that SsSSVP1 and Ptr ToxA have similar cell entry mechanism. In the case of Ptr ToxA, one motif Arg-Gly-Asp (RGD) was predicted to be involved in its interaction with a putative integrin-like receptor in the host [59,60]. However, neither an RGD-like motif nor an RXLR-like motif was found in SsSSVP1. The exact molecular mechanism of SsSSVP1 crossing the plant plasma membrane from the apoplastic space to the interior of plant cells in the absence of a pathogen should be explored in future. Additionally, the cell-to-cell movement of SsSSVP1 is likely the result of the internalization and translocation of SsSSVP1 in the host apoplastic space, because the fluorescent signal could be clearly detected in the apoplastic space of the surrounding cells of the invaded host cells (Fig 4).
QCR8 is a subunit of the cytochrome b-c₁ complex comprising 10 different polypeptide subunits in plants [61]. The cytochrome b-c₁ complex is the center component of the mitochondrial respiratory chain, coupling the transfer of electrons from ubihydroquinone to cytochrome c with the generation of a proton gradient across the mitochondrial membrane [37]. We found that SsSSVP1ΔSP could hijack QCR8 into the cytoplasm of plant cells and disturb the native localization of QCR8 in mitochondria. This character of SsSSVP1ΔSP is similar to that of a rice stripe virus (RSV) specific protein RSV SP, which hijacks host PsbP into cytoplasm from chloroplast [62]. Although we do not know that if the deletion of QCR8 is lethal to plants, our results showed silencing of QCR8 caused obvious plant cell death. This phenomenon indicated a link between the SsSSVP1ΔSP-QCR8 interaction and the biological function loss of QCR8. Alteration of QCR8 native subcellular localization or lack of QCR8 may eventually affect the energy metabolism of plant cells, because knock-down of QCR8 significantly affected plant growth and development. Obviously, the interaction model of SsSSVP1ΔSP and QCR8 is very different from that of classic effectors and R genes. The ‘gene for gene’ and reverse ‘gene for gene’ model might not apply to this typical necrotrophic fungi-host interaction system, as there are almost no resistant hosts to these canonical necrotrophic pathogens. On the other hand, the components of the cytochrome b-c₁ complex are highly conserved in almost all plant cells. Our study provides an intriguing example that the necrotrophic pathogen secretes a small protein which might attack the well conserved component of mitochondrial respiratory chain in plant cells. This hypothesis is also consistent with the broad host range of S. sclerotiorum.

In summary, we screened for small, secreted proteins that were significantly up-regulated during infection and identified a "toxin-like" and "effector-like" protein SsSSVP1 in S. sclerotiorum. SsSSVP1 is essential for the full virulence of S. sclerotiorum. SsSSVP1ΔSP interacts with QCR8 and hijacks QCR8 into the cytoplasm in plant cells. The SsSSVP1ΔSP-QCR8 interaction disturbs the location of QCR8 and hence might interfere with the biological functions of QCR8. The functional loss of QCR8 may seriously affect the plant energy metabolism and caused significant cell death. Two cysteine residues at 38 and 44 of SsSSVP1 are crucial for its structure and functions. These findings further enhance our understanding of the pathogenic mechanism of S. sclerotiorum, highlighting the necessity for large-scale screening and function analyses of the effector candidates in typical necrotrophic fungi with broad host ranges.

Materials and Methods

Bacterial and fungal strains, plants, culture conditions and transformation of S. sclerotiorum and N. benthamiana

The virulent S. sclerotiorum wild-type strain Ep-1PNA367 [63] and B. cinerea wild-type strain B05.10 were used in this study. Fungal cultures were grown on potato dextrose agar (PDA, Difco, Detroit, MI, USA) or inoculated in CM liquid medium at 20°C. S. sclerotiorum and B. cinerea transformants were cultured on PDA amended with 80 μg/ml hygromycin B (Calbiochem, San Diego, CA) to stabilize the transformants. Escherichia coli strain JM109 and DH5α was used to propagate all plasmids, and A. tumefaciens strains EHA105 and GV3101 were used for the transformation of fungi and plants, respectively. Seedlings from A. thaliana (ecotype Columbia-0) and N. benthamiana were grown in the greenhouse at 20 ± 2°C under a 12 h light/dark cycle with 70% relative humidity. The canola cultivar used for virulence assay was zhongyou 821 [64], which is slightly resistant to S. sclerotiorum. The Agrobacterium-mediated transformation method was used to transform S. sclerotiorum as previously described [65], with a modification related to Agrobacterium cultivation: the A. tumefaciens cells were not diluted in minimal medium and directly cultured in induction medium for co-cultivation.
*Agrobacterium*-mediated transformation method was performed to transform *N. benthamiana* via infiltration according to published protocols [66].

**Bioinformatics analysis**

The publicly available genomic sequence database of *S. sclerotiorum* 1980 UF-70 (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiDownloads.html) was used to characterize all *S. sclerotiorum* genes examined in this study. SignalP was used to identify secreted proteins and their SPs [67]. BlastP analysis was done on the website of NCBI (http://www.ncbi.nlm.nih.gov/). The amino acid sequences were aligned using COBALT [68] and viewed and edited in Jalview [69]. The DGE analysis and the identification of differentially expressed genes were performed according to our previous study [35].

**Vector construction for gene expression and gene silencing in *S. sclerotiorum* and *N. benthamiana***

To generate SsSSVP1-FLAG fusion construct (S9A Fig), the promoter PEF-1α was PCR amplified using the primers PEF-1αF/R and subsequently digested with XhoI and SacI. The PCR products of SsSSVP1 were amplified with the primers SsSSVP1-FLAG F/R and subsequently digested with SacI and SmaI. These two fragments were sequentially ligated into the pCH vector [65] through the formation of intermediate constructs. Based on our experience in gene silencing in *S. sclerotiorum*, the silencing efficiency of different RNAi strategies varies from gene to gene. To obtain knockdown transformants with a higher silencing efficiency, two RNAi strategies described by Nguyen [70] and Yu et al. [65] were adopted to construct the *S. sclerotiorum* RNAi vectors: a 320 bp fragment from SsSSVP1 was amplified with the primers RNAi1-2 vector or (ii) digested with suitable enzymes and subsequently ligated into pCIT [27] between PtrpC, the intron and TtrpC in the opposite orientation via intermediate vectors. Subsequently, the PtrpC-intron-TtrpC fragment containing the two *S. sclerotiorum* gene fragments in the opposite orientation was digested with SacI and XhoI and subsequently ligated into pCH to produce pRNAi-2 vector (S9C Fig). Both of these two different RNAi strategies were used to silence SsSSVP1 and similar results were obtained. The transformants used in this study were produced using pRNAi-2 vector. All the constructs were confirmed through sequencing analysis. The primers are shown in S2 Table. These constructs were then introduced into the *A. tumefaciens* strain EHA105 through electroporation [71].

For SsSSVP1 constitutive expression in *N. benthamiana*, the recombinant TRV-based *A. tumefaciens* binary virus vectors pTRV1 and pTRV2 [39] were used for gene expression and gene silencing in *N. benthamiana* in this study. To generate the constitutive expression constructs, (i) SsSSVP1 with and without the SP-encoding sequences were amplified using the primers pTRV-SsSSVP1 F/R and pTRV-SsSSVP1ΔSP F/R, respectively; (ii) GFP with and without the SP-encoding sequences were amplified using the primers pTRV-GFP F/R and pTRV-SP-GFP F/R, respectively; (iii) SsSSVP1ΔSP and GFP-encoding sequence were amplified using the primers SsSSVP1ΔSP F/R and GFP F/R, respectively. The PCR products were subsequently digested with the appropriate restriction enzymes, followed by ligation to the intermediate vector pBl121 [72], and then the PCR product of the SsSSVP1ΔSP-GFP fusion was amplified from the recombinant pBl121 using the primers pTRV-SsSSVP1ΔSP-GFP F/R; (iv) The PCR product of the SsSSVP1-GFP fusion was amplified using the primers pTRV-SsSSVP1-GFP F and GFP R from the pTRV2-SsSSVP1ΔSP-GFP constructs. The final fragments from (i) to (iv) were directly cloned into pTRV2 digested with XcmI to construct pTRV2-SsSSVP1ΔSP, pTRV2-SsSSVP1,
pTRV2-GFP, pTRV2-SP-GFP, pTRV2-SsSSVP1Δ-SP-GFP and pTRV2-SsSSVP1-GFP vectors, respectively (S9D–S9I Fig). For the co-localization assay, SsSSVP1Δ-SP-C38A-C44A-mCherry fusion protein encoding sequence was constructed by spliced overlap extension PCR. SsSSVP1Δ-SP-mCherry fusion protein encoding sequence was amplified using the primers pTRV-SsSSVP1Δ-SP-mCherry F/R from the construct containing SsSSVP1-mCherry-NLS. QCR8 and GFP encoding sequences were amplified using the primers QCR8-F/R and GFP F/R, respectively, and then cloned into the intermediate vector pBI121, from which the QCR8-GFP fusion protein encoding sequence was amplified using the primers pTRV-QCR8-GFP F/R. The amplified SsSSVP1Δ-SP-C38A-C44A-mCherry, SsSSVP1Δ-SP-mCherry and QCR8-GFP fusion protein encoding sequences were finally cloned into the pTRV2 vector, respectively, using the same method described as above (S9J and S9K Fig). For validating the expression of SsSSVP1Δ-SP-C38A-C44A and SsSSVP1Δ-SP in tobacco leaf cells using western blotting analysis, the primers pTRV2-SsSSVP1-3×FLAG F/R were used to amplify SsSSVP1Δ-SP-C38A-C44A and SsSSVP1Δ-SP from the constructs containing these two fragments, respectively, before they were cloned into the pTRV2 vector (S9L Fig). Constructs containing these fragments in the correct orientation were PCR screened using the primer pTRV F and corresponding downstream primers respectively. To generate the VIGS-pTRV2-QCR8 silencing constructs (S9M and S9N Fig), partial coding regions of the three QCR8 genes were amplified from the cDNA library of N. benthamiana using the primers RNAi-QCR8-1F/R, RNAi-QCR8-2 F/R and RNAi-QCR8-3 F/R, respectively, and then digested with EcoRІ and BamHІ prior to ligated into VIGS-pTRV2 vector digested with the same pair of restriction enzymes. The pTRV1 construct (S9O Fig) from Liu Y et al. [39] was directly used. All the constructs were confirmed through sequencing analysis. The primers are shown in S2 Table. These constructs were then introduced into the A. tumefaciens strain GV3101-pM90 through electroporation [71]. Equal amounts of agrobacterium containing the constructs and pTRV1 were mixed respectively for infiltration performed with N. benthamiana leaves as previously described [28].

**Protein precipitation, dialysis and condensation**

To determine whether SsSSVP1 was secreted into the liquid cultures, the positive SsSSVP1–FLAG engineered strains were cultured in liquid CM medium at 20°C for 3 days, with shaking at 200 rpm. The culture broth was filtered with 4 layers of Calbiochem Miracloth and centrifuged at 10,000 rpm for 5 min to remove the hyphal fragments. Secreted proteins in the fermentation liquid were precipitated with solid ammonium sulfate (100% saturated). The precipitated secreted proteins were dissolved in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4) and desalinated through dialysis. The protein extracts in dialysis bag were further condensed using saccharose at 4°C and lyophilized overnight before being dissolved in 0.1ml of 4×protein loading buffer for western blot analysis after quantification.

**Western blotting**

To screen the positive SsSSVP1–FLAG engineered strains, total proteins extracted from the mycelia of SsSSVP1–FLAG transformants by cell lysis buffer (Beyotime, Wuhan, Hubei, China) were used for immunoprecipitation (IP) and western blot analysis. About 5 μl ANTI-FLAG M2 monoclonal antibody (Sigma, Saint Louis, Missouri, USA) was added to 1 ml protein extracts and then was incubated at room temperature for 2 hours. Afterwards, protein A+G agarose (Beyotime, Wuhan, Hubei, China) was added to the protein extracts and was incubated at room temperature for 1 hour before it was collected by centrifugation and washed for five times by the cell lysis buffer, and then protein loading buffer was added for following western blot analysis. Proteins were separated by SDS-PAGE gel (12%) before they were transferred
onto a 0.22 μm PVDF membrane (Millipore) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). A monoclonal α-Anti FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) and a goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) were used as a primary antibody and a secondary antibody respectively. To validate the secretion of SsSSVP1, total proteins obtained from the liquid CM medium through protein precipitation, dialysis and condensation described as above were directly used for western blot analysis using the same method without the IP procedure. The secondary antibody used in this experiment was a goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich, St. Louis, MO, USA). The signals of blots were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific).

**Nuclear targeting assay**

To generate the SsSSVP1-mCherry-NLS and SP-mCherry-NLS fusion constructs, SsSSVP1 and mCherry were PCR amplified using the primers SsSSVP1 F/R and mCherry F/R respectively. The PCR products were digested with appropriate restriction enzymes and then ligated into the pCXH (a fungal expression vector constructed by our lab) through the formation of intermediate constructs. The SP-mCherry-NLS and SsSSVP1-mCherry-NLS fragments with a stop codon were amplified from the pCXH vector with SsSSVP1-mCherry fusion using the primers SP-mCherry-NLS F/R and SsSSVP1-mCherry-NLS F/R before they were finally cloned into pDL2 vector [73], respectively, by the yeast gap repair approach [74]. The SP-mCherry-NLS and SsSSVP1-mCherry-NLS fusion constructs were transformed into the B. cinerea B05.10 strain using the PEG-mediated transformation method [75]. Tissues from onion bulb lower epidermal cells infected with the B. cinerea engineered strains expressing SP-mCherry-NLS and SsSSVP1-mCherry-NLS fusion proteins were examined at 48 hpi, respectively.

**Confocal microscopy**

To observe fluorescence, the tobacco tissues were harvested from infiltrated tobacco leaves at 3 dpi and the onion tissues were harvested from inoculated lower epidermis at 36 hpi, and then directly imaged under a confocal laser scanning microscope (OLYMPUS microscope FV1000). The 488-, 587, 514- and 458-nm absorption laser lines with corresponding appropriate specific emission filter sets were used when images of GFP, mCherry, YFP and chloroplast autofluorescence were recorded, respectively.

**Nucleic acid isolation and transcript level determination**

Genomic DNA was isolated as previously described [76] and used for the validation of T-DNA insertion in the transformants through PCR with the primers Hyg F/R (S2 Table). To evaluate the expression levels of SsSSVP1 in different transformants, the transformants were inoculated on cellophane placed on PDA plates before pure mycelia of the transformants were collected for RNA isolation. To evaluate the expression levels of SsSSVP1 during different infection stages of the wild-type strain, pure fresh mycelia without culture medium were inoculated on A. thaliana leaves. The inoculated leaves were collected at 0, 3, 6, 9, 12 hpi and frozen in liquid nitrogen and ground to a powder for RNA extraction. To evaluate the expression levels of QCR8, the upper and middle N. benthamiana leaves were sampled one month after A. tumefaciens infiltration. Total RNA was extracted using the TRIZOL Reagent (Huashun Biotechnology Co, Shanghai, China) according to the manufacturer’s instructions and treated with DNase I (RNase free, Takara, Dalian, China). Synthesis of first-strand cDNA and qRT-PCR were conducted according to Zhu et al. [27]. The expression levels of SsSSVP1 were examined through qRT-PC using the primers QPCR-SsSSVP1 F/R. The expression levels of the S. sclerotiorum
β-tubulin gene (SS1G_04652) [77] were used to normalize the expression of SsSSVP1 in each corresponding qRT-PCR sample using the primers Tub F/R. The expression levels of the three genes encoding the homologs of QCR8 in N. benthamiana were examined through qRT-PCR using the primers QPCR-QCR8-1 F/R, QPCR-QCR8-2 F/R and QPCR-QCR8-3 F/R, respectively. The expression levels of the N. benthamiana actin gene (AY179605.1) [27] were used to normalize the expression of QCR8 in each corresponding qRT-PCR sample using the primers Actin F/R. The qRT-PCR assay was repeated at least twice for each gene, with three replicates. The primers used for qRT-PCR are shown in S2 Table.

Characterization of the SsSSVP1-silenced and SsSSVP1-overexpressed transformants

The detached B. napus (zhongyou 821) leaves under the same physiological conditions were used for the virulence assay of S. sclerotiorum wild-type strain and transformants. To evaluate virulence, at least six individual detached B. napus leaves or in vivo A. thaliana leaves were inoculated with a single 0.5-cm diameter mycelium-colonized agar plug obtained from the expanding margins of PDA-cultured colonies. Inoculated leaves were maintained at 100% relative humidity at 20°C for 48 h (for B. napus leaves) or 36 h (for A. thaliana leaves). Disease severity was measured using the average lesion diameter. To assay growth rates, the wild-type strain and the transformants were cultivated on PDA at 20°C for 3 days. Mycelial agar discs were collected from the active colony edge and inoculated in the center of the PDA Petri dish at 20°C before the hyphal growth was examined. Each experiment was performed independently at least three times.

Protein-protein interaction assays

Y2H analysis was performed using a GAL4-based Y2H system (Matchmaker Gold Systems; Clontech, Palo Alto, CA). The construction of Y2H library, autoactivation and toxicity test and the screening of Y2H library were performed according to the manufacturer’s instructions. The primers used to create the corresponding constructs are listed in S2 Table. The bait and prey plasmids were co-transformed into a yeast strain Y2HGold (Clontech, Palo Alto, CA). Yeast transformation was performed according to the manufacturer’s instructions. The transformants were assayed for growth on synthetic dropout (SD)/-Trp-Leu plates, and cultured on liquid synthetic SD/-Trp-Leu medium for 36 hours before being collected by centrifugation. The concentration of collected yeast cells were adjusted to 10^6 (cells/ml) using sterile water, and then 5 μl yeast suspension was assayed for growth on SD/-Trp-Leu-His-Ade plates containing the X-α-gal and Aureobasidin A (AbA).

For Co-IP assay, to construct pCNF3-SsSSVP1ΔSP-GFP and pCNF3-QCR8-3×FLAG (S9P and S9Q Fig), the full-length of the SsSSVP1ΔSP-GFP and QCR8-3×FLAG were amplified using the specific primers COIP-SsSSVP1ΔSP-GFP F/R and COIP-QCR8-3×FLAG F/R (S2 Table), respectively, and then cloned into the pCNF3 vector (a plant expression vector constructed by our lab). A. tumefaciens containing the pCNF3-SsSSVP1ΔSP-GFP and pCNF3-QCR8-3×FLAG constructs were co-infiltrated into N. benthamiana leaves using the same method described as above. Total protein was isolated by homogenizing tissues with RIPA lysis buffer (Beyotime, Wuhan, Hubei, China) with a modification [50 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EDTA, 0.5 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1% proteinase inhibitor cocktail (Sigma, Saint Louis, Missouri, USA)]. Approximately 3 g plant tissues were lysed by 10 ml RIPA lysis buffer. The total protein was then centrifuged at 13000 rpm for 1 h to remove residues. For anti-GFP IP, approximately 2 ml supernatant RIPA lysis buffer containing the total protein was incubated with 10 μl of anti-
GFP monoclonal antibody (sigma, Saint Louis, Missouri, USA) and 50 μl of protein G plus-Agarose (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) for 8 h at 4°C on a rotary shaker. The beads were then collected and washed five times with RIPA lysis buffer. The bound protein was eluted from beads by boiling in protein sample buffer. One third of the immunoprecipitated protein was subjected to immunoblot analyses with anti-FALG monoclonal antibody (sigma, Saint Louis, Missouri, USA). Approximately 25 μl of RIPA lysis buffer containing the total protein was loaded as input control.

BiFC assay was used to study the interaction of SsSSVP1ΔSP and QCR8 based on a previously described method [78]. To construct the pBISPYNE-SsSSVP1ΔSP and pBISPYCE-QCR8 vectors (S9R and S9S Fig), respectively, the full-length cDNAs of the SsSSVP1ΔSP and QCR8 were amplified using the specific primers BiFC-SsSSVP1ΔSP F/R and BiFC-QCR8 F/R (S2 Table), respectively, recombined with the N- and C-termini of YFP, respectively, and subsequently cloned into the pBI121 vector through intermediate vectors pUC-SPYNE and pUC-SPYCE. The constructs were verified by sequencing. All plasmids were transformed into N. benthamiana leaves via the A. tumefaciens strain GV3101-pM90.

Site-directed mutagenesis
The eight cysteine residues of SsSSVP1ΔSP were substituted by alanine respectively according to the manual of QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The double-point mutant SsSSVP1ΔSP-C38A-C44A was constructed by fusion PCR using the primers MutC38A-C44A-1 F/R and MutC38A-C44A-2 F/R. The coding sequences of SsSSVP1ΔSP mutants were cloned into the pGBKT7 and pGADT7 vector respectively for Y2H analysis and cloned into the pTRV2 vector respectively for functional analysis. Mutations were confirmed by sequencing analysis. The primers used in this experiment were listed in S2 Table.

Supporting Information
S1 Fig. The occasional subcellular localization of SsSSVP1ΔSP in plant cells and the co-localization of SsSSVP1ΔSP and QCR8. (A) Laser confocal micrograph showing SsSSVP1ΔSP occasionally localizes in nuclei and cytoplasmic compartments in a particle-like form. These photos were taken from different areas in the same N. benthamiana leaf. Red particles showed chloroplast autofluorescence. Photos were taken 3 days after agroinfiltration. Maximum projections of 4 confocal images captured along the z-axis are shown. (B) SsSSVP1ΔSP and QCR8 occasionally co-localize in nuclei or cytoplasm in a particle-like form. These photos were taken from different areas in the same N. benthamiana leaf. Fluorescence was monitored 3 days after agroinfiltration using confocal laser scanning microscopy. The images show maximum projections of 4 confocal images captured along the z-axis.

S2 Fig. The ER-like fluorescence distribution of SP-GFP and SsSSVP1-GFP. Both SP-GFP (which was used as control) and SsSSVP1-GFP localized in ER-like structure in plant cells. The left column fluorescence images, which are higher magnification images of the areas marked by the red boxes in the right column, indicated ER-like structure. The SP refers in particular to the SP of SsSSVP1. Photos were taken 3 days after agroinfiltration.

S3 Fig. The fluorescence distribution of SsSSVP1-mCherry-NLS in different layers of host cells. Details for this nuclear targeting assay see Fig 4. (A) The diagram of laser layer-by-layer scanning around z-axis by a confocal microscope. (B) Divided layer images of laser scanning. All the divided layer images were merged finally. Different layers of the intact surrounding
cells were checked independently to ensure there were no hyphae in these cells. Areas within yellow dotted line indicate hyphal invaded onion epidermal cells.

(S4 Fig. SsSSVP1-silenced transformants showing reduced virulence on A. thaliana leaves. (A) Virulence test of SsSSVP1-silenced transformants on in vivo A. thaliana leaves. (B) Virulence was evaluated according to the lesion diameter at 36 hpi. Six independent replicates were performed. The values are presented as the means±s.d. Different letters on the graph indicate statistical differences, P = 0.05.

(S5 Fig. The biological characteristics of the SsSSVP1 over-expression transformants SsSSVP1. (A) The colony morphology of the SsSSVP1 over-expression transformants. Colonies were grown on PDA for 10 days at 20°C. (B) No significant virulence reduction is observed in over-expression transformants of SsSSVP1. Virulence is evaluated on detached oilseed rape leaves (B. napus zhongyou 821) according to the lesion diameter at 20°C for 48 h. (C) The relative expression of SsSSVP1 in different over-expression transformants is analyzed through qRT-PCR. β-tubulin expression levels is used to normalize the expression levels of SsSSVP1 in different samples, and the expression level in the wild-type strain was set as 1.0. (D) Western blot analysis with proteins isolated from mycelia of the wild-type strain and the SsSSVP1-FLAG engineered strains respectively. SDS-polyacrylamide gel electrophoresis shows the equal loading amount of proteins used for the west blot analysis. Alkaline phosphatase conjugated secondary antibody detected an approximate 17 kDa band in OEsSsSSVP1-3, but not in Ep-PNA367. (E) Comparison of the lesion diameter of over-expression transformants and the wild-type strain. In all experiments, three independent replicates were performed. The values are presented as the means±s.d. Different letters on the graph indicate statistical differences, P = 0.05.

(S6 Fig. Y2H assay showed SsSSVP1ΔSP interacted with the other homolog of QCR8 (AT-QCR8, AT5G05370) in A. thaliana and all the homologs of QCR8 (NB-QCR8) in N. benthamiana. pGBK7T7-53 and pGADT7-T were used as positive controls (Clontech). "−" means there is an empty vector. The negative controls indicated SsSSVP1ΔSP and QCR8 were not self-activated. Photos were taken 2 dpi.

(S7 Fig. Y2H assay and single site-directed mutagenesis of the eight cysteines in SsSSVP1ΔSP were combined to screen double-point mutants losing the capability to form homo-dimer. The coding sequences of different single-point mutants of SsSSVP1ΔSP were cloned into pGBK7T7 and pGADT7 vector, respectively, before performing Y2H assay. Bottom left of the slash shows the growth of co-transformed Y2H strain on SD/-Leu-Trp medium and top right of the slash shows the growth of co-transformed Y2H strain on SD/-Leu-Trp-His-Ade+X-α-Gal+AbA medium. Red rectangle indicates SsSSVP1ΔSP-C38A cannot interact with SsSSVP1ΔSP-C44A anymore. Photos were taken 2 dpi.

(S8 Fig. The validation of the effectiveness of the TRV-VIGS system. The phenotype of PDS-silenced N. benthamiana lines using TRV based VIGS system. Silencing of the PDS in N. benthamiana plants caused photobleaching phenotype. No obvious phenotype was observed in the control. Photos were taken one month after A. tumefaciens infiltration.

(TIFF)
S9 Fig. Graphical representations of the vectors and constructs used in this study. (A) Graphical representation of SsSSVP1-FLAG fusion construct used for immunolocalization. The SsSSVP1-FLAG fusion was expressed under the control of the PEF-1α promoter and the TtrpC terminator. (B) Construction of pRNAi-1 vector targeted against SsSSVP1. The SsSSVP1 fragment was amplified using the corresponding primers from the S. sclerotiorum cDNA library and subsequently inserted between the Neurospora crassa trpC promoter PtrpC and the Aspergillus nidulans gpd promoter Pgpd. The PptrpC and Pgpd are in an opposite directions in this vector. (C) Construction of pRNAi-2 vectors targeted against SsSSVP1. The fused SsSSVP1-intron-SsSSVP1 fragment was inserted between the A. nidulans trpC promoter PtrpC and terminator TtrpC. The two fragments of SsSSVP1 are same but in a reverse orientation in this vector. The intron is from Gibberella zeae. (D-L) Construction of binary virus vectors pTRV2-SsSSVP1ΔSP, pTRV2-SsSSVP1, pTRV2-GFP, pTRV2-SP-GFP, pTRV2-SsSSVP1ΔSP-GFP, pTRV2-SsSSVP1-GFP, pTRV2-SsSSVP1ΔSP-C38A-C44A_mCherry, pTRV2-SsSSVP1ΔSP-mCherry, pTRV2-QCR8-GFP, pTRV2-SsSSVP1ΔSP-C38A-C44A_3xFLAG and pTRV2-SsSSVP1ΔSP-3xFLAG. Corresponding fragments were cloned and inserted into the TA cloning site in the pTRV2 vector under the control of Csp, which is the promoter of the TRV coat protein. The open reading frame (ORF) originating from the virus correspond to a coat protein (CP). (M and N) Graphical representation of the VIGS-pTRV2 vectors used for silencing plant genes. The Csp promoter was removed from the pTRV2 vector to produce VIGS-pTRV2 vector. Partial fragments of PDS and QCR8 amplified from the N. benthamiana cDNA library were cloned into the VIGS-pTRV2 vector, respectively, to silence corresponding endogenous genes in N. benthamiana plants. (O) Graphical representation of pTRV1 vectors. The open reading frames (ORFs) of pTRV1 originating from the virus correspond to 134 and 194 kDa replicases (RPs), a movement protein (MP) and a 16-kDa cysteine-rich protein, respectively. All TRV cDNA clones were placed between the duplicated CaMV 35S promoter (2×35S) and the nopaline synthase terminator (NOSt) in a T-DNA vector. Rz, self-cleaving ribozyme. (P and Q) Graphical representation of the pCNF3-SsSSVP1ΔSP-GFP and pCNF3-QCR8-3xFLAG vectors used for Co-IP. (R and S) Graphical representation of the pBISPYNE-SsSSVP1ΔSP and pBISPYCE-QCR8 vectors used for BiFC. The SsSSVP1ΔSP-YFP N-terminal fusion and the QCR8-YFP C-terminal fusion were expressed respectively under the control of the CaMV 35S promoter and the NOS terminator. SP indicates the SP of SsSSVP1. The Gly linker encodes three glycines in tandem. LB and RB refer to the left and right borders of the T-DNA, respectively. (TIFF)

S1 Table. The potential effector candidates in S. sclerotiorum identified in this study. Δ The 314 potential effector candidates are designated as predicted secreted proteins, the gene expression of which is significantly up-regulated during infection of S. sclerotiorum. * indicates different types of predicted secreted proteins, please refer to Fungal Secretome Database (FSD) for details [79]. ** indicates the gene expression change folds detected by DGE. (XLSX)

S2 Table. Primers used in this study. (XLS)

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Author Contributions
Conceived and designed the experiments: JC DJ. Performed the experiments: XL CS. Analyzed the data: XL CS DJ JC. Contributed reagents/materials/analysis tools: XL CS YF JX DJ GL JC. Wrote the paper: XL JC.

References
1. Boland G, Hall R (1994) Index of plant hosts of Sclerotinia sclerotiorum. Can J Plant Pathol 16: 93.
2. Laluk K, Mengiste T (2010) Necrotroph attacks on plants: wanton destruction or covert extortion? Arabidopsis Book 8: e0136. doi: 10.1199/tab.0136 PMID: 22303261
3. Koeck M, Hardham AR, Dodds PN (2011) The role of effectors of biotrophic and hemibiotrophic fungi in infection. Cell Microbiol 13: 1849–1857. doi: 10.1111/j.1462-5822.2011.01665.x PMID: 21848815
4. Khang CH, Berruyer R, Giraldo MC, Kankanala P, Park SY, et al. (2010) Translocation of Magnaporthe oryzae effectors into rice cells and their subsequent cell-to-cell movement. Plant Cell 22: 1388–1403. doi: 10.1105/tpc.109.069666 PMID: 20435900
5. Ravensdale M, Nemri A, Thrall PH, Ellis JG, Dodds PN (2011) Co-evolutionary interactions between host resistance and pathogen effector genes in flax rust disease. Mol Plant Pathol 12: 93–102. doi: 10.1111/j.1364-3703.2010.00657.x PMID: 21118351
6. Schirawski J, Mannhaupt G, Munch K, Brefort T, Schipper K, et al. (2010) Pathogenicity determinants in smut fungi revealed by genome comparison. Science 330: 1546–1548. doi: 10.1126/science.1195330 PMID: 21148393
7. Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, et al. (2010) Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. Science 330: 1543–1546. doi: 10.1126/science.1194573 PMID: 21148392
8. Riou C, Freyssinet G, Fevre M (1991) Production of cell wall-degrading enzymes by the phytopathogenic fungus Sclerotinia sclerotiorum. Appl Environ Microbiol 57: 1478–1484. PMID: 16348487
9. Dutton MV, Evans CS (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can J Microbiol 42: 881–895.
10. Williams B, Kabbage M, Dickman MB, Low PS (2000) Oxalic acid, a pathogenicity factor for Sclerotinia sclerotiorum, suppresses the oxidative burst of the host plant. Plant Cell 12: 2191–2200. PMID: 11090218
11. Williams B, Kabbage M, Kim HJ, Britt R, Dickman MB (2011) Tipping the balance: Sclerotinia sclerotiorum secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathog 7: e1002107. doi: 10.1371/journal.ppat.1002107 PMID: 21738471
12. Kim KS, Min JY, Dickman MB (2008) Oxalic acid is an elicitor of plant programmed cell death during Sclerotinia sclerotiorum disease development. Mol Plant Microbe Interact 21: 605–612. doi: 10.1094/MPMI-21-5-0605 PMID: 18393620
13. Kabbage M, Williams B, Dickman MB (2013) Cell death control: the interplay of apoptosis and autophagy in the pathogenicity of Sclerotinia sclerotiorum. PLoS Pathog 9: e1003287. doi: 10.1371/journal.ppat.1003287 PMID: 23992997
14. Faris JD, Zhang Z, Lu H, Lu S, Reddy L, et al. (2010) A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. Proc Natl Acad Sci U S A 107: 13544–13549. doi: 10.1073/pnas.1004090107 PMID: 20624958
15. Hammond-Kosack KE, Rudd JJ (2008) Plant resistance signalling hijacked by a necrotrophic fungal pathogen. Plant Signal Behav 3: 993–995. PMID: 19704431
16. Lorang J, Kidarsa T, Bradford CS, Gilbert B, Curtis M, et al. (2012) Tricking the guard: exploiting plant defense for disease susceptibility. Science 338: 659–662. doi: 10.1126/science.1226743 PMID: 23087901
17. Nagy ED, Bennetzen JL (2008) Pathogen corruption and site-directed recombination at a plant disease resistance gene cluster. Genome Res 18: 1916–1923. doi: 10.1101/gr.078766.106 PMID: 18719093
18. Liu ZH, Faris JD, Meinhardt SW, Ali S, Rasmussen JB, et al. (2004) Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by Stagonospora nodorum. Phytopathology 94: 1056–1060. doi: 10.1094/PHYTO.2004.94.10.1056 PMID: 18943793
19. Liu Z, Friesen TL, Ling H, Meinhardt SW, Oliver RP, et al. (2006) The Tsn1-ToxA interaction in the wheat-Stagonospora nodorum pathosystem parallels that of the wheat-tan spot system. Genome 49: 1265–1273. PMID: 17213908
20. Friesen TL, Stuenkenbrock EH, Liu Z, Meinhardt S, Ling H, et al. (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. Nat Genet 38: 953–956. PMID: 16832356
21. Friesen TL, Meinhardt SW, Faris JD (2007) The Stagonospora nodorum-wheat pathosystem involves multiple proteinaceous host-selective toxins and corresponding host sensitivity genes that interact in an inverse gene-for-gene manner. Plant J 51: 681–692. PMID: 17573802
22. Friesen TL, Zhang Z, Solomon PS, Oliver RP, Faris JD (2008) Characterization of the interaction of a novel Stagonospora nodorum host-selective toxin with a wheat susceptibility gene. Plant Physiol 146: 682–693. PMID: 18065563
23. Abeysekara NS, Friesen TL, Keller B, Faris JD (2009) Identification and characterization of a novel host-toxin interaction in the wheat-Stagonospora nodorum pathosystem. Theor Appl Genet 120: 117–126. doi: 10.1007/s00122-009-1163-6 PMID: 19816671
24. Zhang Z, Friesen TL, Xu SS, Shi G, Liu Z, et al. (2011) Two putatively homoeologous wheat genes mediate recognition of SnTox3 to confer effector-triggered susceptibility to Stagonospora nodorum. Plant J 65: 27–38. doi: 10.1111/j.1365-313X.2010.04407.x PMID: 21175887
25. El Oirdi M, El Rahman TA, Rigano L, El Hadrami A, Rodriguez MC, et al. (2011) An Effector-Like Virulence Factor in Stagonospora nodorum. An Effector-Like Virulence Factor in Stagonospora nodorum. PLoS One 8: e53901. doi: 10.1371/journal.pone.0053901 PMID: 23342034
26. Xiao X, Xie J, Cheng J, Li G, Yi X, et al. (2014) Novel secretory protein Ss-Caf1 of the plant-pathogenic fungus Sclerotinia sclerotiorum that suppresses host resistance. PLoS One 8: e53901. doi: 10.1371/journal.pone.0053901 PMID: 23342034
27. Zhu W, Wei W, Fu Y, Cheng J, Xie J, et al. (2013) A secretory protein of necrotrophic fungus Sclerotinia sclerotiorum that suppresses host resistance. PLoS One 8: e53901. doi: 10.1371/journal.pone.0053901 PMID: 23342034
28. Iwata S, Lee JW, Okada K, Lee JK, Iwata M, et al. (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. Science 281: 64–71. PMID: 9651245
29. Nelson BK, Cai X, Nebenfuhr A (2007) A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J 51: 1126–1136. PMID: 17666025
30. Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J 30: 415–429. PMID: 12028572
31. Stergiopoulos I, de Wit PJ (2009) Fungal effector proteins. Annu Rev Phytopathol 47: 233 PMID: 26263551
32. Rep M (2005) Small proteins of plant-pathogenic fungi secreted during host colonization. FEMS Microbiol Lett 253: 19–26. PMID: 16216445
33. Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J 30: 415–429. PMID: 12028572
34. Rep M (2005) Small proteins of plant-pathogenic fungi secreted during host colonization. FEMS Microbiol Lett 253: 19–26. PMID: 16216445
35. Guyon K, Balague C, Roby D, Raffaele S (2014) Secretome analysis reveals effector candidates associated with broad host range necrotrophy in the fungal plant pathogen Sclerotinia sclerotiorum. BMC Genomics 15: 336. doi: 10.1186/1471-2164-15-336 PMID: 24886033
36. Stergiopoulos I, de Wit PJ (2009) Fungal effector proteins. Annu Rev Phytopathol 47: 233–263. doi: 10.1146/annurev.phyto.112408.132637 PMID: 19400631
37. Kalderon D, Roberts BL, Richardson WD, Smith AE (1984) A short amino acid sequence able to specify nuclear location. Cell 36: 483–493. PMID: 6096007
38. Ikawa S, Lee JW, Okada K, Lee JK, Ikawa M, et al. (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. Science 281: 64–71. PMID: 9651245
39. Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J 30: 415–429. PMID: 12028572
40. Severis C, Kaiser CA (2002) Formation and transfer of disulphide bonds in living cells. Nat Rev Mol Cell Biol 3: 836–847. PMID: 12415301
41. Marianayagam NJ, Sunde M, Matthews JM (2004) The power of two: protein dimerization in biology. Trends Biochem Sci 29: 618–625. PMID: 15501681
42. Laskowski M Jr., Kato I (1980) Protein inhibitors of proteinases. Annu Rev Biochem 49: 593–626. PMID: 6996568
43. Ridout CJ, Skamnioti P, Porritt O, Sacriscan S, Jones JD, et al. (2006) Multiple avirulence paralogues in cereal powdery mildew fungi may contribute to parasite fitness and defeat of plant resistance. Plant Cell 18: 2402–2414. PMID: 16905653

44. Block A, Li G, Fu ZQ, Alfano JR (2008) Phytopathogen type III effector weaponry and their plant targets. Curr Opin Plant Biol 11: 396–403. doi: 10.1016/j.pbi.2008.06.007 PMID: 18657470

45. Gohre V, Robatzek S (2008) Breaking the barriers: microbial effector molecules subvert plant immunity. Annu Rev Phytopathol 46: 189–215. doi: 10.1146/annurev.phyto.46.120407.110050 PMID: 18422429

46. Wang Q, Han C, Ferreira AO, Yu X, Ye W, et al. (2011) Transcriptional programming and functional interactions within the Phytophthora sojae RXLR effector repertoire. Plant Cell 23: 2064–2086. doi: 10.1105/tpc.111.086082 PMID: 21653195

47. Whisson SC, Boevink PC, Moleleki L, Avrova AO, Morales JG, et al. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450: 115–118. PMID: 17914356

48. Birch PR, Boevink PC, Gilroy EM, Hein I, Pritchard L, et al. (2008) Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. Curr Opin Plant Biol 11: 373–379. doi: 10.1016/j.mib.2008.04.005 PMID: 18511334

49. Wawra S, Belmonte R, Lobach L, Saraiva M, Willems A, et al. (2012) Secretion, delivery and function of oomycete effector proteins. Curr Opin Microbiol 15: 685–691. doi: 10.1016/j.mib.2012.10.008 PMID: 23177095

50. Panstruga R, Dodds PN (2009) Terrific protein traffic: the mystery of effector protein delivery by filamentous plant pathogens. Science 324: 748–750. doi: 10.1126/science.1171652 PMID: 19423815

51. Ellis J, Catanzariti AM, Dodds P (2006) The problem of how fungal and oomycete avirulence proteins enter plant cells. Trends Plant Sci 11: 61–63. PMID: 16406302

52. Birch PR, Rehmany AP, Pritchard L, Kamoun S, Beynon JL (2006) Trafficking arms: oomycete effector proteins enter host plant cells. Trends Microbiol 14: 8–11. PMID: 16356717

53. Kale SD, Gu B, Capelluto DG, Dou D, Feldman E, et al. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142: 284–295. doi: 10.1016/j.cell.2010.06.006 PMID: 20695469

54. Dou D, Kale SD, Wang X, Jiang RH, Bruce NA, et al. (2008) RXLR-mediated entry of Phytophthora sojae effector Avr1b into soybean cells does not require pathogen-encoded machinery. Plant Cell 20: 1930–1947. doi: 10.1105/tpc.107.056093 PMID: 18621946

55. Kale SD, Tyler BM (2011) Entry of oomycete and fungal effectors into plant and animal host cells. Cell Microbiol 13: 1839–1848. doi: 10.1111/j.1462-5822.2011.01659.x PMID: 21819515

56. Gu B, Kale SD, Wang Q, Wang D, Pan Q, et al. (2011) Rust secreted protein PsbP is conserved in diverse fungal pathogens and contains a RXLR-like motif sufficient for translocation into plant cells. PLoS One 6: e27217. doi: 10.1371/journal.pone.0027217 PMID: 22076138

57. Tyler BM, Kale SD, Wang Q, Tao K, Clark HR, et al. (2013) Microbe-independent entry of oomycete RXLR effectors and fungal RxLR-like effectors into plant and animal cells is specific and reproducible. Mol Plant Microbe Interact 26: 611–616. doi: 10.1094/MPMI-02-13-0051-IA PMID: 23550528

58. Manning VA, Ciuffetti LM (2005) Localization of Ptr ToxA produced by Phytophthora sojae ToxA. An RGD-containing effector-like virulence factor in S. sclerotiorum reveals protein import into wheat mesophyll cells. Plant Cell 17: 3203–3212. PMID: 16199615

59. Meinhardt SW, Cheng W, Kwon CY, Donohue CM, Rasmussen JB (2012) Role of the arginyl-glycyl-aspartic motif in the action of the fungal RxLR effector Avr1b in soybean cells. J Gen Virol 87: 2491–2496. doi: 10.1099/jgv.0.043781-0 PMID: 22804012

60. Sarma GN, Manning VA, Ciuffetti LM, Karplus PA (2005) Structural model of PtoXa receptor complex: a key target of the bacterial PtoXa effectors. J Mol Biol 345: 1200–1208. doi: 10.1016/j.jmb.2004.11.082 PMID: 15650363

61. Braun HP, Kruft V, Schmitz UK (1994) Molecular identification of the ten subunits of cytochrome-c reductase from potato mitochondria. Planta 189: 100–105. PMID: 7604824

62. Kong L, Wu J, Lu L, Xu Y, Zhou X (2014) Interaction between Rice stripe virus disease-specific protein and host PsbP enhances virus symptoms. Mol Plant 7: 691–708. doi: 10.1093/mp/ssu158 PMID: 24214493

63. Xie J, Wei D, Jiang D, Fu Y, Li G, et al. (2006) Characterization of debilitation-associated mycovirus infecting the plant-pathogenic fungus Sclerotinia sclerotiorum. J Gen Virol 87: 241–249. PMID: 16361437

64. Li Y, Chen J, Bennett R, Kiddie G, Wallsgrass R, et al. Breeding, inheritance, and biochemical studies on Brassica napus cv. Zhongyou 821: tolerance to Sclerotinia sclerotiorum (stem rot); 1999.

65. Yu Y, Jiang D, Xie J, Cheng J, Li G, et al. (2012) Ss-Sl2, a novel cell wall protein with PAN modules, is essential for sclerotial development and cellular integrity of Sclerotinia sclerotiorum. PLoS One 7: e34962. doi: 10.1371/journal.pone.0034962 PMID: 22558105
66. Yuan M, Chu Z, Li X, Xu C, Wang S (2010) The bacterial pathogen Xanthomonas oryzae overcomes rice defenses by regulating host copper redistribution. Plant Cell 22: 3164–3176. doi: 10.1105/tpc.110.078022 PMID: 20852017

67. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8: 785–786. doi: 10.1038/nmeth.1701 PMID: 21959131

68. Papadopoulos JS, Agarwala R (2007) COBALT: constraint-based alignment tool for multiple protein sequences. Bioinformatics 23: 1073–1079. PMID: 17332019

69. Clamp M, Cuff J, Searle SM, Barton GJ (2004) The Jalview Java alignment editor. Bioinformatics 20: 426–427. PMID: 14960472

70. Nguyen QB, Kadotani N, Kasahara S, Tosa Y, Mayama S, et al. (2008) Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, Magnaporthe oryzae, using a high-throughput RNA-silencing system. Mol Microbiol 68: 1348–1365. doi: 10.1111/j.1365-2958.2008.06242.x PMID: 18433453

71. Wise AA, Liu Z, Binns AN (2006) Three methods for the introduction of foreign DNA into Agrobacterium. Methods Mol Biol 343: 43–53. PMID: 16988332

72. Chen PY, Wang CK, Soong SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breeding 11: 287–293.

73. Zhou X, Li G, Xu JR (2011) Efficient approaches for generating GFP fusion and epitope-tagging constructs in filamentous fungi. Methods Mol Biol 722: 199–212. doi: 10.1007/978-1-61779-040-9_15 PMID: 21590423

74. Bruno KS, Tenjo F, Li L, Hamer JE, Xu JR (2004) Cellular localization and role of kinase activity of PMK1 in Magnaporthe grisea. Eukaryot Cell 3: 1525–1532. PMID: 15590826

75. Hamada W, Reignault P, Bompeix G, Boccara M (1994) Transformation of Botrytis cinerea with the hygromycin B resistance gene, hph. Curr Genet. 26: 251–255. PMID: 7859308

76. Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of Aspergillus nidulans by using a trpC plasmid. Proc Natl Acad Sci U S A 81: 1470. PMID: 6324193

77. Harel A, Bercovich S, Yarden O (2006) Calcineurin is required for sclerotial development and pathogenicity of Sclerotinia sclerotiorum in an oxalic acid-independent manner. Mol Plant Microbe Interact 19: 682–693. PMID: 16779301

78. Walter M, Chaban C, Schutze K, Batistic O, Weckermann K, et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J 40: 428–438. PMID: 15469500

79. Choi J, Park J, Kim D, Jung K, Kang S, et al. (2010) Fungal secretome database: integrated platform for annotation of fungal secretomes. BMC Genomics 11: 105. doi: 10.1186/1471-2164-11-105 PMID: 20146824