The Contribution of *Sclerotinia sclerotiorum* Effectors to Sclerotinia Stem Rot Development

By

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ABSTRACT

Sclerotinia stem rot (SSR) is the disease caused by the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. SSR has a major impact on yield in crop production worldwide. Recent advances in genome sequencing and other computational tools revealed the presence of hundreds of proteinaceous effectors in the genome of this broad range pathogen. Our latest transcriptome analysis from *S. sclerotiorum*-infected soybeans further showed that at least 57 fungal effectors are highly upregulated *in planta*, suggesting they are potentially deployed during infections. One of the effector candidates from this list, SsCM1, which encodes *S. sclerotiorum* secreted chorismate mutase was functionally characterized. SsCM1was shown to enhance plant susceptibility to necrotrophic infection by upregulating salicylic acid (SA), which is likely driven by the ability of this enzyme to block downstream routes of chorismate as an inefficient CM or by directly acting on chorismate as an isochorismate pyruvate lyase (IPL) in plant plastids. In addition, the chloroplast localization feature is shared by SsCM1-homologs in other fungi with similar lifestyles, suggesting that SsCM1-like effectors represent a novel group of secreted CMs broadly associated with plant necrotrophy. Ultimately, *S. sclerotiorum* effectors that are proven to be important for plant colonization can be utilized to develop genetic SSR resistance tools. As a proof of concept, host-induced gene silencing (HIGS) targeting the biosynthesis of a key *S. sclerotiorum* virulence factor, oxalic acid (OA), was assessed as potential SSR control strategy. The work here showed that *S. sclerotiorum* was able to take up environmental RNAs in the form of double-stranded/small-interfering RNAs (dsRNA/siRNAs) both *in vitro* and *in planta*, and this uptake successfully reduced OA production and disease levels. Taken together, this project provided insights into the contribution of *S. sclerotiorum* effectors to SSR and further expanded

the repertoire of *S. sclerotiorum* virulence determinants that can be targeted via HIGS and other RNAi approaches to enhance resistance to this broad host range pathogen.

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CHAPTER 1: Effectors of Plant Necrotrophic Fungi

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ABSTRACT

Plant diseases caused by necrotrophic fungal pathogens result in large economic losses in field crop production worldwide. Effectors are important players of plant-pathogen interaction and deployed by pathogens to facilitate plant colonization and nutrient acquisition. Compared to biotrophic and hemibiotrophic fungal pathogens, effector biology is poorly understood for necrotrophic fungal pathogens. Recent bioinformatics advances have accelerated the prediction and discovery of effectors from necrotrophic fungi, and their functional context is currently being clarified. In this review we examine effectors utilized by necrotrophic fungi and hemibiotrophic fungi in the latter stages of disease development, including plant cell death manipulation. We define "effectors" as secreted proteins and other molecules that affect plant physiology in ways that contribute to disease establishment and progression. Studying and understanding the mechanisms of necrotrophic effectors is critical for identifying avenues of genetic intervention that could lead to improved resistance to these pathogens in plants.

INTRODUCTION

Plant pathogens are often categorized based on their relationship with host plants. These categories include biotrophs, which infect living plants with the objective of suppressing the plant immune system and acquiring nutrients from living cells; necrotrophs, which infect living plants

with the objective of killing the plant upon or shortly after infection and acquiring nutrients from dead or dying tissues; and hemibiotrophs, which infect living plant tissues akin to biotrophs to first establish infection before "switching" to necrotrophy and killing the plant (Giraldo and Valent, 2013). Despite these seemingly distinct definitions, arguments can be made that trophic designations of some of these pathogens are convoluted as we learn more about the complexities of their interactions with a given host plant (Kabbage et al., 2013; Kabbage et al., 2015; Toruño et al 2016). A common theme that unifies plant-associated fungal pathogens despite their different colonization and nutrient acquisition strategies, is the use of effectors during plant colonization (Giraldo and Valent, 2013). While more frequently studied in biotrophic and hemibiotrophic fungi, effectors in necrotrophic fungi have only recently begun to receive significant attention. This review discusses the roles of effectors from necrotrophic and late-stage hemibiotrophic fungi in disease development, including induction of plant cell death, suppression of plant immunity, and the activation of susceptibility genes (inverse gene-for-gene relationship).

As efficient plant killers, necrotrophic fungi are infamous for causing significant losses in the field and in storage worldwide, and the diseases they cause annual threats to food security (Fones et al., 2020). Managing diseases caused by necrotrophic fungi requires multiple approaches, though fungicide applications are the most common approach currently used. The heavy reliance on spray regimes is costly, poses environmental challenges, and can lead to the emergence of resistant populations (Brent and Holloman, 2007). Therefore, control strategies through improving genetic resistance are desirable, yet our understanding of the molecular intricacies between the plant and necrotrophs lags behind their biotrophic counterparts. Notably, a broad understanding of the role of effectors in the pathogenic development of these fungi is

lacking. As their roles in this pathogenic system are clarified, necrotrophic effectors and their plant targets will become targets for manipulation to confer resistance to these pathogens.

Effector proteins are narrowly defined as small, cysteine rich, secreted proteins used by pathogens to manipulate plant cellular responses to the benefit of the pathogen. Effectors from plant biotrophic/hemibiotrophic fungi have been studied by generations of plant pathologists since the gene-for-gene theory was put forward by Harold Flor (1971), and their ability to suppress plant immunity, manipulate plant physiology, and be recognized by host defense mechanisms is well documented (Koeck et al., 2011). In contrast, necrotrophs were previously thought to use a brute-force approach, including the deployment of large repertoires of cell wall degrading enzymes (CWDE) and broad-spectrum toxins to kill plant cells in advance of fungal growth, obtaining nutrients from dead tissue. However, recent advances in genome and transcriptome sequencing, and computational prediction tools suggest there are hundreds of putative effectors present in necrotrophic genomes (Derbyshire et al., 2017; Le Marquer et al., 2019; Lopez et al., 2018; Sperschneider et al., 2016). The surprisingly large number of effectors identified in necrotrophs indicates that the mechanisms deployed by necrotrophic fungi are more intricate than initially hypothesized. Indeed, functional studies of effectors referenced in this review support this notion, and suggest a clear contribution of these molecules to the pathogenic success of fungal necrotrophs.

Herein, we broadly discuss the participation of effectors from necrotrophic and late stage hemibiotrophic fungi in disease development, plant cell death modulation, and both the suppression and the hijacking of plant immune responses. In addition to proteinaceous effectors, we also discuss the recent discovery of small RNAs produced by necrotrophic fungi and their role in disease establishment.

1. Plant Cell Death-inducing Effectors

By definition, necrotrophs kill host cells and acquire nutrients from dead cells. It is thus reasonable to speculate that necrotrophic fungi may utilize effectors to coopt host programmed cell death (PCD). Indeed, many examples of effectors from necrotrophic fungi have been reported for their cell death-inducing activities.

1.1 Necrotrophic effectors hijack R-mediated resistance.

The hypersensitive response (HR) is a localized cell death program triggered by the recognition of effectors by plant resistance (R) proteins to confer resistance against biotrophs and hemibiotrophs, and is commonly referred to as Effector-Triggered Immunity (ETI) (Jones and Dangl, 2006). Remarkably, this genetic program can be hijacked by necrotrophs to their own benefit, considering their trophic lifestyle. This phenomenon is termed effector-triggered susceptibility (ETS) due to the activation of plant defense responses often including cell death, leading to susceptibility to necrotrophs (**Table 1**) (Williams and Dickman, 2008). These necrotrophic effectors were initially termed "host selective toxins" (HST), and are typically effective in a narrow range of plant hosts (Tan et al., 2010).

An excellent example of this is found in the tan spot pathogen of wheat, *Pyrenophora tritici-repentis*. *P. tritici-repentis* secretes two effectors, PtrToxA (Tuori et al. 1995) and PtrToxB (Friesen and Faris 2004), that are critical for disease development. Susceptibility in wheat is conferred by the Tsn1 gene, a classical plant R gene with conserved features such as a serine/threonine protein kinase (S/TPK) domain, nucleotide binding site (NBS), and leucine rich repeat (LRR) domain. Tsn1 is required for plant susceptibility to PtrToxA, although Tsn1 does not directly interact with PtrToxA (Faris et al., 2010). PtrToxA translocates into plant cells possibly

through the recognition of arginine-glycine-aspartic acid (RGD) motif in the protein by a plant surface receptor (Manning and Ciuffetti, 2005) and eventually localizes to chloroplasts leading to light-dependent reactive oxygen species (ROS) accumulation and cell death (Ciuffetti et al., 2010). To date, the precise role of Tsn1 in ToxA sensitivity is unknown, though the evidence is clear that Tsn1 plays a strong role in sensitivity to PtrToxA. PtrToxA sensitivity in plants lacking Tsn1 have also been reported (Manning and Ciuffetti, 2015), suggesting the presence of other susceptibility factors linked to PtrToxA. Similarly, PtrToxB also requires wheat genotypes which possess the single dominant susceptibility locus Tsc2 (Friesen and Faris, 2004), though the specific PtrToxB sensitivity gene in this locus remains elusive (Corsi et al., 2020). In contrast to PtrToxA, PtrToxB appears to function extracellularly in the plant apoplast (Figueroa et al., 2015). However, the extracellular interactors and specific mode of action of PtrToxB are yet to be identified.

Parastagonospora nodorum is another necrotroph with host-selective effectors that target specific host genotypes of wheat and other cereals, causing *P. nodorum* leaf blotch (Solomon et al., 2006). Nine host-specific effectors from *P. nodorum* and their corresponding host susceptibility loci have been identified and characterized (reviewed by Cowger et al., 2020). SnTox1 was the first HST identified in *P. nodorum* that requires a single host susceptibility locus Snn1 to confer susceptibility (Liu et al., 2004). Snn1 genes allow recognition of SnTox1 in a light-dependent manner, triggering hallmarks of PCD including an oxidative burst, strong up-regulation of pathogenesis-related (PR) genes, and DNA laddering (Liu et al., 2012). Notably, SnTox1 was also shown to have a chitin binding domain, which is thought to play a role in protecting fungal cell walls from degradation by plant chitinases (Liu et al., 2016). This feature may also be important to prevent the release of chitin monomers, which are recognized by pattern recognition receptors (PRRs) and subsequent activation of immune responses (discussed in section 2: Plant

Immunity-suppressing Effectors). Another *P. nodorum* effector, SnToxA, was shown to be almost identical to PtrToxA from *P. tritici-repentis* and shares the same host susceptibility gene, Tsn1 (Friesen et al., 2006), suggesting that *P. tritici-repentis* acquired ToxA from *P. nodorum* by horizontal gene transfer (Friesen et al., 2004). Seven additional *P. nodorum* HSTs, SnTox2, SnTox3, SnTox4, SnTox5, SnTox6, SnTox7, and SnTox8, have been identified along with their corresponding susceptibility loci, Snn2 (Friesen et al., 2007), Snn3 (Liu et al., 2009), Snn4 (Abeysekara et al., 2009), Snn5 (Friesen et al., 2012), Snn6 (Gao et al., 2015), Snn7 (Shi et al., 2015), and Snn8 (Faris et al., 2007), respectively. Due to the complex nature of the wheat genome, specific genes within susceptibility loci that are responsible for the susceptibility to *P. nodorum* effectors remain unknown. However, these studies report that the SnTox2-Snn2, SnTox4-Snn4, SnTox5-Snn5, and SnTox6-Snn6 interactions are all dependent on light and very likely share the similar downstream pathways leading to the induction of PCD, thus subverting this plant resistance mechanism for the benefit of the necrotrophic pathogen.

In the maize pathogen *Cochliobolus heterostrophus*, a ToxA-like gene (ChTOXA) was found to share both sequence and structure similarities with PtrToxA, with the exception of the putative translocation RGD motif (Lu et al., 2015). It induces light-dependent cell death in sensitive maize lines, however, the maize gene responsible for susceptibility to ChToxA is yet to be identified (Lu et al., 2015). Recent genome analyses of three *C. sativus* isolates (anamorph *Bipolaris sorokiniana*), the causal agent for multiple diseases in wheat and barley, have also revealed the presence of a ToxA-like gene that shares homology with PtrToxA and SnToxA (McDonald et al., 2018). Pathogenicity assays show that isolates harboring ToxA genes are more virulent on Tsn1 wheat genotypes (McDonald et al., 2018), suggesting ToxA from *C. sativus* functions similarly to other ToxA effectors.

Corynespora cassiicola is a necrotroph that produces a proteinaceous HST known as cassiicolin, a small, secreted, cysteine-rich phytotoxic protein (de Lamotte et al., 2007). *C. cassiicola* is the causal agent of leaf spot or leaf fall disease on many economically important crops, including rubber tree, cocoa, and soybean (Silva et al., 2007). At least seven Cas genes encoding isoforms of cassiicolin have been identified in various *C. cassiicola* isolates (Cas1 to Cas7) (Déon et al., 2014; Lopez et al., 2018). In a recent study, a *cas1* mutant generated from a highly aggressive isolate lost all virulence in susceptible rubber tree clones (Ribeiro et al., 2019). Quantitative trait loci (QTL) analysis has identified potential loci associated with the rubber tree susceptibility to cassiicolin and other *C. cassiicola* effectors (Ribeiro et al., 2019), however, specific sensitivity genes within these QTL are yet to be explored.

One example of a non-traditional effector is victorin, produced by *Cochliobolus victoriae*, the causal agent of Victoria blight in oats. Victorin functions within a narrow range of plant hosts, so it is considered a HST. Victorin is non-traditional because it is a mixture of hexapeptides that were previously thought to be synthesized independent of ribosomes. Surprisingly, recent evidence revealed that victorin is in fact ribosomally synthesized (Kessler et al., 2020). The Vb gene in oat (Wolpert et al., 1985) and LOV1 gene in *Arabidopsis thaliana* determines the sensitivity to victorin (Lorang et al., 2007). Similar to susceptibility genes against *S. nodorum* and *P. tritici-repentis*, LOV1 in Arabidopsis also encodes a characteristic R gene with NBS-LRR domains (Lorang et al., 2007) and the recognition of victorin elicits apoptotic-like cell death which facilitates the infection of *C. victoriae* as a necrotroph. The Pc-2 gene in oat confers disease resistance against rust pathogen *Puccinia coronata*, and is tightly linked to the Vb gene, complicating breeding efforts against both rust and blight diseases (Meehan and Murphy, 1946; Litzenberger 1949). Pc-2 and Vb genes are now considered to be the same gene that confers resistance to *Puccinia coronata*, but

susceptibility to C. *victoriae* (Wolpert et al., 2002; Lorang et al., 2012). This constitutes a remarkable example of how necrotrophs can evolve mechanisms to highjack R genes against biotrophs to their own benefit. Additional non-traditional HSTs are discussed in section 1.6.

1.2 Effectors that hijack PCD pathways

Besides inducing PCD via an inverse gene-for-gene manner, necrotrophic effectors can also target or hijack specific components of the PCD pathway. Upon pathogen attack, plants often increase the level of cytosolic calcium, which stimulates calcium-dependent signaling mechanisms that mediate plant defense responses, often culminating in HR or PCD (Lam et al., 2001). Thus, mimicking such responses can be beneficial to necrotrophs.

Endopolygalacturonases are commonly secreted by fungi as (CWDE), releasing oligogalacturonides as by-products. However, one endopolygalacturonase purified from *Sclerotinia sclerotiorum* also increases intracellular calcium and triggers hallmarks of apoptosis-type cell death, including cytochrome c release from mitochondria and activation of caspase 9-like and caspase 3-like enzymes in soybean cells (Zuppini et al., 2005). In contrast, the oligogalacturonides byproducts were unable to initiate PCD (Binet et al., 2001), revealing that the *S. scleortiorum* endopolygalacturonase alone is the trigger of PCD, independent of its presumed enzymatic activity (Zuppini et al., 2005).

Sspg1d is another endopolygalacturonase from *S. sclerotiorum* that contributes to PCD induction in plants (Wang et al., 2009). Instead of functioning as a CWDE, Sspg1d specifically interacts with the C2 domain of the canola IPG-1 protein. C2 domain proteins have calcium-binding affinity and are likely involved in calcium-dependent signal transduction (Evans et al., 2004) and defense responses (Laxalt et al., 2001). IPG-1 subcellular localization is determined by calcium concentrations, indicating a role in calcium dependent signaling. IPG-1 is also highly

upregulated during a compatible interaction with *S. sclerotiorum*, leading to susceptibility. As several C2 domain proteins were reported to be PCD regulators (Yang et al., 2007) and another known endopolygalacturonase of *S. sclerotiorum* induced PCD in host plant (Zuppini et al., 2005), Sspg1d is proposed to promote PCD by interfering with the activity of IPG-1 as a negative regulator of PCD (Wang et al., 2009). However, not all endopolygalacturonases have the capacity to coopt plant cell death components, and likely function as classical CWDEs. For instance, endopolygalacturonases from *Botrytis cinerea* (BcPG1 and BcPG2) induce necrotic cell death by provoking a loss of cell wall integrity, as is the case of many CWDEs produced by fungal pathogens (Kars et al., 2005).

1.3 Cell death inducing effectors containing conserved domains

The term "necrosis-inducing" can be misleading, as necrosis typically refers to cell death involving non-physiological or non-programmed events due to overwhelming injury. Effectors that contain the necrosis and ethylene-inducing peptide (NEP) domain have been shown to induce cell death in plants, and despite necrosis in the name, this mechanism appears to be physiological for some NEPs (**Table 2**).

The first NEP (Nep1) was isolated from *Fusarium oxysporum* infecting a coca plant (*Erythroxylum coca*) (Bailey, 1995), and Nep1 also triggers cell death in *Arabidopsis* (Bae et al., 2006). Two *F. oxysporum* Nep1 homologs were identified in *S. sclerotiorum* and referred to as SsNep1 and SsNep2 (Dallal-Bashi et al., 2010). SsNep2 was highly expressed during infection on *Brassica napus* leaves and caused PCD that is dependent on calcium signaling (Dallal-Bashi et al., 2010). Nep-like proteins (NLPs) appear to be conserved in many filamentous fungi, some of which have cell death-inducing qualities and others do not (Oome and Van den Ackerveken 2014). Many NLPs have a calcium binding pocket and may also be influenced by intracellular calcium

concentrations (Oome and Van den Ackerveken 2014), suggesting that these molecules may influence plant cells into committing suicide. In the lily pathogen *Botrytis elliptica*, two NLPs (BeNEP1 and BeNEP2) were identified and display cell death-inducing activity in a range of dicots, but not monocots, including its host lily (Staats et al., 2007). Therefore, NLPs are not essential virulence factors or HSTs for *B. elliptica* on lily, but may be a horizontally acquired feature that led to a competitive advantage allowing colonization of additional hosts (Staats et al., 2007). In hemibiotrophic fungus *Colletotrichum higginsianum*, six NLPs were identified (ChNLPs) based on the presence of NEP domains. Among them, only ChNLP1 and ChNLP2 were expressed exclusively during the transition to necrotrophy and induced potent cell death when overexpressed in *N. benthamiana* (Kleemann et al., 2012). Additional NLPs from *Colletotrichum orbiculare* and *Fusarium virguliforme* are also reported to induce cell death in tobacco and soybean, respectively (Yoshino et al. 2012; Chang et al., 2016). The postharvest pathogen *Penicillium expansum* contains two NLP effectors, PeNLP1 and PeNLP2 that cause cell death when transiently expressed in *N. benthamiana* (Levin et al., 2019). PeNLP1 is highly induced compared to PeNLP2 during infection of apple fruit, and deletion of PeNLP1, but not PeNLP2, led to a significant reduction in lesion size (Levin et al., 2019).

Cerato-platanin family proteins (CPPs) are another secreted protein family found only in filamentous fungi and reported to cause phytotoxicity, including in *B. cinerea* (Frías et al., 2011), *S. sclerotiorum* (G. Yang et al., 2018), *Heterobasidion annosum* (Chen et al., 2015) and *Magnaporthe oryzae* (Yang et al., 2009). BcSpl1, a CPP from *B. cinerea*, is the sixth most abundant protein in the secretome of this fungus (Frías et al., 2011), and mutants lacking BcSpl1 show reduced virulence compared to wild-type strains. The infiltration of purified BcSpl1 protein causes rapid cell death in tomato, tobacco, and *Arabidopsis* leaves in a dose-dependent manner,

and this cell death response was accompanied by hallmarks of the HR response; autofluorescence, ROS burst, electrolyte leakage, and cytoplasm shrinkage (Frías et al., 2011). Furthermore, the BcSpl1 activity requires the membrane-bound co-receptor BAK1 for full activity, which is known to enhance immune signaling responses in plants (Schwessinger and Ronald, 2012). The detection of cell surface receptors associated with PCD suggest a physiological and subtle cell death induction by necrotrophs and their effectors. This supports a growing number of hypotheses that some necrotrophic fungi have evolved mechanisms to hijack and induce plant PCD for their own benefit of colonizing and feeding off the dead plant tissues (Kabbage et al. 2013).

Another CPP was recently identified in *S. sclerotiorum* (SsCP1), a close relative of *B. cinerea*, as an important contributor to virulence (Yang et al., 2018). Similar to BcSpl1, SsCP1 induces cell death when transiently overexpressed in *N. benthamiana* in a dose-dependent manner (Yang et al., 2018). Interestingly, SsCP1 also interacts with PR1 in the apoplast, presumably inhibiting the antifungal activity of PR1 and promoting infection (Yang et al., 2018). A CPP-like protein HaCPL2 was also found in the basidiomycete *Heterobasidion annosum sensu stricto*, which is a necrotrophic fungal pathogen of Scot pine (*Pinus sylvestris*) (Chen et al., 2015). HaCPL2 is highly induced during infection of *P. sylvestris*, and induces cell death, phytoalexin production, and upregulation of multiple defense genes in the non-host plant *Nicotiana tabacum* (Chen et al., 2015). Recombinant HaCPL2 also induces cell death and inhibits root growth in *P. sylvestris*, accompanied by an upregulation of genes related to jasmonic acid (JA) / ethylene (ET) signaling pathways in *P. sylvestris* (Chen et al., 2015). However, whether HaCPL2 triggers programmed or necrotic-like cell death in its host requires further investigation.

Lipase domain containing proteins constitute another protein family with a role in cell death induction. In AG1IA anastomosis group of *Rhizoctonia solani*, the causal agent of rice sheath

blight, the effector AGLIP1 contains this lipase domain and elicits cell death, with or without a functional lipase domain in non-host *N. benthamiana*, but not *Arabidopsis* (Li et al., 2019). Ectopic expression of AGLIP1 in transgenic *Arabidopsis* led to a suppression of defense-response genes, suggesting AGLIP1 also suppresses immune signaling (Li et al., 2019). Whether cell death elicited by AGLIP1 in plants is due to recognition of AGLIP1 by certain plant receptors, direct phytotoxicity, or lipase activity remains to be investigated.

There is a special group of "necrosis-inducing" proteins (NIP) referred to as catalytic NIPs due to their glycosyl hydrolase activities. Another group of NIPs which lack a catalytic domain will be discussed in the next section. Catalytic NIPs not only degrade plant cell walls like CWDEs, but also induce plant defense responses that are often unrelated to their catalytic activity (Kars et al., 2005). The latter property contrasts with endopolygalacturonases, like BcPG1 and BcPG2 discussed above, whose necrotizing activity is dependent on enzymatic activity. Several catalytic NIPs have been reported in *B. cinerea* (Noda et al., 2010; Zhang et al., 2015; Frías et al., 2016; González et al., 2017; Zhu et al, 2017; Y. Yang et al., 2018; Bi et al., 2020). One of these catalytic NIPs, BcGs1, is a glucan 1,4-alpha-glucosidase, and infiltration of BcGs1 in tobacco and tomato leaves induces strong cell death, accumulation of ROS, and upregulation of PR genes, suggesting an HR-like PCD (Zhang et al., 2015). Interestingly, BcGs1-treated plants induce disease resistance against tobacco mosaic virus, *Pseudomonas syringae* pv. tomato DC3000, and against *B. cinerea* itself (Zhang et al., 2015). This study demonstrates that BcGs1 acts as an elicitor that induces a strong, localized immune response that likely primes plant defenses for subsequent infections. However, *B. cinerea* actively secretes BcGs1, which may trigger HR-like PCD at the point of infection, allowing *B. cinerea* to take advantage of the subsequent cell death leading to susceptibility.

B. cinerea xylanase BcXyn11A (Noda et al., 2010; Frías et al., 2019) and BcXyl1 (Y. Yang et al., 2018) are catalytic NIPs with xylan degrading activity, but also induce HR-like cell death including ROS production and electrolyte leakage, which is independent of their xylan degrading activity. In addition, a 26-amino acid peptide derived from BcXy1, and a 25-amino acid peptide derived from BcXyn11a are sufficient for eliciting HR-like cell death (Y. Yang et al., 2018; Frías et al. 2019). Recognition of BcXy1 is mediated by plant apoplastic LRR receptor-like kinases BAK1 and SOBIR1 (Y. Yang et al., 2018). Similarly, a xyloglucanase BcXYG1 was reported to have cell death- and immune response-eliciting activities which were also independent of xyloglucanase activity (Zhu et al., 2017).

BcIEB1 from *B. cinerea* is another elicitor that induces HR-related symptoms of cell death, ROS burst, autofluorescence, and electrolyte leakage (Frías et al., 2016). Additional work on BcIEB1 found that this protein, specifically a 35-amino acid conserved region called ieb35, interacts with a plant osmotin, which belongs to family 5 of PR proteins in plant cells and the interaction protected *B. cinerea* from the antifungal osmotin (González et al., 2017). Moreover, osmotin was shown to interfere with the cell death induced by BcIEB1, but was not directly involved in the elicitation of defense responses (González et al., 2017).

1.4 Effectors with unknown protein domains

Recent bioinformatic studies have revealed repertoires of small secreted proteins (SSPs) in several phytopathogens that might contribute to virulence, and many of them are cysteine-rich, small-sized proteins without known protein domains (Wen et al., 2019; Denton-Giles et al., 2020). Predicted effectors with no known domains from a transcriptome study of *S. sclerotiorum* were recently screened for cell death-inducing activity (Seifbarghi et al., 2020). One such effectors, SsNE2, induces cell death in *N. benthamiana* (Seifbarghi et al., 2020). Interestingly, this cell death

activity requires plant receptor-like kinases, suggesting a hijacking of immune signaling (Seifbarghi et al., 2020). Similarly, two NIPs without catalytic domains, ZtNIP1 and ZtNIP2, were also identified as putative effectors from *Zymoseptoria tritici* (Ben M'Barek et al., 2015). Further characterization of ZtNIP1 and ZtNIP2 indicated that infiltration of both proteins induces cell death, but only in select wheat cultivars. The genetic basis for susceptibility to these effectors is yet to be determined (Ben M'Barek et al., 2015).

In the conifer pathogen *Heterobasidion parviporum*, a SSP without any known protein domains, HpSSP35.8, was identified as a necrotrophic effector protein secreted during infection (Wen et al., 2019). Overexpression of HpSSP35.8 induces a strong cell death phenotype and activates PR genes, the "WRKY" transcription factors, genes involved in JA/ET-signaling, and chitinase genes, all of which are known defense responses (Wen et al., 2019). This response suggests that the cell death observed is an HR-like response, though HpSSP35.8 also had significant effects on chlorophyll and photosynthesis, which could indicate that the cell death observed is truly necrosis. It is interesting to note that one of the HpSSP3.5-triggered plant responses, the induction of chitinase genes, was also observed in *P. nodorum* SnTox1 - wheat Snn1 interaction, where SnTox1 has chitin binding activity for protecting *P. nodorum* from degradation of induced plant chitinases (Liu et al., 2016). Whether HpSSP35.8 has the same chitin-binding activity is yet to be investigated, though it has no predicted chitin-binding domain.

A comparative analysis of secretomes of three closely related members of Sclerotiniaceae, *B. cinerea*, *S. sclerotiorum*, and *Ciborinia camelliae*, revealed an expansion of a class of cysteine-rich SSPs in the genome of *C. camelliae* (73 CcSSPs) compared to only one homolog in both *B. cinerea* (BcSSP2) and *S. sclerotiorum* (SsSSP3). These are called *C. camelia*-like SSPs (CCL-SSPs) due to their abundance in *C. camelliae* (Denton-Giles et al., 2020). This SSP family is

conserved in many other plant pathogens, but for the most part, have not been characterized. Interestingly, recombinant BcSSP2 and SsSSP3 induce significantly stronger and faster cell death than any CcSSP tested (Denton-Giles et al., 2020). Furthermore, both BcSSP2 and SsSSP3 are capable of inducing cell death in plant hosts (camelliae) and non-hosts (*N. benthamiana*) (Denton-Giles et al., 2020), suggesting that CCL-SSPs might act as non-host-specific, broad cell death inducers in a wide range of plants.

1.5 Effectors that play roles in biotrophy-necrotrophy switch (BNS)

Because hemibiotrophic fungal pathogens display both biotrophic and necrotrophic qualities, stage specific effectors play a key role in the establishment of infection. Some effectors secreted from hemibiotrophs induce cell death at the later stages of infection, but others facilitate the biotrophy-necrotrophy switch (BNS). During infection of lentil (*Lens culinaris*) by the hemibiotroph *Colletotrichum truncatum*, effector CtNUDIX (NUcleoside DIphosphate linked to some other moiety X) regulates the BNS (Bhadauria et al., 2013). In bacteria and mammals, proteins containing Nudix hydrolase domains catalyze hydrolysis of mutagenic nucleotides and toxic components and function as cellular surveillance enzymes to maintain physical homeostasis (Safrany et al., 1999; Xu et al., 2001; McLennan, 2006). During infection, CtNUDIX is exclusively expressed during the late biotrophic stage precisely at the BNS. CtNUDIX localizes to the plant plasma membrane and induces severe HR-like cell death when overexpressed in *N. benthamiana* (Bhadauria et al., 2013). CtNUDIX-overexpressing strains of *C. truncatum* and *M. oryzae* fail to induce disease symptoms on lentil and barley, respectively, but light-brown discoloration is observed, which is suspected to be HR-like cell death on infected plants (Bhadauria et al., 2013). These results suggest that the timely secretion of CtNUDIX is key to normal disease progression,

and its premature secretion likely triggered cell death in the biotrophic phase, preventing these two hemibiotrophs from establishing infection.

Another example of an effector involved in BNS is found in *Colletotrichum graminicola*, the causal agent of anthracnose of maize (Vargas et al., 2012). The *C. graminicola* effector, Cgfl, is a metalloprotease fungalysin that is highly induced during the switch to the necrotrophic stage (Vargas et al., 2012). Sanz-Martín et al. (2016) further characterized this metalloprotease as a predicted chitinase degrading enzyme, and deletion mutants of Cgfl displayed severely reduced virulence on plants. This suggests that Cgfl may be involved in suppressing chitin-triggered immunity as a virulence mechanism, particularly during the BNS when it is most highly expressed.

In *C. lentis*, the effector ClToxB is also induced during the BNS in virulent races of *C. lentis* (Bhadauria et al., 2015). Intriguingly, ClToxB shares extensive sequence similarity with PtrToxB from *P. tritici-repentis*, suggesting ClToxB is a potential HST on lentils. Indeed, RNAi strains of *C. lentis* with reduced expression of ClToxB show impaired virulence compared to the wild-type strain, and its transient overexpression failed to induce cell death in the non-host tobacco *N. tobacum* (Bhadauria et al., 2015). Interestingly, if susceptibility to ClToxB is conditioned by an R-like protein, as is the case in the *P. tritici-repentis* pathosystem, this would constitute a remarkable example where R-mediated processes can participate in both resistant and susceptible outcomes in this hemibiotroph depending on the phase of infection. However, further work is required to identify the plant target(s) of ClToxB, and to better understand the biochemical context in which it functions.

1.6 Non-traditional effectors and host-selective toxins (HSTs)

Fungal secondary metabolism has seen a resurgence of research attention in recent years (Keller, 2019). Many well-studied HSTs are fungal secondary metabolites (SM), and have strong

disease-inducing effects in plants (Stergiopoulos et al., 2013; Tsuge et al., 2013). These fungal SM range from small modified peptides to molecules with complex chemical structures, and have various modes of action specific to susceptible host genotypes.

For example, HC-toxin is a cyclic tetrapeptide from *Cochliobolus carborum*, the causal agent of Northern corn leaf spot, that suppresses expression of host defense genes by targeting histone-deacetylases in susceptible plants (Brosch et al., 1995, Walton, 2006). The chlorinated peptides Peritoxin A and B produced by the sorghum pathogen *Periconia circinata* induce apoptotic cell death (Dunkle and Macko, 1995) in sorghum plants harboring NBS-LRR type of R gene *Pc-B* (Nagy and Bennetzen, 2008). This suggests that *P. circinata* deploys peritoxins to exploit PCD mechanisms in sorghum similar to that of victorin in oats (Lorang et al. 2012), though a direct interaction between peritoxins and R-genes has yet to be determined.

Mycotoxins like aflatoxins, trichothecenes, and structurally related toxins are major concerns in food contamination because of their high toxicity to mammals (Arunachalam and Doohan, 2013; Fasoyin et al., 2019; Luciano-Rosario et al., 2020). However, many of these fungal SM also contribute to plant colonization and pathogenicity. For instance, *Aspergillus flavus* and *Penicillium expansum* are common post-harvest pathogens of a broad range of storage crops, and also strong producers of toxic SM (Kelley et al., 2012; Luciano-Rosario et al., 2020). Patulin and citrinin produced by *P. expansum* contribute to aggressiveness and establishment in some plant cultivars, although neither is required for infection (Snini et al., 2016; Luciano-Rosario et al., 2000; Touhami et al., 2018). These post-harvest pathogens rely heavily on secreted CWDE (Fountain et al. 2014), though other proteinaceous effectors and virulence factors have also been identified and are covered in section 1.3 (Levin et al., 2019; Luciano-Rosario et al., 2020).

Additional examples of SM that contribute to virulence are found in *Alternaria* and *Fusarium* species. *Alternaria alternata* f. sp. *lycopersici* deploys AAL toxins that induce apoptotic-like phenotypes in sensitive tomato plants (Wang et al., 1996), but not in plants harboring the Asc1 (*Alternaria* stem canker resistance gene 1) gene (Brandwagt et al., 2000). Additional *A. alternata* pathotypes produce similar toxins that have a wide range of negative effects on plant cells, and have been summarized elsewhere (Meena and Samal, 2019). Deoxynivalenol, a mycotoxin produced by *Fusarium graminearum*, accumulates to high concentrations in the necrotic phase of infection and elicits strong ROS burst (Mudge et al. 2006; Desmond et al. 2008), but can actually inhibit PCD at low concentrations (Diamond et al. 2013).

2. Plant Immunity-suppressing Effectors

Accumulating evidence suggests that necrotrophs interact with their hosts in a more subtle and complex way, beyond cell death induction. Recent studies show that some necrotrophic effectors do in fact suppress plant immunity (**Table 3**), a commonly accepted feature of effectors from biotrophic and early-stage hemibiotrophic pathogens.

2.1 Effectors Preventing Pathogen Recognition and Immunity

The lysin motif (LysM) domain is a widespread protein motif found in many organisms, including fungi and plants, and is known for binding peptidoglycans and chitin (Kaku et al. 2006; Miya et al., 2007; Buist et al., 2008; Shimizu et al., 2010). During infection, secreted plant chitinases lead to degradation of fungal cell walls, releasing fungal cell wall fragments like chitin or chitosan oligomers. These oligomers are detected by plant extracellular receptors with LysM domains, thus triggering an immune response (Thomma et al., 2011). Many biotrophic and hemibiotrophic fungal effectors also contain the LysM motif and competitively bind to chitin or

its oligomers, interfering with plant recognition of these oligomers (de Jonge et al., 2010; Marshall et al., 2011; Kombrink et al., 2017). A recent study in the necrotrophic fungus *R. solani* found that RsLysM, the only putative LysM effector present in the genome of *R. solani* AG2-2IIIB (Wibberg et al., 2016), suppresses chitin-triggered immunity by binding to chitin (Dölfors et al., 2019). Notably, RsLysM does not protect hyphae from chitinolytic activity of plant chitinases (Dölfors et al., 2019), as some other chitin-binding effectors like *Cladosporium fulvum* Avr4 does, but likely prevents recognition by plant chitin receptors (van den Burg et al., 2006). A new family of chitin-related effectors were recently identified in the biotrophic fungus *Podosphaera xanthii* which causes powdery mildew in cucurbits, and termed effectors with chitinase activity (EWCA) (Martínez-Cruz et al., 2021). These EWCA degrade chitin oligomers, therefore suppressing chitin-triggered immunity. Phylogenetic analyses revealed that EWCA orthologs are present in many necrotrophic fungi too, including *B. cinerea*, *S. sclerotiorum*, and *R. solani* (Martínez-Cruz et al., 2021). Whether these necrotrophic EWCA orthologs also act as inhibitors of chitin-triggered immunity is yet to be determined.

Another *R. solani* AG2-2IIIB effector protein, RsRlpA, encodes a rare lipoprotein A-like that has immunity suppressing activity (Charova et al., 2020). RsRlpA is highly induced during early infection of sugar beets, and its overexpression in *N. benthamiana* suppresses HR imposed by *C. fulvum* Avr4 (Charova et al., 2020). RsRlpA shares sequence homology to papain-like inhibitors which are known for blocking activity of papain-like cysteine proteases (PLCPs) that help induce PCD and other immune responses in plants (reviewed by Misas-Villamil et al., 2016). Indeed, the HR inhibition by RsRlpA is associated with protease inhibitor activity, inhibiting a plant cathepsin that is a known PLCP (Bárány et al., 2018). Various other plant protease activities, including caspase-like activities, have been shown to function in the execution of plant PCD

(Kabbage et al. 2017), so suppression of HR-like cell death through other protease inhibition mechanisms may also be present in plant-pathogen interactions. AGLIP1 from *R. solani* strain AG1IA, which was mentioned in the previous section, also inhibits plant immunity, but by currently unknown mechanisms (Li et al., 2019).

In the apple Valsa canker fungus, *Valsa mali,* seven out of 70 randomly selected candidate effectors (referred to as VmEPs) were shown to suppress BAX-induced PCD in *N. benthamiana* (Li et al., 2015). The Bax protein belongs to the cell death antagonist Bcl-2 family of proteins and was demonstrated to induce cell death resembling defense-related HR in plants (Lacomme and Cruz, 1999, Kabbage et al., 2017). Suppression of Bax-associated PCD by VmEPs indicates that these effectors inhibit plant HR-related resistance during infection.

Overall, the suppression of cell death in these examples are counterintuitive considering the trophic lifestyle of these pathogens. However, we speculate that early stages of infection by necrotrophs may require a suppression of cell death to establish infection before triggering the opposite, in a manner that is analogous to hemibiotrophic infections. Indeed, the prototypical necrotroph *S. sclerotiorum* is proposed to have a brief biotrophic phase before quickly switching to necrotrophy (Kabbage et al., 2015). Alternatively, it may be possible that not all cell deaths are created equal, and the type of cell death triggered by the plant may differ from the one imposed by the pathogen, and therefore needs to be suppressed. In accordance, an interplay between autophagic and apoptotic cell deaths with opposing outcomes was reported in necrotrophic fungal infections (Kabbage et al., 2013), and a deeper understanding of the native plant PCD mechanisms is needed (Kabbage et al., 2017).

2.2 Effectors Altering Hormone, Calcium Signaling, and Oxidative Burst for Improper Immune Responses

Phytohormones play critical roles in disease resistance and in general, salicylic acid (SA) signaling pathways are induced for successful resistance against biotrophs (Glazebrook 2005), while induction of JA signaling pathways typically lead to successful defense against necrotrophs (Gfeller et al., 2010). As extensive crosstalk between the SA and JA signaling pathways have been demonstrated (Kunkel and Brooks, 2002), it's reasonable to expect pathogen effectors to manipulate hormone signaling pathways to suppress hormone mediated resistance (El Oirdi et al., 2011). For example, fungal integrin-like (ITL) proteins are important for fungal development (Corrêa et al., 1996; Zhu et al., 2013), but SsITL from *S. sclerotiorum* is secreted during plant infection and interacts with chloroplast-localized calcium sensing receptor, promoting SA biosynthesis (Nomura et al., 2012; Tang et al., 2020). This influx of SA leads to a suppression of JA signaling, culminating in enhanced susceptibility to *S. sclerotiorum*. In a hypovirulent strain of *S. sclerotiorum*, SsITL is significantly downregulated, supporting its role in virulence (Li et al., 2008).

A non-traditional effector produced by *S. sclerotiorum* that leads to improper immune responses is oxalic acid (OA). The effects of OA are broad, initially creating a strong reducing environment that dampens immune responses like ROS burst, callose deposition, and autophagy (Williams et al. 2011; Kabbage et al. 2013). Later in infection, accumulation of OA can induce ROS and trigger apoptotic-like PCD, contributing to necrotrophic success (Kabbage et al. 2013).

3. sRNA Effector-like molecules

Recent studies have revealed that non-coding small RNAs (sRNAs) derived from necrotrophic fungi can also be delivered into host cells, hijack plant RNAi machinery, and silence

plant genes that are involved in immunity (Weiberg et al., 2013; Wang et al., 2016; Wang et al., 2017). As small, secreted molecules that affect plant physiology, sRNAs are now often considered effector-like molecules, despite being non-proteinaceous (Weiberg and Jin, 2015). Pathogen sRNAs are mainly derived from gene-poor, repeat-rich regions in genomes (Weiberg et al., 2013) and plant targets of sRNAs are typically associated with immune responses (Dong et al., 2015).

To date, sRNA effectors from necrotrophic fungi have largely been reported from *B. cinerea*. The sRNAs Bc-siR3.1, Bc-siR3.2, and Bc-siR5 are the most abundant sRNAs during infection of tomato and *Arabidopsis* (Weiberg et al., 2013). These sRNAs specifically target a peroxiredoxin (oxidative stress-related gene), mitogen activated protein kinases (MPK1, MPK2, MPKKK4), and a cell wall-associated kinase (WAK) (Weiberg et al., 2013). These plant targets of fungal sRNAs are known components of immune responses; oxidative burst and signal transduction pathways. Thus, their silencing results in enhanced susceptibility to the fungus (Weiberg et al., 2013). Another sRNA, Bc-siR37, can lead to silencing of *Arabidopsis* WRKY transcription factors, receptor-like kinases, and cell wall modifying enzymes, all leading to suppression of plant immunity against the fungus (Wang et al., 2017).

In *S. sclerotiorum*, sRNA sequencing of the fungus *in vitro* and during infection of *Arabidopsis* and common bean (*Phaseolus vulgaris*) revealed a group of fungal sRNAs that were secreted specifically *in planta* (Derbyshire et al., 2019). These sRNAs are predicted to target and suppress plant genes that are associated with quantitative disease resistance during infection (Derbyshire et al., 2019). In particular, mutations of two sRNA targets, encoding kinase genes SERK2 and SNAK2, increased susceptibility to *S. sclerotiorum*, suggesting that these sRNA targets contribute to disease resistance (Derbyshire et al., 2019). The role of sRNA in plant-pathogen interactions is an emerging field of plant pathology, and continued advances in

sequencing technologies followed by functional characterization will likely reveal a broad utilization of these effector-like molecules across fungal taxa.

CONCLUSIONS AND FUTURE PERSPECTIVES

Necrotrophic fungal pathogens of plants were previously considered to cause cell death with simplistic mechanisms by secreting phytotoxic molecules and degrading plant cell walls. Recent bioinformatic advances and functional studies have accelerated the discovery of virulence factors in necrotrophs, including proteinaceous effectors, HSTs, and sRNA effectors, revealing that necrotrophs utilize a broad range of sophisticated virulence mechanisms during infection of plants (**Figure 1**). Our understanding of necrotrophic effectors is improving and studies have revealed that these molecules are capable of both subverting and hijacking plant physiological processes to their advantage, including PCD. While PCD mechanisms are successfully deployed by plants that recognize biotrophic and hemibiotrophic effectors, necrotrophic effectors evolved to manipulate plant PCD and other immune responses to promote susceptibility. As necrotrophic fungi continue to cause significant crop losses worldwide, it is essential to improve our understanding of these molecules and their plant targets to identify novel modes of resistance against these pathogens.

To date, qualitative and robust genetic resistance to necrotrophic fungi is lacking in plants, aside from a few plant genotypes that are insensitive to fungal HSTs. Similarly, quantitative resistance against necrotrophs has shown limited efficiency and complicate breeding efforts. However, the recent discovery of sRNA cross-talk between plants and fungi has opened new avenues for disease control. Multiple fungal pathogens have now been shown to uptake environmental RNAs, leading to the silencing of specific fungal genes. Thus, plants can be

weaponized to target virulence factors of necrotrophic fungi, including necrotrophic effectors. Spray-induced gene silencing is also showing promise as an RNAi tool.

Lastly, while many necrotrophic effectors manipulate plant PCD, the biochemical context of PCD in plants is poorly understood (Kabbage et al. 2017). We propose that necrotrophic effectors can be used as a valuable tool to uncover plant PCD components. A mechanistic understanding of how effectors trigger PCD, and how PCD can be prevented, is likely to have implications beyond plant-fungal interactions. In conclusion, there is a wealth of potential applications of plant-fungal necrotrophic effector research in disease control, basic plant physiology, and fungal biology, so expanding our understanding of these molecules will greatly expedite these applications.

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FIGURES AND TABLES

Figure 1. Schematic representation of effector functions during plant-fungal necrotroph interactions.

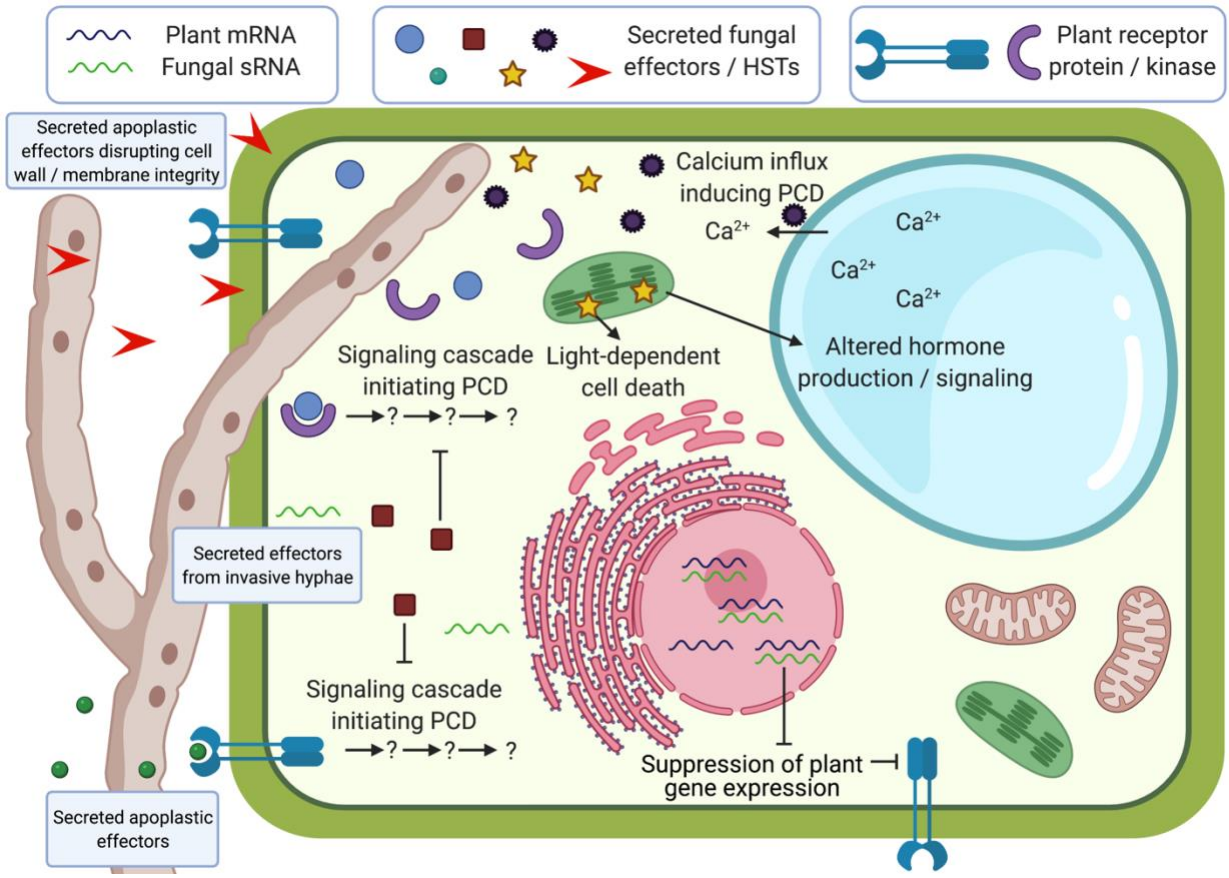

Figure 1. Schematic representation of effector functions during plant-fungal necrotroph interactions. Effectors can be secreted into the apoplast or into plant cells from invasive hyphae, and can disrupt cell wall / membrane integrity, initiate programmed cell death (PCD), suppress PCD, alter hormone signaling, affect signaling cascades that use calcium ion fluxes, or suppress plant gene expression through small non-coding RNAs (sRNAs). Some of these processes have been shown to be light-dependent. Created with BioRender.com.

Table 1. Effectors that induce plant cell death in an inverse gene-for-gene manner.

Pathogen name	Effector	Protein domain/function	Plant susceptibility gene	Reference(s)
Pyrenophora tritici-repentis	PtrToxA	arginine-glycine-aspartic acid (RGD) motif	Tsn1	Faris et al., 2010 Manning and Ciuffetti, 2005 Manning et al., 2007 Manning and Ciuffetti, 2015
	PtrToxB	N/A	Tsc2	Friesen and Faris 2004 Figueroa et al., 2015
Parastagonospora nodorum	SnToxA	arginine-glycine-aspartic acid (RGD) motif	Tsn1	Friesen et al., 2006
	SnTox1	N/A	Snn1	Liu et al., 2004 Liu et al., 2012 Liu et al., 2016
	SnTox2	N/A	Snn2	Friesen et al., 2007
	SnTox3	N/A	Snn3	Liu et al., 2009

	SnTox4	N/A	Snn4	Abeysekara et al., 2009
	SnTox5	N/A	Snn5	Friesen et al., 2012
	SnTox6	N/A	Snn6	Gao et al., 2015
	SnTox7	N/A	Snn7	Shi et al., 2015
	SnTox8	N/A	Snn8	Faris et al., 2007
Cochliobolus heterostrophus	ChToxA	N/A	unknown	Lu et al., 2015
Cochliobolus sativus (Bipolaris sorokiniana)	BsToxA	N/A	Tsn1	McDonald et al., 2018
Corynespora cassiicola	cassiicolin	N/A	unknown	de Lamotte et al., 2007
Cochliobolus victoriae	victorin	N/A	Vb in oat, LOV1 in *Arabidopsis*	Wolpert et al., 1985 Lorang et al., 2007

Table 2. Effectors with necrosis-inducing or other known domains.

Pathogen name	Effector	Protein domain/function	Plant target	Reference(s)
Sclerotinia sclerotiorum	SsNep1	necrosis and ethylene-inducing peptides (NEP)	unknown	Dallal-Bashi et al., 2010
	SsNep2	NEP	unknown	Dallal-Bashi et al., 2010
	SsCP1	Cerato-platanin protein	unknown	G. Yang et al., 2018
Botrytis cinerea	BcSp1	Cerato-platanin protein	unknown	Frías et al., 2011
	BcGs1	glucan 1,4-alpha-glucosidase	unknown	Zhang et al., 2015
	BcXyn11A	xylanase	unknown	Noda et al., 2010 Frías et al., 2019
	BcXyl1	xylanase	unknown	Y. Yang et al., 2018
	BcXYG1	xyloglucanase	unknown	Zhu et al., 2017

	BcCrh1	Congo red hypersensitivity transglycosylase	unknown	Bi et al., 2020
	BcIEB1	IgE binding protein	osmotin	Frías et al., 2016 González et al., 2017
Botrytis elliptica	BeNEP1	NEP	unknown	Staats et al., 2007
	BeNEP2	NEP	unknown	Staats et al., 2007
Colletotrichum higginsianum	ChNLP1	NEP	unknown	Kleemann et al., 2012
	ChNLP2	NEP	unknown	Kleemann et al., 2012
Heterobasidion annosum	HaCPL2	Cerato-platanin protein	unknown	Chen et al., 2015
Rhizoctonia solani	AGLIP1	lipase domain	unknown	Li et al., 2019

Table 3. Plant immunity-suppressing effectors in necrotrophic fungi.

Pathogen name	Effector	Class / Domain	Function	Reference(s)
Cochliobolus carborum	HC-toxin	Secondary metabolite	Disrupt plant histone deacetylaces	Brosch et al. 1995 Walton, 2006
Colletotrichum graminicola	Cgfl	Metalloproteas e	Degrade plant chitinases	Vargas et al., 2012 Sanz-Martín et al. 2016
Sclerotinia sclerotiorum	SsITL	Integrin-like domain protein	Disruption of calcium signaling, induce SA / suppress JA signaling	Nomura et al., 2012 Tang et al., 2020
	Oxalic acid	Elicitor	Suppress ROS burst and callose deposition	Williams et al. 2011
	SsEWCA	Chitinase	Suppress chitin-triggered immunity	Martínez-Cruz et al. 2021
Botrytis cinerea	Bc-siR3.1	Small RNA	Reduce expression of key immune signaling genes	Weiberg et al., 2013

	Bc-siR3.2	Small RNA	Reduce expression of key immune signaling genes	Weiberg et al., 2013
	Bc-siR5	Small RNA	Reduce expression of key immune signaling genes	Weiberg et al., 2013
	Bc-siR37	Small RNA	Reduce expression of key immune signaling genes	Wang et al., 2017
	BcEWCA	Chitinase	Suppress chitin-triggered immunity	Martínez-Cruz et al. 2021
Rhizoctonia solani	RsLysM	LysM containing protein	Chitin binding	Wibberg et al., 2016 Dölfors et al., 2019
	RsRlpA	Lipoprotein	Protease inhibition	Charova et al., 2020
	RsEWCA	Chitinase	Suppress chitin-triggered immunity	Martínez-Cruz et al. 2021
Valsa mali	VmEP	Unknown	Unknown	Li et al., 2015

CHAPTER 2: Host-induced gene silencing of a *Sclerotinia sclerotiorum oxaloacetate acetylhydrolase* using bean pod mottle virus as a vehicle reduces disease on soybean

This chapter is a research article published in *Frontiers in Plant Sciences.* Megan McCaghey and Dandan Shao serve as co-first authors on this collaborative manuscript.

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ABSTRACT

A lack of complete resistance in the current germplasm complicates the management of Sclerotinia stem rot (SSR) caused by *Sclerotinia sclerotiorum* in soybean. In this study, we used bean pod mottle virus (BPMV) as a vehicle to down-regulate expression of a key enzyme in the production of an important virulence factor in *S. sclerotiorum*, oxalic acid (OA). Specifically, we targeted a gene encoding oxaloacetate acetylhydrolase (*Ssoah1*), because *Ssoah1* deletion mutants are OA deficient and non-pathogenic on soybean. We first established that *S. sclerotiorum* can uptake environmental RNAs by monitoring the translocation of Cy3-labeled double-stranded and small interfering RNA (ds/siRNAs) into fungal hyphae using fluorescent confocal microscopy. This translocation led to a significant decrease in *Ssoah1* transcript levels *in vitro*. Inoculation of soybean plants with BPMV vectors targeting *Ssoah1* (pBPMV-OA) also led to decreased expression of *Ssoah1*. Importantly, pBPMV-OA inoculated plants showed enhanced resistance to *S. sclerotiorum* compared to empty-vector control plants. Our combined results provide evidence supporting the use of HIGS and exogenous applications of ds/siRNAs targeting virulence factors

such as OA as viable strategies for the control of SSR in soybean and as discovery tools that can be used to identify previously unknown virulence factors.

INTRODUCTION

Sclerotinia sclerotiorum is a broad host range fungal pathogen that infects many dicotyledonous plants worldwide. It is the causal agent of Sclerotinia stem rot (SSR; or white mold) on soybean, a challenging and significant yield-limiting disease (Allen *et al.,* 2017*)*. SSR development is heavily influenced by the weather, and disease onset is favored by cool and wet conditions during flowering (Peltier et al., 2012; Willbur and McCaghey, 2019; Workneh and Yang, 2000). SSR causes substantial yield losses to soybean globally (Wrather et al., 2010), and between 2010 and 2014 SSR resulted in yield losses between 150 million and 1.1 billion kgs in the United States, annually (Allen et al., 2017). Current SSR management strategies have limited efficacy and comprise of integrated cultural, chemical, and biological control practices (Peltier et al., 2012; Willbur et al., 2019). Disease control is complicated by the lack of complete genetic resistance to SSR, however, breeding efforts have identified partially resistant soybean genotypes in the laboratory and through field trials (Boland and Hall, 1986; Chun et al., 1987; Iquira et al., 2015; Kim and Diers, 2000; McCaghey et al., 2017).

RNA interference (RNAi) is a gene silencing process that utilizes complementary RNA particles to target mRNA and inhibit gene expression and translation. RNAi has emerged as a promising technology to control pests and pathogens within agricultural systems and has been demonstrated to reduce the aggressiveness of plant pathogenic filamentous fungi both through host-induced gene silencing (HIGS) and exogenous applications of double-stranded (dsRNA) onto the surface of plants (Andrade et al., 2016; Gilbert et al. 2018; Koch et al., 2016; Tiwari et al., 2017; Wagner et

al., 2020; Wang et al., 2016; Yin et al., 2010). Spray-induced gene silencing (SIGS), involving ectopic application of dsRNAs targeting pathogen genes, has the added benefit of circumventing the genetic manipulation of plants, and thus further avoiding the regulatory expenses and public concern surrounding genetically modified organisms (GMOs). SIGS has been tested on different plant-pathosystems and was effective against various groups of plant pests and pathogens (Dubrovina and Kiselev, 2019) including viruses (Carbonell et al., 2008), insects (Li et al., 2015; Gogoi et al., 2017), and fungi (Hu et al., 2020; Koch et al., 2016; McLoughlin et al., 2017; Wang et al., 2016). Furthermore, applied dsRNAs targeting pathogen genes can be taken up and processed by plants (Koch et al., 2016 and Mitter et al. 2017). For instance, spraying dsRNAs targeting a gene involved in *Fusarium graminearum* ergosterol biosynthesis on detached barley leaves inhibits fungal growth both at the application site and on distal parts of leaves (Koch et al., 2016). Due to the wide array of target options, the specificity required for RNA binding, and advances in protective materials such as clay nanosheets to adhere and protect dsRNAs on the plant surface (Mitter et al., 2017), HIGS and exogenous applications of dsRNA constitute promising crop protection technologies.

The efficacy of HIGS and SIGS approaches against fungal pathogens is dependent, in part, on the nature of the selected target sequence. Genes encoding pathogenicity determinants can constitute ideal targets of such approaches, because they are essential for pathogens to cause disease. In previous studies, silencing of pathogenicity genes by HIGS in multiple pathogens suppressed disease development (Panwar et al., 2013; Yin et al., 2014; Pliego et al., 2013; Shanmugam et al., 2017; Yin et al., 2015). In *S. sclerotiorum*, oxalic acid (OA) is a key factor governing its pathogenic success. OA can contribute to pathogenesis in a variety of ways, including the manipulation of the

redox status of the host (Williams et al., 2011, Ranjan et al., 2017), the induction of programmed cell death (Kim and Dickman, 2008, Kabbage et al, 2013), and the activation of degradative enzymes (Dutton and Evans, 1996). Thus, the use of HIGS to target OA production could provide a valuable tool to manage SSR and reduce fungicide use. Previous work has targeted a chitin synthase in *S. sclerotiorum* and reduced disease severity through HIGS in transgenic tobacco plants (Andrade et al., 2016). However, it is important to test this approach on specific crops of interest, identify additional useful targets that can be stacked to ensure durable resistance in a particular host, and importantly, identify and target unique sequences such as virulence factors to avoid sufficiently conserved genes across taxa to prevent off-target effects, including within beneficial fungal communities.

Herein, we target a *S. sclerotiorum oxaloacetate acetylhydrolase* (*Ssoah1*) gene that encodes an enzyme that converts oxaloacetate to oxalate and acetate. The deletion of this gene in *S. sclerotiorum* abolished OA accumulation and resulted in restricted lesions in which the infectious hyphae gradually lose viability on tomato, soybean, and *Arabidopsis* (Liang et al., 2015 and Xu et al., 2015). Additionally, *Ssoah1* expression appears to play a particularly critical role during infection of soybean, compared to other *S. sclerotiorum* hosts (Westrick et al., 2019 and Xu et al., 2015). Using a combination of confocal microscopy, gene expression studies, small RNA (sRNA) sequencing, and virus-induced gene silencing, we show that *S. sclerotiorum* mycelia take up environmental double-stranded and small interfering (ds/siRNAs) targeting *Ssoah1*, leading to the downregulation of *Ssoah1* gene expression both *in vitro* and *in planta*. *In planta,* silencing assays were conducted using bean pod mottle virus (BPMV)-mediated HIGS. Importantly, our HIGS assays significantly suppressed *S. sclerotiorum* development and disease onset. This work

demonstrates that RNAi strategies provide an additional tool for improving soybean resistance to *S. sclerotiorum* and managing SSR.

MATERIALS AND METHODS

In vitro synthesis of dsRNA and siRNA

RNA was extracted from actively growing *S. sclerotiorum* mycelia using a Maxwell RSC Plant RNA Kit (Promega) and cDNA was synthesized using a ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) following manufacturers' instructions. A 365-base pair fragment corresponding to *Ssoah1* was amplified and added with a T7 promoter sequence by PCR using specific primers, T7_OA_F and T7_OA_R (Supplementary File 1). PCR reactions (50 µl) were set up using a KAPA HiFi PCR Kit (Roche Life Science) following the manufacturer's instructions and placed in a thermal cycler using the following conditions: 95°C for 3 min, 35 cycles of: 98°C for 30 s, 62°C for 15 s, and 72°C for 30 s, and last-step extension at 72°C for 30 s. The resulting PCR product was purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research) and further used for *in vitro* transcription using a MEGAscript RNAi Kit (Life Technologies) according to the manufacturer's instructions. *Ssoah1*-siRNA was processed from *Ssoah1*-dsRNA using ShortCut RNase III (New England Biolabs) according to the manufacturer's specifications. To perform fluorescent microscopy, *Ssoah1*-dsRNA and siRNA were covalently labeled with Cy3 dye using a Silencer siRNA Labeling Kit (Life Technologies). Both non-labeled and labeled *Ssoah1*-dsRNA and siRNA were subsequently quantified by spectrophotometer (NanoDrop 2000, Thermo Scientific) following the protocols provided in the user guides for the MEGAscript RNAi Kit and Silencer siRNA Labeling Kit. GFP-dsRNA was used as a negative control, and a 353 bp-fragment of the GFP gene was also selected for *in vitro* transcription of GFP-dsRNA using

procedures mentioned above and the primer pair, T7_GFP_F and T7-GFP-R (Supplementary File 1).

Detection of fluorescently labeled ds/siRNAs in *Sclerotinia sclerotiorum*

A 5-mm hyphal plug of *S. sclerotiorum* from the leading edge of a 2-day old fungal culture was placed on a cellophane layer on the surface of a Petri plate containing potato dextrose agar (PDA). After two days, 1 ml of potato dextrose broth (PDB) was added to the Petri plate, and fungal mycelia were collected from cellophane and transferred into a 1.5 ml Eppendorf tube. After centrifugation at 4000 rpm for 5 min, the supernatant was removed, and mycelia were resuspended in 200 µl of PDB. Next, 4 µg of Cy3-labeled *Ssoah1*-dsRNA/siRNA were mixed into the PDB containing *S. sclerotiorum* mycelia and incubated on a shaker at room temperature at 100 rpm for 13 hours. Cy3-*Ssoah1*-dsRNA/siRNA treated *S. sclerotiorum* mycelia were washed by adding 800 µl of sterile H_2O, and mycelia were then collected by centrifugation, at 4000 rpm for 5 min, for microscopy. To produce protoplasts from Cy3-*Ssoah1*-dsRNA/siRNA treated fungal material, mycelia collected from cellophane on PDA were suspended in 1 ml PDB and transferred into a 25 ml flask. After 18 hours of incubation with 20 µg dsRNA or siRNA, mycelia were collected by centrifugation, and protoplasts were processed by adding 2.4 ml protoplasting buffer containing 10 mg/ml lysing enzyme (modified from Rollins, 2003). To determine if *S. sclerotiorum* takes up dsRNAs non-selectively, 4 µg of Cy3-labeled siRNA with no targets in *S. sclerotiorum* (Silencer Cy3-labeled Negative Control No. 1 siRNA, Thermo Fisher Scientific) was also applied to the fungus exogenously. Water, applied to *S. sclerotiorum*, was used as negative control for microscopy. Mycelia and protoplasts were visualized by confocal microscopy using a Zeiss LSM 710.

RNA extraction, cDNA synthesis, and RT-qPCR

A 5-mm *S. sclerotiorum* hyphal plug was placed into a 25-ml flask with 7 ml PDB at pH 8. Non-labeled *Ssoah1*-dsRNA were applied to PDB to make a final dsRNA concentration of 500 ng/ml, and the flasks were kept at room temperature on a shaker at 100 rpm. The application of GFP-dsRNA in PDB with *S. sclerotiorum* hyphal plugs, maintained in the same conditions as above, was considered a negative control for this experiment. Mycelia were collected at 96 hours after placement in the PDB. RNA extraction and cDNA synthesis were performed as described previously. The transcriptional level of *Ssoah1* was determined by RT-qPCR using synthesized cDNA as a template. Reactions (20 µl) were set up using SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. Quantitative PCR (qPCR) reactions were conducted using the Bio-Rad CFX96 Real-Time System and following program: 95°C for 30 s, followed by 40 cycles of: 95°C for 5 s, and 58°C for 5 s. Melt curves were generated by heating from 65°C to 95°C with 0.5°C increments. Relative expression of *Ssoah1* to *S. sclerotiorum H3* were calculated as $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) prior to calculating expression relative the GFP negative control. Experiments were repeated three times with at least three biological replicates for each repetition. Primers used for qPCR are listed as follows: Ssoah1_F1, Ssoah1_R1, H3_F1, and H3_R1 (Supplementary File 1). In conjunction with expression assays, PDB was collected from 7 ml cultures containing 500 ng/ml of dsRNA or GFP at 24, 48, and 72 hours to analyze oxalate production using the method described in Willbur et al. (2017) (Supplementary File 2). Plates were read at 590 nm using a Bio-Rad iMark (Bio-Rad Laboratories) microplate reader.

Construction of BPMV silencing vectors

In order to silence the fungal gene *Ssoah1*, a 365 base pair sequence (Supplementary File 3) was selected within the mRNA of *S. sclerotiorum* strain 1980 (GenBank Accession XM_001590428). Total RNA was extracted from *S. sclerotiorum* using Trizol, and cDNA was synthesized via reverse transcription-PCR (RT-PCR) using an AMV first strand cDNA synthesis kit (New England Biolabs, Catalog # E6550). Through PCR with a high-fidelity DNA polymerase (KAPA HiFi®, Kapa Biosystems), BAMH1 restriction sites (to form 'sticky ends') were incorporated onto the double-stranded cDNA using specific primers (BAMH1_OA_F and BAMH1_OA_R, Table S1), and the following PCR conditions: 95 °C for 2 min followed by 35x at 98 °C for 20 s, 68 °C for 30 s, 72°C for 15 s, and a final extension at 72 °C for 4 min. After digestion and gel purification (QIAquick Gel Extraction Kit®, QIAGEN), the construct was ligated into the viral vector RNA2 plasmid, pBPMV-IA-V1 to form pBPMV-OA (Zhang et al. 2010). The vector plasmids were then transformed into DH5α competent cells using 5 ul of the purified ligation product per 60 ul of competent cells, a 30 min ice incubation, 45 s heat shock in a 42 °C water bath, and incubation in 500 ul of Luria broth (LB) for 1 h at 37 °C. Colony PCR with MyTaq™ Red Mix (Bioline) was used to confirm successfully transformed clones. After an initial step for cell breakage at 95 °C for 6 min, 35 cycles (95°C for 15 s, 68°C for 15 s, and 72 °C for 10 s) were performed to confirm successful transformations, and colonies from a mini preparation were sent for sequencing to confirm antisense insertion. Using glycerol stocks, midi preparations were conducted (Fast Ion Plasmid Midi Kit®, IBI Scientific) for subsequent biolistic inoculations.

Plant inoculation with BPMV vectors

The modified system, used in this work and developed by Zhang (2010), uses bean pod mottle virus (BPMV) which is bipartite. The two viral segments consist of a 6 kb RNA1 segment (pBPMV-IA-R1M) encoding viral proteins needed for genome replication and a 3.6 kb RNA2 segment (pBPMV-IA-V1) that encodes viral proteins needed for capsid assembly and movement and contains the cloning site where the silencing construct was inserted. As described in the protocol by Whitham et al. (2016), the RNA1 and RNA2 segments were co-bombarded into 10-day old Williams 82 (PI 518671) seedlings using gold particles coated with plasmid DNA. Two inoculations were made per plant, with one on each unifoliate leaf. Prior to inoculations, unifoliate seedlings were forced to etiolate in the dark for 24 h. After inoculations, plants were sprayed with water and kept in plastic bags for 24 h to maintain humidity. The inoculated plants were placed in a growth chamber at 22 °C during the day and 20 °C at night on a 16 h photoperiod. Soil was checked daily to determine if water was needed, and the plants were fertilized once weekly with Miracle-Gro® (Scotts Miracle-Gro Co.).

Successful infection of Williams 82 soybean plants was confirmed visually, as phenotypes resembled those of plants infected with BPMV, including rugosity and mottling (Supplementary File 4). Further confirmation using RT-PCR was also performed (Supplementary File 5), using the primers for silencing construct development, and one corresponding to the RNA2, VS_R2_F and VS_R2_R (Supplementary File 1). Williams 82 soybean plants confirmed to contain the viral construct were lyophilized and stored at -80 °C to be used in subsequent rub inoculations of more soybean plants. These inoculations were performed by grinding 50 mg of lyophilized tissue in 50 mM potassium phosphate buffer and rubbing the plant serum onto the soybean variety Traff (PI

470930), as described in Whitham et al. 2016. The Traff-BPMV HIGS system was previously validated by Ranjan et al. (2018). Traff has more tolerance to BPMV than Williams 82 and *Sclerotinia* and BPMV are able to co-infect in this system. Observations suggest that BPMV-infected Williams 82 inhibits *S. sclerotiorum* infection (Grau, data not shown). Viral symptoms of mottling and rugosity were apparent but milder in Traff than in Williams 82 (Supplementary File 6). RNA was extracted from the third trifoliates of plants with viral symptoms, from one experimental replicate, and plants were confirmed to contain the silencing constructs via RT-PCR and sequencing

Characterization of processed sRNAs

To confirm the processing of siRNA from pBPMV-OA, a high concentration of total RNA was extracted from three leaves in three biological replicates from the pBPMV-OA and pBPMV-EV-containing plants. Leaves for RNA extractions were collected from the variety Traff, prior to inoculating soybeans symptomatic for BPMV with *S. sclerotiorum* in one experimental replicate of the expression experiment. The third trifoliate leaves were immediately frozen in liquid nitrogen. Two grams of leaf tissue per biological replicate was ground into fine powder using liquid nitrogen in a mortar and pestle and used in RNA extractions. A phenol chloroform extraction with a LiCl purification was performed as described in Aragão et al. (2013) to yield high RNA concentrations of 1.2-2.8 ug/ul. A cDNA library was constructed by Novogene (CA, USA) using a Small RNA Sample Pre Kit, and Illumina sequencing was conducted according to company workflow, using 20 million reads. Raw data were filtered for quality as determined by reads with a quality score > 5, reads containing N < 10%, no 5' primer contaminants, and reads with a 3'

primer and insert tag. The 3' primer sequence was trimmed and reads with a poly A/T/G/C were removed.

Pathogen aggressiveness assays

In order to assess the aggressiveness of *S. sclerotiorum* on plants containing pBPMV-OA and plants containing pBPMV-EV constructs, BPMV-symptomatic Traff plants were inoculated using the cut petiole method as detailed by Peltier and Grau (2008), and lesions were measured 24 -120 h post inoculation (HPI). Five pots, completely randomized per biological replicate and treatment, were thinned to one to three symptomatic plants per pot. Viral symptoms were apparent by the V3-V5 (vegetative stage at which the plant had three to five trifoliates) growth stage.

At the V4-V5 growth stage, the third trifoliate was excised at 2 cm and inoculated with *S. sclerotiorum,* strain 1980. All cultures were grown in the same manner prior to inoculations. The isolate was obtained from sclerotia harvested from plants grown in a growth chamber, surface disinfested for one minute in 95% ethanol and one minute in 10% concentrated household bleach (8.25% NaClO, prior to dilution), and grown on standard, 15-mm deep Petri plates containing PDA, before sub-culturing onto 20-mm Petri plates with a thicker depth of PDA prior to inoculations. After 3 days of growth on these latter plates, a plug from the leading edge of mycelia was cut using an inverted one-ml pipette tip. The inverted pipette tip with agar plug, was then slid onto the excised petiole, which was cut to 2 cm prior to inoculation. Lesions were measured with digital calipers 24-120 HPI. These data were used to compare lesion size, across time points, and used to calculate the area under the disease progress curve transformation (Shaner and Finney, 1977.) This experiment was repeated three times.

Plant expression assays

Similarly, for expression assays, Traff soybean plants were challenged with *S. sclerotiorum* strain 1980 using the cut petiole technique at the third petiole when plants were at the V4 or V5 growth stage. Expression assays were conducted in experiments independent of the pathogen aggressiveness assay. However, the 120 h time point used in expression assays included plant samples from the pathogen aggressiveness assay, since lesions in the aggressiveness assay were last measured at 120 h. In order to understand whether expression decreased in plants containing the silencing construct compared to plants with pBPMV-EV, 6 cm of stem tissue (3 cm above and 3 cm below the inoculation site) was collected 48-120 HPI and immediately frozen in liquid nitrogen. One-to-three stems per pot were combined for RNA extraction and three biological replicates were used for extractions. Expression experiments were repeated three times. Tissues were ground in liquid nitrogen using a mortar and pestle, and RNA was extracted using a Maxwell® RSC Plant RNA Kit. Two primer pairs were used for RT-qPCR to determine whether expression was reduced in plants containing pBPMV-OA (as described previously for expression analyses with exogenous applications of RNA). One primer pair (Ssoah1_F1 and Ssoah1_R1) corresponded to *Ssoah1*, outside of the region used for the silencing precursor, as to not amplify sequences from the silencing vector, and another corresponding to the *S. sclerotiorum* endogenous control, *Histone H3* (H3_F1 and H3_R1). Relative expression of *Ssoah1* to *S. sclerotiorum H3* were calculated as $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) prior to calculating expression relative to plants containing the empty vector negative control, pBPMV-EV.

Statistical analyses

Expression, AUDPC, and lesion size differences were evaluated using a mixed-model analysis of variance (ANOVA) using PROC GLIMMIX in the SAS statistical software package (v 9.4, SAS Institute, Inc. Cary, NC, United States). Significance was reported at the α=0.05 significance level. For the lesion size differences in aggressiveness assays, a lognormal distribution and compound symmetry covariance structure were used in the model for data analyses.

RESULTS

Uptake of external RNAs by *Sclerotinia sclerotiorum*.

Effective HIGS strategies are reliant on the ability of fungal organisms to uptake external ds/siRNAs. Accordingly, we tested whether such molecules can translocate into *S. sclerotiorum* hyphae. Double-stranded RNAs (dsRNAs) corresponding to a *S. sclerotiorum Ssoah1* were transcribed *in vitro,* processed into small interfering RNAs (siRNAs) and labeled with Cy3 fluorescent dye. Fluorescent signals were detected in *S. sclerotiorum* hyphae, after a two-day growth period in potato dextrose broth (PDB) supplemented with Cy3-labeled *Ssoah1*-dsRNA/siRNAs. Clear, fluorescent signals were detected in Cy3-*Ssoah1*-dsRNA/siRNA treated samples using fluorescent confocal microscopy, but not in the negative control (Figure 1). To further confirm that RNAs were within the fungal cellular space, rather than on the surface of the hyphae, protoplasts were generated from Cy3-*Ssoah1*-dsRNA/siRNA treated mycelia. Similarly, fluorescent signals were clearly visible within protoplasts, indicating their translocation across cell walls and plasma membranes (Supplementary File 7). The uptake of environmental RNAs appears to occur readily in *S. sclerotiorum,* as Cy3-labeled siRNAs with no specific targets in this fungus were also observed in fungal mycelia and protoplasts (Figure 1 and Supplementary File 7). No

fluorescence was observed in water-treated *S. sclerotiorum* mycelia or protoplasts (Figure 1 and Supplementary File 7). Taken together, these results indicate that environmental RNAs can freely translocate into *S. sclerotiorum* cells, and can thus potentially be used to target gene expression within the pathogen.

External application of RNAs targeting *Ssoah1* reduced transcript levels *in vitro*.

We next tested whether the external application of dsRNAs leads to the silencing of the target gene. In three independent experiments, actively growing *S. sclerotiorum* hyphal plugs were placed in potato dextrose broth (PDB) containing *in vitro* transcribed *Ssoah1*-dsRNAs; GFP-dsRNA treatment served as a negative control. Transcript levels of *Ssoah1* were assessed by RT-qPCR at 96 hours post-inoculation. Compared to the GFP-dsRNA controls, *Ssoah1* gene expression was markedly reduced (by > 60%) in mycelia exposed to *Ssoah1*-dsRNAs (Figure 2) ($P<0.01$), suggesting the uptake of *Ssoah1*-dsRNAs resulted in the downregulation of *Ssoah1* transcripts in *S. sclerotiorum* and thus potentially depriving the fungus from a key virulence factor during infection.

Overall, our *in vitro* assays show that *S. sclerotiorum* uptakes environmental RNAs targeting *Ssoah1*, and this uptake leads to reduced expression of the target gene. Accordingly, we reasoned that dsRNA/siRNAs generated as part of a HIGS strategy targeting a vital virulence factor might hinder *S. sclerotiorum* pathogenic development in soybean.

Accumulation of siRNAs corresponding to *Ssoah1* in soybean mediated by BPMV

With evidence that *S. sclerotiorum* could uptake exogenous siRNAs, we evaluated whether plants could express silencing constructs for uptake by *S. sclerotiorum* through HIGS. To express siRNAs targeting *Ssoah1*, a 365 bp *Ssoah1* fragment was cloned into the viral vector pBPMV-IA-V1 (Zhang et al., 2010) after the viral translation stop codon to create pBPMV-OA. Soybeans were inoculated with pBPMV-OA and an empty vector control (pBPMV-EV, negative control) using a combination of particle bombardment and rub inoculation. Small RNAs (sRNAs), sequenced from plant total RNA, were mapped to the 365 bp region carried by the pBPMV viral vector (Figure 3). Of the sRNAs profiled, 21 nt sequences were particularly abundant, with over 10,000 copies (reads per millions X .0235) estimated to be contained in leaf extracts tested (Figure 3A). This size corresponds to the 20-25 nt size characteristic of siRNAs, knowing that 21 nt siRNAs are the most efficient at mRNA degradation (Elbashir et al., 2001). These sRNAs mapped to various regions across the 365 bp cDNA silencing construct (Figure 3B, C; Supplementary File 3). The location distribution was similar in all three biological replicates from which RNA was sequenced (Figure 3, Supplementary File 8, and Supplementary File 9). As expected, reads corresponding to the silencing target were only present in the plants containing the pBPMV-OA vector, and not in plants containing the empty vector, except for five, negligible reads in one of the three samples sequenced for sRNAs. Therefore, HIGS plants were confirmed to generate abundant siRNAs corresponding to the *S. sclerotiorum* gene, *Ssoah1*.

Targeted silencing of *Ssoah1 in planta* using BPMV-mediated HIGS

We tested whether expression of the target gene, *Ssoah1*, could be reduced *in planta* using a modified BPMV vector. Plant containing pBPMV-OA and empty vector controls were challenged with *S. sclerotiorum* strain 1980 (ATCC18683) in a time-course experiment. Stem tissues were

collected at 48, 72, 96, and 120 HPI for RNA extractions and subsequent RT-qPCR. Across three independent experimental repetitions, the expression of *Ssoah1* was significantly reduced in plants inoculated with the pBPMV-OA silencing vector compared to the empty vector control (Figure 4). The most significant reduction in expression was observed at 48 HPI (60% reduction, $P<.01$) and 96 HPI (42% reduction, $P=.02$; Figure 4) and all-time points trended towards a reduction in *Ssoah1* expression. Although differences were not statistically significant at 72 and 120 HPI. These results suggest that BPMV-mediated HIGS targeting *Ssoah1*, effectively reduced the expression of this *S. sclerotiorum* gene in our time course.

Disease development was impeded in BPMV-mediated HIGS plants

To test whether the reduced expression of *Ssoah1* resulted in reduced pathogen aggressiveness, pBPMV-OA was introduced into the soybean variety, Traff. Symptomatic Traff seedlings were similarly challenged with *S. sclerotiorum* strain 1980 (ATCC18683) using a cut petiole method (Peltier and Grau 2008; Ranjan et al, 2018), and lesions were measured 24-120 HPI. Visual differences in lesions were apparent, with plants containing pBPMV-EV having large, girdling lesions 96 HPI compared to markedly restricted lesions in pPBMV-OA plants (Figure 5A). Significant differences in lesion size were detected at 72 HPI and onward (Figure 5B, $P<.01$). Additionally, the area under the disease progress curve (AUDPC) in plants containing the silencing construct was reduced by 73% compared to pBPMV-EV plants (Figure 5C, $P<.01$), indicating a delay and reduction in SSR disease development. Reduced pathogen development using BPMV-mediated HIGS in soybean challenged with *S. sclerotiorum* suggest that such a strategy is effective in controlling SSR.

DISCUSSION

The first application of *in planta* HIGS as a disease control strategy was patented in 2008 for control of pathogenic fungi and oomycetes in *Glycine max* and *Arabidopsis* (Roberts et al., 2008). Since, various strides have been made in the application of HIGS (Andrade et al., 2016; Dou et al. 2019; Ghag et al., 2014; Koch et al., 2016; Mahto et al., 2020; Nowara et al., 2010; Panwar et al., 2013; Pleigo et al. 2013; Tinoco et al., 2010; Yin et al., 2010) and SIGS (Koch et al., 2019; McLoughlin et al., 2018; Wang et al., 2016) in the control of a range of phytopathogens. The aforementioned works demonstrate that RNAi can be an effective gene silencing strategy in many plant-fungal interactions. Extending such methods to other fungal pathosystems could constitute an exciting alternative to chemical management, particularly in crops where breeding efforts have failed to produce highly resistant commercial cultivars. This study targets an essential pathogenicity factor of *S. sclerotiorum*, oxalic acid (OA) to enhance the resistance of soybean to Sclerotinia stem rot (SSR). Oxalic acid is an important virulence factor in *S. sclerotiorum*, where OA deficient mutants show reduced colonization and disease development (Liang et al., 2015; Xu et al. 2015). Accordingly, we targeted *Ssoah1*, a gene encoding an oxaloacetate acetylhydrolase enzyme that catalyzes the last step in OA biosynthesis, and the deletion of which abolishes OA production (Liang et al., 2015; Xu et al., 2015). Several lines of evidence presented within this study are consistent with the following conclusions: (i) environmental dsRNAs/siRNAs translocate into *S. sclerotiorum* hyphae; (ii) transcripts encoding *Ssoah1* are markedly reduced when *S. sclerotiorum* is exposed to the corresponding dsRNAs in *vitro* and *in planta*; and (iii) RNAi of *Ssoah1* using HIGS can reduce pathogen aggressiveness and SSR disease development.

We first determined whether *S. sclerotiorum* can uptake environmental RNAs, as uptake of dsRNA is not a ubiquitous feature of fungi. While exogenous dsRNA uptake occurs in the closely related *Botrytis cinerea*, recent findings indicate that the wheat pathogen *Zymoseptoria tritici* does not uptake environmental dsRNA despite encoding key components of the RNAi pathway (Wang et al., 2016; Kettles et al., 2019). After applying fluorescently labelled dsRNA/siRNA to fungal cultures, we detected fluorescent signals within fungal hyphae treated with both *Ssoah1* derived-dsRNA/siRNAs and non-targeting siRNA (Figure 1), indicating that *S. sclerotiorum* readily uptakes these molecules in a non-specific manner.

While the mechanism by which RNA molecules are able to translocate across fungal cell walls and membranes is yet to be determined, it is clear that the nonselective movement of these molecules occurs in the predominantly necrotrophic pathogen *S. sclerotiorum*. Within fungi, these translocated dsRNAs are processed to siRNA after uptake and both dsRNA and siRNA efficiently silence gene expression (Dang et al., 2011 and Koch et al., 2016). Though exceptions have been noted, ds/siRNA uptake is likely a common occurrence in the fungal kingdom considering the successful utilization of HIGS against several fungal species (Andrade et al., 2016; Cooper and Campbell., 2017; Dou et al., 2019; Ghag et al., 2014; Hu et al., 2020; Koch et al., 2016; Mahto et al., 2020; Nowara et al., 2010; Panwar et al., 2013; Pleigo et al. 2013; Tinoco et al., 2010; Yin et al., 2010). Thus, RNA spray regimes or HIGS has the potential to be utilized across many fungal pathogens including *S. sclerotiorum*. Interestingly, these fungal targeted RNAs also translocate, and are amplified within plant cells as well. Long dsRNA and dsRNA-derived siRNA efficiently translocate in barley (Koch et al., 2016). Song et al. (2018) also reported the successful *in planta* amplification of siRNA targeting *Fusarium* in wheat through plant RdRp, providing evidence for

the potential of sustained dsRNA/siRNA-based SIGS in a field setting. This may also alleviate concerns about the stability of such molecules in nature and may confer prolonged protection under field conditions. Spray-induced gene-silencing studies are needed to evaluate the effectiveness of exogenously-applied dsRNAs for silencing *Ssoah1* at local and distal locations, relative to the application site, in *S. sclerotiorum* hosts and to evaluate the effectiveness of SIGS targeting *Ssoah1* to manage disease in a field setting and

To confirm that these dsRNA/siRNAs were not only absorbed but actively utilized in gene silencing, *Ssoah1* transcripts in *S. sclerotiorum* were analyzed following treatment with corresponding dsRNAs *in vitro*. Oxalic acid production in *S. sclerotiorum* is a pH-responsive process and *Ssoah1* transcripts are concordantly induced in alkaline conditions (Kim et al., 2007 and Rollins et al., 2003). To ensure that *Ssoah1* was highly expressed and a reduction of transcripts in *S. sclerotiorum* would be observable after taking up *Ssoah1*-dsRNA, the treated liquid media was alkalized to a pH of 8. As anticipated, uptake of *Ssoah1*-dsRNA did occur and resulted in significant reduction of *Ssoah1* mRNA level at 96 hours after dsRNA treatment (Figure 2). These results were encouraging, and clearly show that the translocation of environmental RNAs in *S. sclerotiorum* significantly affect transcript abundance of corresponding sequences.

In planta assays were performed to evaluate the use of HIGS targeting *Ssoah1* to reduce the aggressiveness of *S. sclerotiorum*. We took advantage of our working virus-based HIGS system (Zhang et al, 2010; Ranjan et al., 2018) that uses BPMV as a vehicle to produce dsRNAs corresponding to our fungal target. Soybeans were inoculated with a BPMV vector containing an *Ssoah1* fragment (pBPMV-OA), and the presence of corresponding sRNAs was determined via

RNA sequencing. Indeed, sRNAs aligning to *Ssoah1* were found within the expected range of siRNAs (21-24 nt, Figure 3A) in pBPMV-OA inoculated plants indicating successful processing by the soybean RNAi machinery. The sRNAs corresponding to *Ssoah1* were not detected in the empty vector (pBPMV-EV) control plants. Importantly, a marked reduction in disease symptoms and reduced AUDPC was observed in pBPMV-OA soybeans compared to control empty vector plants (Figure 5). Small lesions did develop on plants expressing *Ssoah1*-dsRNAs, but in most replicates, these lesions were not girdling the stems at 96 HPI, as occurred in plants inoculated with pBPMV-EV. This reduction in symptom development was accompanied by reduced transcript levels of *Ssoah1*, indicating that HIGS is an effective strategy for reducing expression of *S. sclerotiorum* genes in soybean and conferring resistance to this pathogen. Future work is needed to generate and evaluate stable, transgenic soybean plants that use HIGS to target virulence factors, such as *Ssoah1*, of *S. sclerotiorum*.

A similar, previous approach, aimed to reduce the aggressiveness of *S. sclerotiorum* by targeting the structural gene chitin synthase in tobacco (Andrade et al., 2016). This study, however, is the first case of HIGS being used to target a pathogenicity rather than developmental factors of *S. sclerotiorum*. Because *Ssoah1* orthologues are primarily present in other plant pathogenic fungi, the risk of off-target effects from this silencing construct on beneficial and non-pathogenic fungi is low. Targeting pathogenicity-related genes with RNAi in cereal crops has provided effective disease resistance to *Puccinia graminis* and the targeting of *Ssoah1* and other *S. sclerotiorum* genes may provide similar resistance in soybean (Panwar et al., 2018). Ongoing research efforts in our lab aim to identify *S. sclerotiorum* specific virulence determinants that can be utilized in this manner.

Our findings pave the way for RNAi-based approaches to achieve enhanced resistance to *S. sclerotiorum* in soybean and potentially other valuable field crops. Virus-based HIGS using pBPMV, as developed by Zhang et al. (2010), can also be deployed in functional studies to both understand the role of *S. sclerotiorum* genes in infecting soybean hosts and to evaluate host susceptibility factors to *S. sclerotiorum*, as utilized by Ranjan et al. 2018. As a novel tool in SSR management, RNAi has the potential to reduce the use of chemical control in soybean systems and to close the gap of incomplete resistance observed in commercial and public soybean cultivars (Conley et al., 2018; Hoffman et al., 2007; Huzar-Novakowiski and Dorrance, 2018; Kim and Diers, 2000; McCaghey et al., 2017).

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FIGURES AND TABLES

Figure 1. External RNAs are taken up by *Sclerotinia sclerotiorum*.

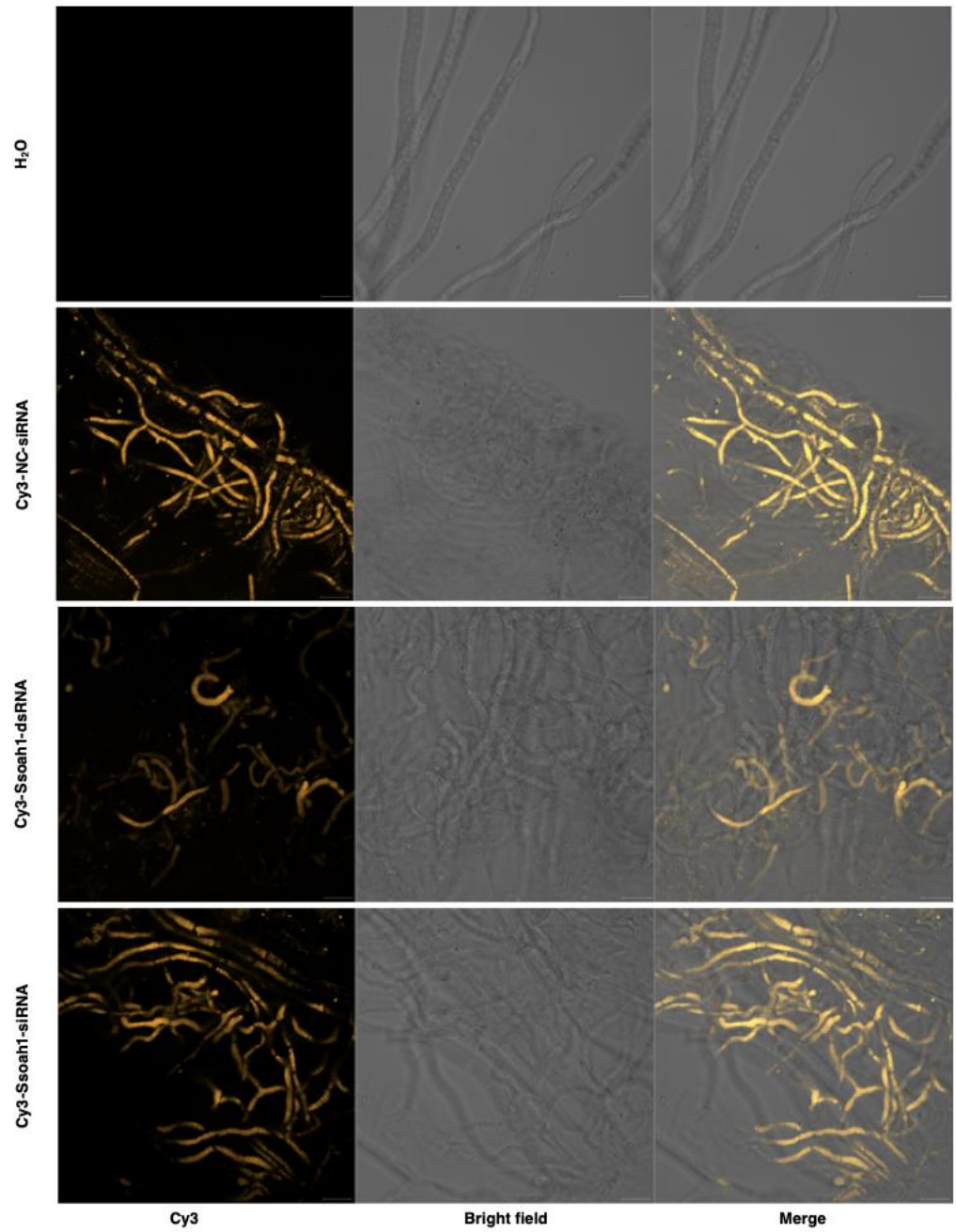


Figure 1. External RNAs are taken up by *Sclerotinia sclerotiorum*. Treatments were added to potato dextrose broth (PDB) containing two day old *S. sclerotiorum* mycelia originally grown on potato dextrose agar (PDA). Fluorescent signals were detected using confocal fluorescent microscopy. (A) An application of water, as a negative control, yielded no signal. (B) The positive control, application of Cy3-labeled-nocoding siRNA (Cy3-NC-siRNA, with no specific targets in *S. sclerotiorum),* (C) 4 ug application Cy3-labeled-*Ssoah1*-dsRNA, and (D) application of 4 ug of Cy3-labeled-*Ssoah1*-siRNA (targeting *Ssoah1* in *S. sclerotiorum*) produced fluorescent signals that were detected 13 hours post inoculation. Scale bars = 20 μm.

Figure 2. External uptake of *Ssoah1*-dsRNA decreased *Ssoah1* transcript levels in *Sclerotinia sclerotiorum*.

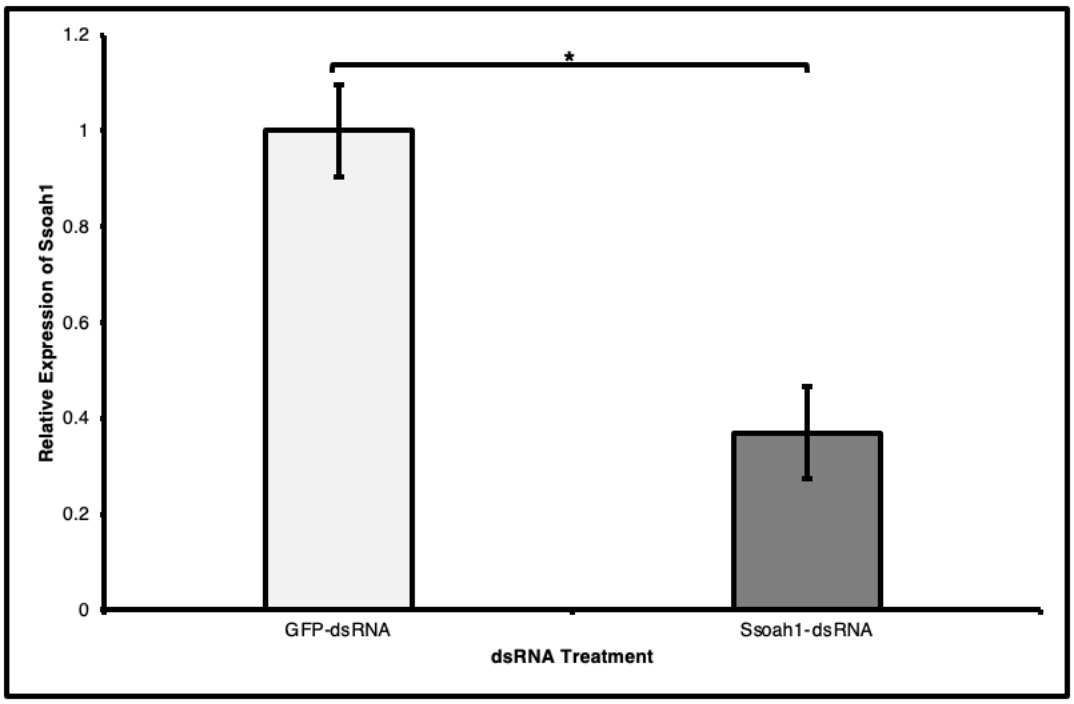


Figure 2. External uptake of *Ssoah1*-dsRNA decreased *Ssoah1* transcript levels in *Sclerotinia sclerotiorum*. A concentration of 500 ng/mL of *Ssoah1*-dsRNA were applied to potato dextrose broth (pH 8) and a potato dextrose agar plug containing mycelia of *S. sclerotiorum* was placed in the broth. Mycelia were collected 96 hours after addition to PDB and the transcript level of *Ssoah1* were analyzed. Data were normalized to *S. sclerotiorum* endogenous *Histone H3* transcript levels. *Ssoah1* transcript levels were significantly reduced across three three experimental replicates ($p<0.01$). Error bars represent ± standard errors of three (for R1 and R2) or four (for R3) biological replicates across three experimental repetitions.

Figure 3. Total sRNAs were profiled from Traff soybean plants bombarded with pBPMV -OA and pBPMV-EV.

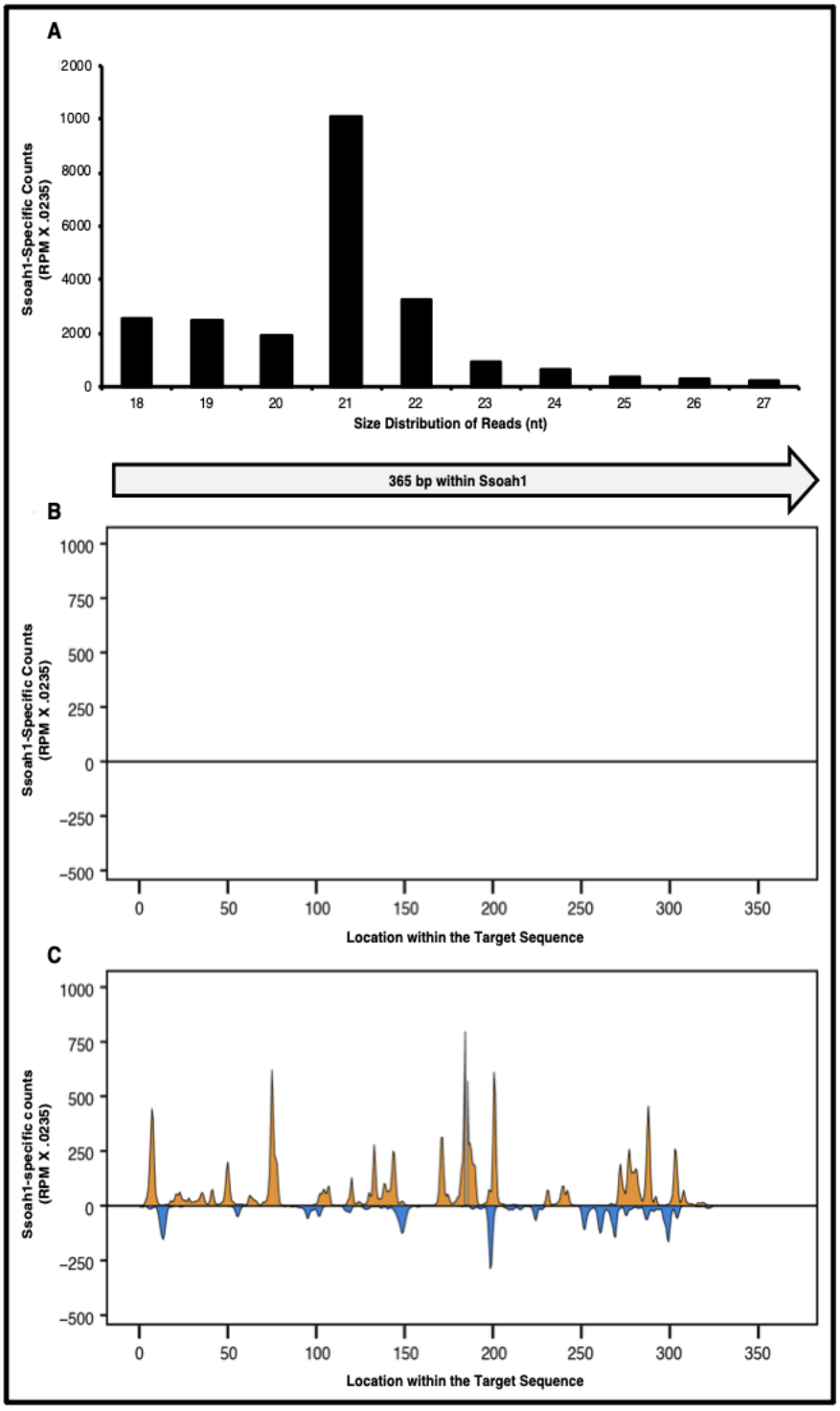


Figure 3. Total sRNAs were profiled from Traff soybean plants bombarded with pBPMV -OA and pBPMV-EV. The size distribution of sRNA reads less than 27 nt and corresponding to the 365 nt target within *Ssoah1* were normalized to a count, using the conversion factor of .0235 X reads per million (RPM). (A) The size distribution of Ssoah1-dsRNA-derived sRNAs corresponding to *Ssoah1* from a pBPMV-OA single plant are presented for sRNAs ranging from 18-27 nt. The 5' end of 18-27 nt sRNAs from plants containing B) pBPMV-EV and (B) pBPMV-OA vectors were aligned to the 365 nt sequence cDNA sequence used for silencing construct development. Antisense reads are presented in orange and sense reads are presented in blue.

Figure 4. Transcript levels of the target gene, *Ssoah1*, were decreased in expression assays.

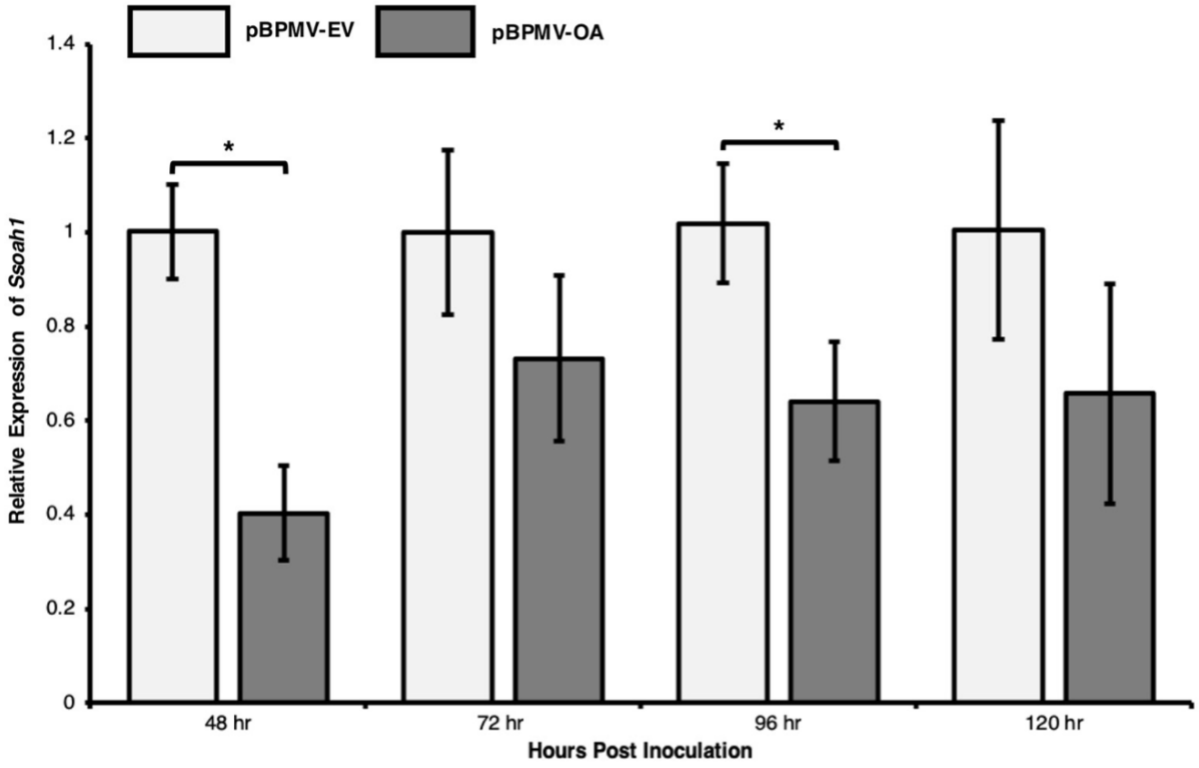


Figure 4. Transcript levels of the target gene, *Ssoah1*, were decreased in expression assays. Plants containing virus-induced gene silencing constructs, pBPMV-OA and empty vectors, pBPMV-EV were challenge with *S. sclerotiorum* strain 1980, using a cut petiole technique. RNA was extracted from stem tissue from three biological replicates and three experimental repetitions. Expression in pBPMV-OA treated plants tended to decrease for all time points, however, the difference was significant at at 48 hours ($P<0.01$) and 96 hours ($P=0.02$).

Figure 5. Pathogen aggressiveness was reduced in virus induced gene silencing (VIGS) assays.

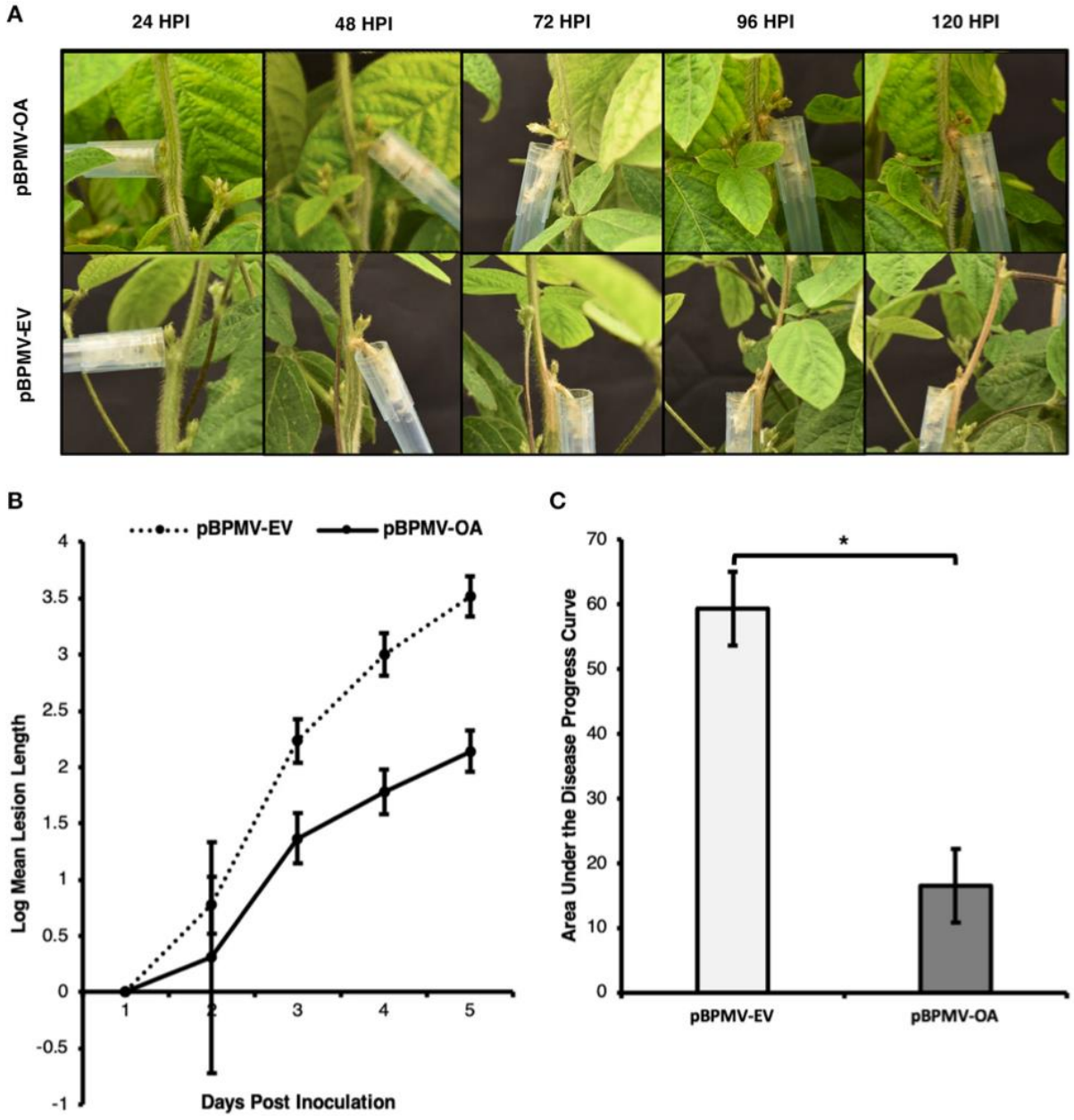


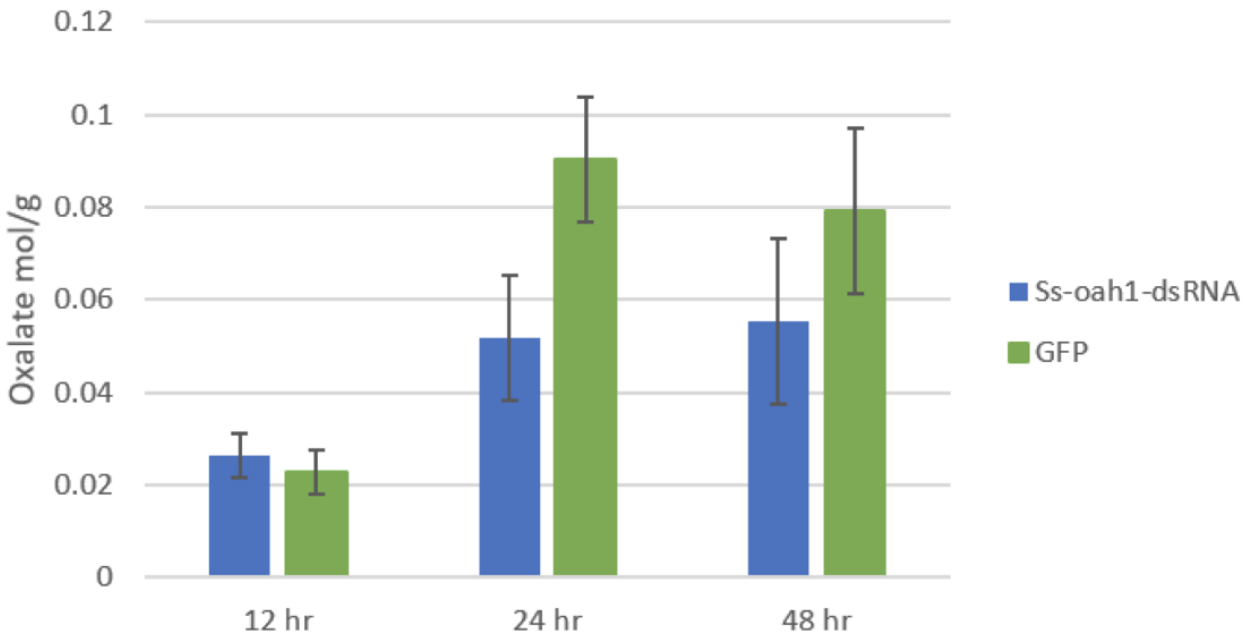
Figure 5. Pathogen aggressiveness was reduced in virus induced gene silencing (VIGS) assays. Plants containing VIGS silencing constructs (pBPMV-OA) and empty vectors (pBPMV-EV) were challenged with *S. sclerotiorum* strain 1980, using a cut petiole technique. Lesions were measured 1-5 days post inoculation (DPI). Data are the result of five biological replicates and three experimental repetitions. (A) Lesion development was delayed, and lesions were smaller in plants

containing pBPMV-OA, while plants containing pBPMV-EV often showed girdling lesions at 96 hours post inoculation (HPI). (B) Lesion size was reduced after two DPI, with an overall reduced lesion size ($P<0.01$). (C) The overall area under the disease progress curve was lower in plants containing pBPMV-OA ($P<0.01$).

Supplementary File 1: List of primers used for construct design and Reverse Transcription PCR.

Primer	**Sequence (5'-3')**
T7_OA_F	TAATACGACTCACTATAGGGAGAGTCTTTGCAGCCATCTCCCTTTG
T7_OA_R	TAATACGACTCACTATAGGGAGAGGACCACCCTTGATTGCTGA
T7_GFP_F	TAATACGACTCACTATAGGGAGAGCAGTGCTTCAGCCGCTA
T7_GFP_R	TAATACGACTCACTATAGGGAGATGTTCTGCTGGTAGTGGTCG
Ssoah1_F1	ATCATGGATGCACTCCCAAT
Ssoah1_R1	CCGGTCTGGTAGACTGTGGT
H3_F1	ATGGCTCGTACCAAGCAAAC
H3_R1	AGAGCACCAATAGCGGAAGA
BAMH1_OA_F	CGCGGATCCGTCTTTGCAGCCATCTCCTTTG
BAMH1_OA_R	CGCGGATCCGGACCACCCTTGATTGCTGA
VS_R2_F	GGTGCTGGTTCACATTCTTCAG
VS_R2_R	GAGTGCGCTGCAAATCAACACT

Supplementary File 2: Oxalate concentrations detected from PDB cultures, using a colorimetric assay, after the application of *Ssoah1*-dsRNA or GFP (negative control).



Supplementary File 2

Oxalate concentrations detected from PDB cultures, using a colorimetric assay, after the application of *Ssoah1*-dsRNA or GFP (negative control). A concentration of 500 ng/mL of *Ssoah1*-dsRNA were applied to potato dextrose broth (pH 8) and a potato dextrose agar plug containing mycelia of *S. sclerotiorum* was placed in the broth (n=3, $p>0.05$). Samples were processed as described in Willbur et al. (2017), and absorbencies were measured at 590 nm.

Supplementary File 3: 365 base pair cDNA sequence, from Ssoah1, used for silencing construct development.

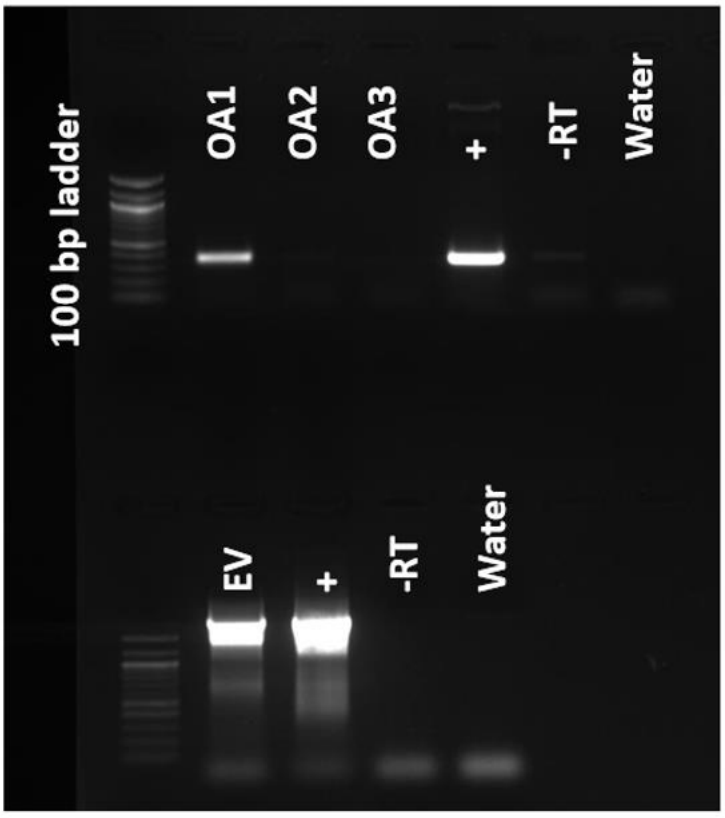
GGACCACCCTTGATTGCTGATATGGACACCGGATACGGTGGACCTCTCATTGTTGACAAAGCCG
TCAAGGCCTACATCAGAGCCGGTGTTGCTGGATTCCATATCGAAGATCAAATTCAAAACAAGCG
TTGTGGCCATCTTGCAGGCAAGAAGGTTGTCCCTGAAGAAGAGTACTACATGAGAATTCGTGC
CGCCAAGGGTGCCAAAGATGCCATGAAATCCGATATTGTGTTGATTGCACGCACAGATGCGCT
CCAACAACTTGGTTATGATGAGTGCGTCAAACGTTTGAAGGTTGCTCGTGAGCTTGGTGCAGAT
GTTGGCTTGCTCGAGGGTTACACTTCAAAGGAGATGGCTGCAAAGAC

Supplementary File 4: Williams 82 (W82) biolistically inoculated with pBPMV-OA and pBPMV-EV displayed viral symptoms such as rugosity and mottling.

Supplementary File 4

Williams 82 (W82) biolistically inoculated with pBPMV-OA and pBPMV-EV displayed viral symptoms such as rugosity and mottling. A) Williams 82 inoculated with pBPMV-OA silencing construct B) and a non-inoculated plant grown under the same conditions.

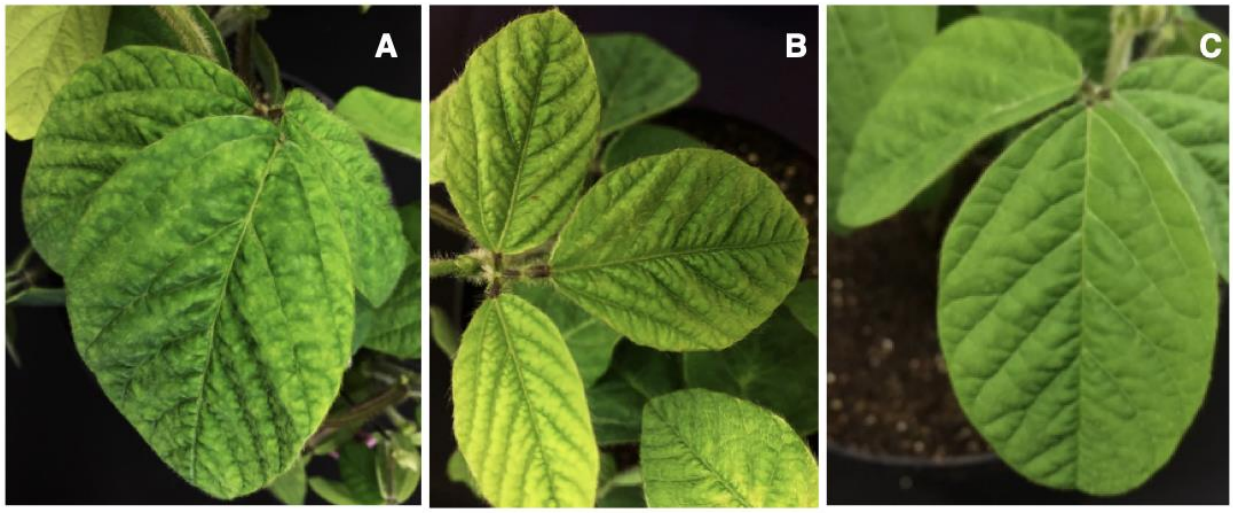
Supplementary File 5: RT- PCR was used to confirm successful biolistic inoculations with pBPMV-OA and pBPMV-EV in order to use tissue for subsequent rub inoculations.



Supplementary File 5

RT- PCR was used to confirm successful biolistic inoculations with pBPMV-OA and pBPMV-EV in order to use tissue for subsequent rub inoculations. Plasmids containing the silencing construct and the empty vector were used for positive controls. Negative controls included RNA from plant-OA1 and the positive control with no reverse transcriptase and water.

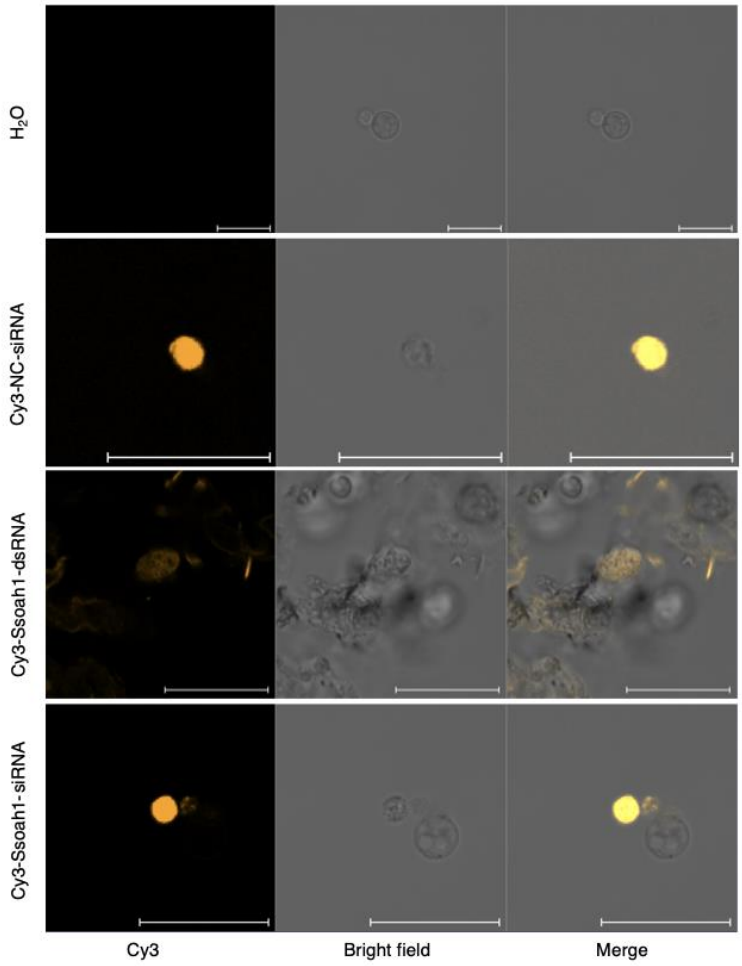
Supplementary File 6: After rub-inoculation of Traff with sap from Williams 82 containing A) pBPMV-OA and B) pBPMV-EV, BPMV symptoms of mottling and rugosity were apparent, compared to C) non-inoculated plants grown under the same conditions.



Supplementary File 6

After rub-inoculation of Traff with sap from Williams 82 containing A) pBPMV-OA and B) pBPMV-EV, BPMV symptoms of mottling and rugosity were apparent, compared to C) non-inoculated plants grown under the same conditions. Symptoms were less severe in Traff than in Williams 82.

Supplementary File 7: Protoplasts demonstrate that external RNAs are taken up by *Sclerotinia sclerotiorum* cells.

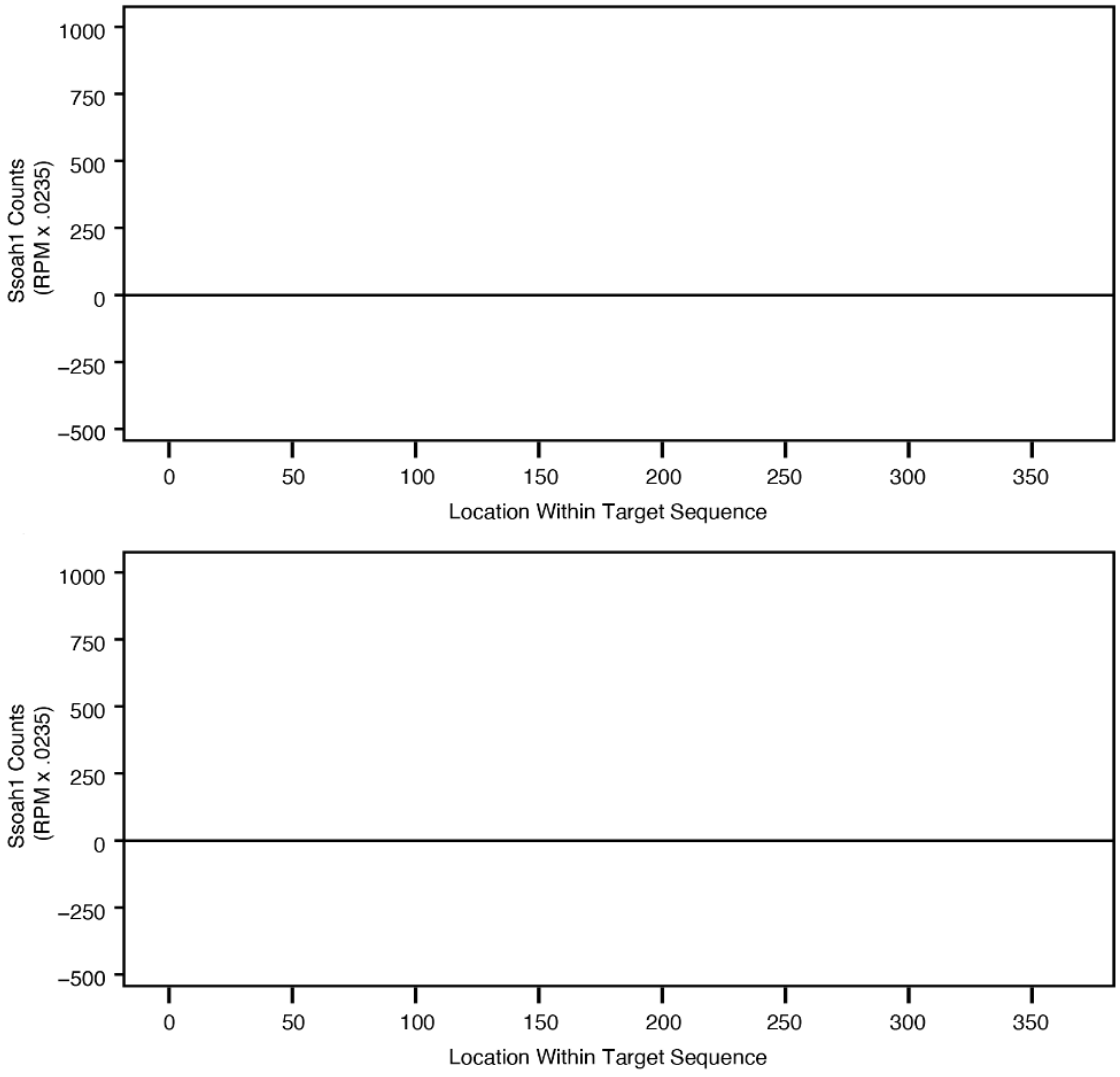


Supplementary File 7

Protoplasts demonstrate that external RNAs are taken up by *Sclerotinia sclerotiorum* cells. Treatments were added to potato dextrose broth (PDB) containing two day old *S. sclerotiorum* mycelia originally grown on potato dextrose agar (PDA). Protoplasts were processed 18 hours post inoculation. Fluorescent signals were detected using confocal fluorescent microscopy. A) An application of water, as a negative control, yielded no signal. B) The positive control, application of Cy3-labeled-nocoding siRNA (Cy3-NC-siRNA, with no specific targets in *S. sclerotiorum),* C)

20 ug application Cy3-labeled-*Ssoah1*-dsRNA, and D) application of 20 ug of Cy3-labeled-*Ssoah1*-siRNA (targeting *Ssoah1* in *S. sclerotiorum*) produced fluorescent signals. Scale bars = 20 μm.

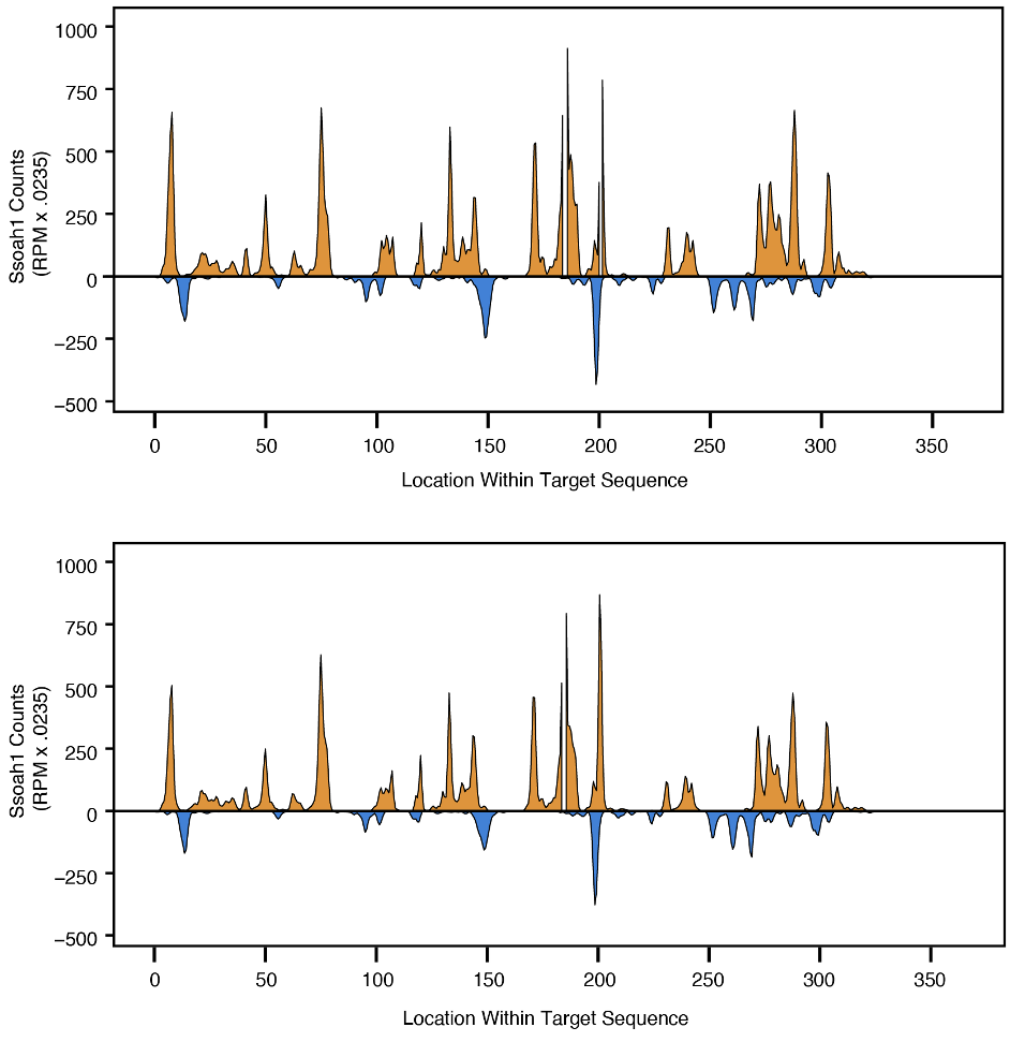
Supplementary File 8: Total sRNAs were profiled from Traff soybean plants bombarded with pBPMV-EV.



Supplementary File 8

Total sRNAs were profiled from Traff soybean plants bombarded with pBPMV-EV. The size distribution of sRNA reads less than 27 nt and corresponding to the 365 nt target within *Ssoah1* were normalized to a count, using the conversion factor of .0235 X reads per million (RPM). The 5' end of 18-27 nt sRNAs from plants containing pBPMV-EV vectors were aligned to the 365 nt sequence cDNA sequence used for silencing construct development. Reads locations with the target sequence were negligible across EV1 (in Fig. 3(B)), EV2, and EV3 and not detectible at this scale.

Supplementary File 9: Total sRNAs were profiled from Traff soybean plants bombarded with pBPMV -OA.



Supplementary File 9

Total sRNAs were profiled from Traff soybean plants bombarded with pBPMV -OA. The size distribution of sRNA reads less than 27 nt and corresponding to the 365 nt target within *Ssoah1* were normalized to a count, using the conversion factor of .0235 X reads per million (RPM). The 5' end of 18-27 nt sRNAs from plants containing pBPMV-OA vectors were aligned to the 365 nt

sequence cDNA sequence used for silencing construct development. Read locations within the target sequence were similar across the plant in OA1 (Fig. 3(C)) and plants, OA2 and OA3.

CHAPTER 3: A broadly conserved fungal chorismate mutase hijacks the plant shikimate pathway to achieve pathogenic success

Dandan Shao will serve as first author on this collaborative manuscript with the Dickman lab. The work on this project is complete, and this manuscript is now in the final stages of preparation.

Authors

Dandan Shao, Nathaniel Westrick, Wende Liu, Oded Yarden, Yuri Kimura, Hiroshi Maeda, Marty Dickman, Mehdi Kabbage

ABSTRACT

The molecular underpinnings of plant-pathogen interactions are complex, with host plants and their pathogens in a constant battle to gain the upper hand. While plants are capable of mobilizing an intricate system of antimicrobial metabolites and physiological alterations to disrupt pathogenesis, pathogens have similarly evolved mechanisms to subvert these processes through the secretion of proteinaceous effectors. A well-studied example of this is the deployment of secreted chorismate mutases (CMs) by biotrophic fungi and nematodes. These CMs coopt the endogenous shikimate pathway of the host by diverting chorismate away from the production of salicylic acid (SA) and subsequently undermine SA-mediated defenses which are typically antagonistic to biotrophic infection. Although previously studied CMs are typically associated with biotrophy, in this study we identify a novel CM, from the broad host range and predominately necrotrophic pathogen *Sclerotinia sclerotiorum*, and demonstrate that orthologs of this secreted

CM are present in a far more diverse range of fungal lifestyles than initially theorized. *S. sclerotiorum* CM (SsCM1) is critical to plant successful infection and localizes to the plant chloroplast where it interacts with plant plastidic CMs. While SsCM1 is a functional, albeit weak CM, it contains a novel domain architecture of bacterial origin, including a putative isochorismate pyruvate lyase (IPL) domain, and transient expression of SsCM1 *in-planta* increases rather than decreases host SA levels. Our results indicate that secreted CMs are broadly conserved in many plant associating fungi outside of canonical biotrophs and likely facilitate infection through a novel manipulation of the plant Shikimate pathway that redirects the flow of this pathway towards increased SA production and away from the biosynthesis of antimicrobial compounds. We propose that SsCM1-like effectors represent a novel class of secreted CMs that may be commonly utilized by plant associated necrotrophs to achieve pathogenic success.

INTRODUCTION

Sclerotinia sclerotiorum is a cosmopolitan necrotrophic fungal pathogen that can infect over 600 plant species (Purdy, 1979; Boland and Hall, 1994; Tu, 1997), causing annual multimillion dollar yield losses on economically important crops (http://www.whitemoldresearch.com). Virulence factors for this aggressive pathogen have largely focused on the phytotoxic compound oxalic acid (Williams et al., 2011; Liang et al., 2015) and cell wall degrading enzymes (CWDEs) (Amselem et al., 2011). However, recent bioinformatic and functional studies have identified other components that are involved in pathogenic success of this pathogen, including small RNAs (Derbyshire et al.,2019), detoxifying enzymes (Peyraud et al., 2019; Westrick et al., 2019), and effectors (Seifbarghi et al., 2017; Westrick et al., 2019). Defined as small secreted, cysteine-rich, molecules that facilitate plant colonization, phytopathogen effectors have been widely studied for their roles in pathogenicity (Presti et al., 2015). During the

past two decades, advances in genome sequencing and computational tools have enabled the discovery of effectors from a wide range of necrotrophic fungal pathogens. However, with a few exceptions, our understanding of these molecules in necrotrophs is limited, and much of what we know about effector biology has been chiefly described in (hemi)biotrophic pathogens (Louet et al., 2021).

The shikimate pathway is used by bacteria, archaea, fungi, and plants for the biosynthesis of carbocyclic aromatic compounds (Bochkov et al., 2011; Maeda and Dudareva 2012). Chorismate, the common branchpoint for the production of these metabolites, is converted by chorismate mutases (CMs) into prephenate, an intermediate in the biosynthesis of the aromatic amino acids phenylalanine and tyrosine, and further downstream to a large number of secondary metabolites including phenylpropanoids and phytoalexins (Maeda and Dudareva 2012). Chorismate also serves as a precursor for the plant hormone salicylic acid (SA) (Dempsey et al., 2011). Some fungal pathogens have both endogenous and secreted CMs, the latter of which are required for full virulence in plants (Djamei et al., 2011). Homologs in plant nematodes have also been shown to be important pathogenicity determinants (Lambert et al., 1999; Jones et al., 2003; Bekal et al., 2003; Wang et al., 2018). These secreted CMs are proposed to contribute to virulence through the manipulation of the plant shikimate pathway and associated defense compounds. The key role of the phenolic hormone SA, a metabolite derived from the shikimate pathway, in the regulation of disease signaling upon pathogen attack is well established. In general, SA-dependent defense responses are often deployed against biotrophic pathogens, while jasmonic acid (JA-) and ethylene (ET-) dependent defenses commonly discourage pathogens with predominately necrotrophic lifestyles (Glazebrook 2005; Bari and Jones, 2009). Unsurprisingly, many pathogens make use of effectors to interfere with phytohormone pathways to their own benefit (Han and

Kahmann, 2019). One such example is found in the smut fungus *Ustilago maydis*, which secretes a CM encoded by *CMU1* and is required for full virulence on maize (Djamei et al., 2011). During infection, Cmu1 interacts with a plant cytoplasmic CM and facilitates the conversion of chorismate into prephenate, funneling chorismate away from the SA biosynthetic pathway, therefore restricting SA-mediated defenses against this biotroph pathogen (Djamei et al., 2011). Initial genome data showed that secreted CMs were largely associated with the biotrophic fungal lifestyle (Djamei at al., 2011). However, more recent sequencing data suggest that CM-like effectors are more widely distributed in fungi with diverse lifestyles, including predominately necrotrophic organisms such as *S. sclerotiorum*.

In this manuscript, we describe a novel group of secreted CMs that possess a chloroplast targeting peptide and have a unique CM domain with greater homology with the bifunctional isochorismate pyruvate lyase (IPL)/CM proteins found in prokaryotes. Using the *S. sclerotiorum* CM ortholog, SsCM1, we show that this protein functions through a mechanism that is distinct from the *U. maydis* Cmu1. After secretion from the pathogen, we propose that SsCM1 translocates to the chloroplast where it upregulates SA levels, therefore decreasing antagonistic JA-mediated defenses against *S. sclerotiorum* and presumably inducing SA-mediated cell death to the benefit of the pathogen. SsCM1 likely increases SA levels in the host by redirecting the flow of the chorismate pathway or by acting directly as an isochorismate pyruvate lyase (IPL) to synthesize SA. Collectively, these results suggest that these secreted SsCM1-like effectors use a distinct and previously unexplored mechanism to facilitate successful plant infection.

RESULTS

SsCM1-like effectors are novel chorismate mutases likely arising from cross-kingdom horizontal gene transfer

While secreted CM effectors have been characterized in various plant pathogens including the fungus *U. maydis* (Cmu1) and nematode *Meloidogyne incognita (*Mi-CM-3*)*, it has been broadly assumed that these effectors are hallmarks of biotrophic plant/pathogen interactions given their canonical SA-suppressing activity (Djamei et al, 2011; Wang et al., 2018). To evaluate this, the predominately necrotrophic fungal pathogen *S. sclerotiorum* was analyzed for the presence of CM-like genes in its genome. Preliminary genomic analysis identified two putative CM genes within the *S. sclerotiorum* genome, hereupon referred to as *SsCM1* (XP_001584707.1) and *SsCM2* (XP_001590829.1). Protein homology and subcellular localization prediction suggest that *SsCM2* is likely an endogenous, cytoplasmic CM, which have been shown in other species to play a pivotal role in the fungal Shikimate pathway (Romero et al., 1995). Unlike *SsCM2*, however, *SsCM1* contains a predicted secretion signal peptide (SP) and an unusual CM domain, suggesting a functional divergence between the two genes (**Fig 1A**). While *SsCM2* contains a monofunctional CM domain (TIGR01802) found commonly in eukaryotes, *SsCM1* has a CM domain containing a predicted isochorismate pyruvate lyase (IPL) motif (PRK07075), sharing greater homology with the bifunctional IPL/CM proteins found in prokaryotes (**Fig 1A**) (Gaille et al., 2002). Unlike CMs which convert chorismate into prephenate as a precursor to aromatic amino acid production, bacterial IPLs convert isochorismate directly into SA (Serino et al., 1995; Mercado-Blanco et al., 2001). The evolutionary history of *SsCM1* was inferred using the maximum parsimony method from an alignment of predicted *SsCM1* homologs (**Fig 1B**). The vast majority of these homologs were proteobacteria, whereas proteins containing the IPL domain were comparatively uncommon

in fungi. The tree was rooted within proteobacteria given the apparent ubiquity of IPL/CM proteins within the phylum, and the resulting tree suggests that the presence of *SsCM1* in the *S. sclerotiorum* genome is likely to be the result of horizontal gene transfer (HGT) first into basidiomycete fungi and then into Leotiomycetes through a second HGT event (**Fig. 1B**). A distinct HGT event leading to the presence of *SsCM1* orthologs within a limited number of *Penicillium* spp. also appears likely (**Fig. 1B**). As Cmu1, from the biotrophic pathogen *U. maydis*, is the only other well characterized CM effector in fungi and is known to localize to the host cytoplasm, all predicted fungal SsCM1 homologs were analyzed bioinformatically for the presence of both secretion SPs and any predicted subcellular localization within plants (**Table 1**). Most of these homologs contained a predicted SP, but surprisingly ~70% of Leotiomycete homologs, including *S. sclerotiorum*, also contained a predicted chloroplast targeting peptide (cTP), a feature which was comparatively rare in other fungal groups (**Fig. 1A, Table 1**). Such localization has not been observed in the secreted CMs found in the biotrophic plant pathogens that have been evaluated to date (Lambert et al., 1999; Djamei et al, 2011; Wang et al., 2018). As the Leotiomycete species containing secreted CMs appear to share a common necrotrophic lifestyle, this subcellular localization may be relevant to this particular lifestyle. Taken together, SsCM1 appears to represent a novel class of secreted CM effectors present throughout the fungal kingdom and is found in fungi with lifestyles seemingly incompatible with the SA-suppressing activity of currently characterized CM effectors.

Inactivation of SsCM1 results in strains with reduced virulence

Transcriptomic analysis of *S. sclerotiorum* infection of both soybean and *Arabidopsis thaliana* have noted that *SsCM1* is highly expressed during infection, but confirmation of this effector's importance in pathogenicity requires further confirmation (Westrick et al, 2019; Peyraud et al., 2019). To confirm its role in virulence, the wild-type *SsCM1* gene was replaced by a

hygromycin phosphotransferase (Hyg) cassette via homologous recombination (**Fig 2A**). Two independent knockout mutants (Δ*sscm*1-1 and Δ*sscm*1-2) were generated. Gene replacement was confirmed at the DNA and transcriptional levels (**Fig 2B**). *SsCM1* knockouts were equivalent to wild type with respect to fungal growth and development (**Fig 2C**). Additionally, sclerotial morphogenesis and oxalic acid were also unchanged between the wild-type and mutant strains (**Fig 2D and E**).

Despite these similarities, when the Δ*sscm*1 mutants were inoculated on detached leaves of multiple host plants (*Arabidopsis thaliana*, *Nicotiana benthamiana*, *Solanum lycopersicum*), a consistent, statistically significant reduction in virulence was observed (**Fig 3A-F**). During the early stage of infection (24 hpi), typical lesions were observed on wild-type inoculated leaves, but not in leaves inoculated with the mutants (upper panels in **Fig 3A, C and E**). At later time points lesions developed in leaves inoculated with the mutant strains, indicating a delayed onset of disease (lower panels in **Fig 3A, C and E**). Moreover, the reduced virulence of *SsCM1* mutants was rescued when the mutants were complemented with the full-length *SsCM1* gene driven by its native promoter (**Fig 3A, C and E** right panels). Together these results suggest that SsCM1 is required for full virulence on multiple hosts.

SsCM1 is secreted and translocates to the host chloroplast during infection

As mentioned previously, SsCM1 is predicted to contain a secretion signal at the N-terminus with a strong prediction score (0.864) based on SignalP. To examine whether SsCM1 is indeed secreted, we used two approaches. We first generated a hemagglutinin (HA)-tagged SsCM1 strain followed by immunoblotting, SsCM1-HA was clearly detected in minimal media supernatant under standard *in vitro* growth conditions (**Fig 4A**), fungal actin served as control. Immunogold electron microscopy was also used to directly visualize the secretion of SsCM1 *in*

vivo using the generated SsCM1-HA strain (**Fig 4B**). Transmission electron microscopy (TEM) showed that gold particles affixed with HA tag antibodies were not only detected in the fungal hyphae but also outside of the fungal cell wall (**Fig 4B**). Together these results indicate that SsCM1 is a secreted protein.

The *in planta* subcellular localization of SsCM1 during infection was also analyzed given the predicted cTP (**Fig 1**). *S. sclerotiorum* expressing HA-tagged *SsCM1* was inoculated onto 4-week old *N. benthamiana* leaves. HA-tagged SsCM1 was observed in intact chloroplasts but not in the mitochondrial fractions, indicating the chloroplast targeting sequence is functional (**Fig 4C**). Additionally, HA-tagged SsCM1 were also distributed in host cytoplasm (**Fig 4C**), suggesting that *S. sclerotiorum* secretes SsCM1 into host cells and presumably cytosolic SsCM1 is then translocated to host chloroplasts. These results were confirmed by the immunogold labeling of SsCM1-HA, where signals were observed in the apoplast, fungal hyphae, and importantly in the host cytoplasm and chloroplast (**Fig 4D**). This suggests that chloroplast localization of SsCM1 may be required for full virulence in this aggressive broad pathogen with a predominantly necrotrophic lifestyle. The functional relevance of the cTP was further examined by generating GFP-tagged constructs harboring *SsCM1* with or without the cTP, and consequently expressed in *N. benthamiana* protoplasts. SsCM1-GFP with the cTP element localized to the chloroplast of plant cells, while the SsCM1-GFP lacking the cTP was only visible in the cytoplasm (**Fig 4E**). Therefore, the cTP of SsCM1 is clearly relevant to the subcellular localization and function of this protein within the host environment.

SsCM1 upregulates salicylic acid levels in plants

We further explored how the chloroplast localization underlies the mode of action of SsCM1. As indicated by bioinformatic analyses (**Fig 1A**), *S. sclerotiorum SsCM1* potentially

encodes a protein with both CM and IPL activities. IPLs are known to convert isochorimsate into salicylate in bacteria (Serino et al., 1995; Mercado-Blanco et al., 2001). In plants, SA is an important defense signal and mediates resistance against phytopathogens (Glazebrook 2005), and its biosynthesis pathway is known to occur in the plastids (Rekhter et al., 2019). In alignment with the chloroplast localization (**Fig 4)**, it is conceivable that SsCM1 acts as an IPL to directly convert isochorimate to SA which is then released into the cytoplasm to mediate downstream processes (Rekhter et al., 2019). Alternatively, SsCM1 can also act as a CM as previously described with the *U. maydis* Cmu1 (Djamei et al., 2011) and potentially affect the conversion of chorismate into prephenate and downstream phenylpropanoids. To verify if SA level in plants is impacted by the presence of SsCM1, we expressed SsCM1 in *N. benthamiana* using an *Agrobacterium*-mediated transient expression system. Two days after Agro-infiltration, leaves were collected and analyzed for their SA content using HPLC. Compared to Cmu1 and a GFP control, SsCM1 expression resulted in a significantly higher SA content (**Fig 5A**). Interestingly, this is in contrast to Cmu1, which was shown to prevent SA accumulation upon pathogen attack (Djamei et al., 2011). Thus, SsCM1 acts in a mechanism that is distinct from that used by Cmu1, and likely functions to increase SA levels in the host upon pathogen challenge.

SsCM1 mediated accumulation of salicylic acid is owed to a weak CM activity

Whether the induction of SA is driven by IPL or CM activities of SsCM1 was further investigated. To test the IPL activity of the protein, *SsCM1* was cloned upstream of a rhamnose-inducible promoter and transformed into *Escherichia coli* DH 10β. *E. coli* is known to utilize isochorimate synthase encoded by *entC* to synthesize isochorismate, but lacks the ability to convert isochorismate into SA (Mishra and Baek 2021). Upon induction by rhamnose and IPTG, a protein with IPL activity expressed in *E. coli* is expected to convert endogenous isochorismate into SA,

which can be further detected using HPLC. PchB, a known *Pseudomonas aeruginosa* IPL (Serino et al., 1995) was used as positive control in this experiment. A strong SA signal was detected from *E. coli* cells expressing PchB, however, SA levels in SsCM1 expressing *E. coli* were statistically indistinguishable from Cmu1 expressing cells or those containing the empty vector control (**Fig 5B**). This suggests SsCM1 likely lacks IPL activity or is not a functional IPL, at least in a bacterial environment.

To explore the alternative mechanism that could explain SA induction by SsCM1, we evaluated the predicted CM activity of SsCM1 (**Fig 1A**) by testing the ability of SsCM1 to complement the yeast CM mutant Δ*aro7*. *Saccharomyces cerevisiae* Aro7 shares similarity in structure with SsCM1 in the CM catalytic domain (**Fig 1A)** and is required for the biosynthesis of the aromatic amino acids tyrosine (Tyr) and phenylalanine (Phe) (Brown and Dawes 1990). *S. cerevisiae* mutants lacking *Aro7* are unable to grow on media lacking these two aromatic amino acids (**Fig 6A**, Δ*aro7*). However, the overexpression of SsCM1 in Δ*aro7* yeast strains restored the ability of these mutants to grow in media lacking Tyr and Phe **(Fig 6A)**. The CM activity of SsCM1 was also determined by using steady-state kinetic assays that monitored the conversion of chorismate to prephenate (Westfall et al., 2014). Compared to the positive control, a purified recombinant His-tagged Cmu1 which exhibited CM enzymatic activities (Djamei et al., 2011), the recombinant SsCM1-His also demonstrated a similar capacity to catalyze chorismate into prephenate (**Suppl. Fig S1**). Accordingly, both the yeast complementation and kinetic assays indicate that *SsCM*1 gene encodes an active functional chorismate mutase. Interestingly however, *in vitro* CM enzyme activity assays showed that although all tested chorismate mutases are able to convert chorismate into prephenate, the catalytic capability of SsCM1 is much weaker compared to plant chorismate mutases (~16% of the activity of AtCM1) (**Fig 6B)**. SsCM1 also has weaker

chorismate catalytic activity compared to Cmu1 (**Suppl. Fig S1**), suggesting SsCM1 is less efficient in converting chorismate than its *U. maydis* counterpart.

Chorismate mutases are known to exist as dimers, we thus reasoned that SsCM1, due to its weak CM activity, may interfere with plant CMs through competitive binding. We next tested the ability of SsCM1 to bind *Arabidopsis* CMs in yeast. The *Arabidopsis* genome contains three predicted chorismate mutase genes – the chloroplast localized *AtCM1* and *AtCM3,* and one cytosolic chorismate mutase *AtCM2* (Eberhard et al., 1996). We cloned *AtCM*1 and *AtCM*2 individually into a prey yeast vector, while SsCM1 was used as bait. Results showed that SsCM1 not only bound to chloroplast-localized AtCM1, but also to the cytoplasmic AtCM2 (**Fig 6C**), suggesting all the two Arabidopsis chorismate mutases are potential targets during infection. Together, we propose that SsCM1 likely acts as a weak CM to slow down the flow of chorismate to prephenate, thus funneling more chorismate towards SA biosynthesis. As upregulation of SA often leads to the downregulation of JA responses (Glazebrook 2005) and likely increased cell death, the manipulation of SsCM1 of plant CM activity is expected to contribute to the pathogenic development of *S. sclerotiorum* and potentially other pathogens with SsCM1-like effectors.

DISCUSSION

Shikimate pathway is utilized by bacteria, archaea, fungi, and plants for production of aromatic amino acids and other compounds that are essential for development, immune responses, and other processes (Bochkov et al., 2011; Maeda and Dudareva 2012). Chorismate is the intermediate branch point of the pathway and its partitioning towards various products is mediated by several chorismate metabolizing enzymes including chorismate mutase (CM) which converts chorismate to prephenate. While endogenous CMs are broadly conserved in organisms which utilize the Shikimate pathway, such as bacteria (Calhoun et al., 2001; Sasso et al., 2005) and fungi

(Djamei et al., 2011), secreted CMs are found in certain phytopathogenic fungi and nematodes (Lambert et al., 1999; Jones et al., 2003; Bekal et al., 2003; Djamei et al., 2011; Wang et al., 2018). Moreover, many of the above secreted CM discovered in phytopathogenic organisms are shown to be involved in pathogenicity/virulence (Lambert et al., 1999; Jones et al., 2003; Bekal et al., 2003; Djamei et al., 2011; Wang et al., 2018). The value of secreted CM effectors in pathogenicity is highlighted by their independent acquisition by phytopathogens through a number of distinct evolutionary paths. Both SsCM1-like effectors and the secreted CMs found in phytopathogenic nematodes are the likely result of HGT from bacteria, whereas the secreted CM from the biotrophic pathogen *U. maydis*, Cmu1, is presumably the result of a gene duplication from an endogenous CM **(Fig. 1A-B)** (Djamei et al., 2011; Wang et al., 2018). While the presence of secreted CM effectors has traditionally been considered a hallmark of biotrophic interactions, phylogenetic analysis places SsCM1 homologs within the genomes of a number of biotrophic, necrotrophic, and even saprotrophic fungi, suggesting a more complex interaction with the host than previously thought (**Fig. 1B**). The chloroplastidic localization of SsCM1 and other secreted CMs from related Leotiomycetes is a relatively unique feature of these proteins when compared to other characterized CM effectors and the CM homologs spread throughout fungi (**Table 1**) (Lambert et al., 1999; Djamei et al., 2011; Wang et al., 2018). Whether this localization contributes to the diverging functionality of SsCM1 compared to other CMs, such as those found in smut fungi, will still need to be explored.

Despites of the sequence variance and the presence of cTP, based on their predicted function of catalyzing chorismate, it's reasonable that most CM-encoding effectors contribute to virulence by targeting chorismate branch of the shikimate pathway, in particular, the production of SA. SA is well known for its pivotal role in plant immunity. In general, SA mediates defense

response against pathogens with biotrophic lifestyles and has antagonistic relationship with JA which involves in defense against necrotrophic pathogens (Glazebrook 2005). Decades of research has been dedicated to investigating how plant biosynthesize SA. Until recently, researchers revealed that in higher plants, pathogen-induced SA is derived from chorismate through biosynthesis routes that occur in both plastids and cytoplasm (Rekhter et al., 2019). In detail, upon pathogen attack, plastidic chorismate is converted to isochorismate by isochorismate synthase and further released into cytosol through chloroplast envelope localized transporter (Serrano et al., 2013; Rekhter et al., 2019). Instead of employing IPL like SA-producing bacteria (Serino et al., 1995; Mercado-Blanco et al., 2001), plants like Arabidopsis convert cytosolic isochorismate into SA through a two-step biosynthesis pathway which involves isochorismoyl-glutamate synthase and isochorismoyl-glutamate A pyruvoyl-glutamate lyase (Torrens-Spence et al., 2019). Many of the components in SA biosynthesis pathway can be potential targets of pathogen virulence factors. For example, isochorismate was shown to be targeted by effectors from oomycete pathogen *Phytophthora sojae* and fungus *Verticilium dahliae*, which are encoded by Pslsc1 and Vdlsc1, respectively. Specifically, these two effectors function as isochorismatase to hydrolyze isochorismate into 2,3-dihyro-2,3-dihydrobenzoate, therefore reducing isochorismate-deriving SA and its related defense (Liu et al., 2014). As CM domain in SsCM1 is predicted to encode an isochorismate pyruvate lyase (IPL) (**Fig 1A**), SsCM1 is also likely to catalyze isochorismate as substrate. Although no *bona fide* IPLs were identified in plants (Zhou et al., 2018), expressing bacterial IPLs has been shown to successfully upregulate SA in plants (Verberne et al., 2000; Torrens-Spence et al., 2019). Similar to phenotypes seen for plant expressing bacterial IPLs, overexpressing SsCM1 in *N. benthamiana* also significantly triggers SA upregulation (**Fig 5A**). The modest induction of SA (**Fig 5A**) is probably due to the singular expression of IPL in this

study, as other groups who achieved drastic increase of SA have co-expressed IPL together with isochorismate synthesis-related genes to enable accumulation of both upstream substrate and the enzyme for an efficient *in-planta* IPL reaction (Verberne et al., 2000; Torrens-Spence et al., 2019). In reality, *S. sclerotiorum* secretes a cocktail of virulence factors that work synergistically to achieve a metabolic profiling that's beneficial to the fungus, therefore, it's very likely that other effectors also possess isochorismate synthesis-related functions to amplify SA induction driven by IPL activity of SsCM1. To verify if SsCM1 is indeed an IPL, we expressed SsCM1 in *E. coli* but failed to detect SA production that's resulted from IPL activity (**Fig 5B**). Nevertheless, SsCM1 is still a potential IPL *in planta* as *E. coli* is likely not an optimal environment for expressing fungus-derived IPL, though the codons encoding SsCM1 was optimized for bacterial expression (data not shown). *In vitro* enzymatic assay using recombinant SsCM1 and isochorismate substrate could potentially solve the problem, however, isochorismate is unstable and its synthesis requires recombinant chorismate and the catalyzing enzyme.

Alternatively, SsCM1 can target chorismate through its predicted CM domain. SsCM1 was verified to be a functional CM, and is able to complement the *S. cerevisiae* aro7 mutant and restore yeast growth on media lacking Phe and Tyr (**Fig 6A**). However, the CM activity of SsCM1 is much weaker compared to plant CMs (**Fig 6B**) or even Cmu1 (**Suppl. Fig S1**). In addition, as shown by confocal and electron microscopy , SsCM1 translocates to chloroplasts during infection (**Fig 4**), whereas Cmu1 acts in plant cytoplasm (Djamei et al., 2011). Considering the homology in bacterial origin and all above aspects, SsCM1 is very likely a CM that is distinct from *U. maydis* Cmul. As confirmed by Y2H (**Fig 6C**), SsCM1 interacts with plant CMs in the chloroplast. However, instead of acting in conjunction with plant CMs like Cmu1 (Djamei et al., 2011), SsCM1 potentially interferes with plant CMs by its weak activity (**Fig 6B**), thus slowing down the

conversion of chorismate to prephenate and increasing the availability of chorismate for SA biosynthesis. This can also have additional consequences on the biosynthesis of secondary metabolites derived from aromatic amino acids. These include antimicrobial compounds with documented roles in plant defenses, such as phenylpropanoids (Ranjan et al., 2019; Singh et al, 2019). Indeed, our recent work suggests that the upregulation of phenylpropanoid intermediates is strongly correlated with resistance against *S. sclerotiorum* (Ranjan et al., 2019). Taken together, we propose that SsCM1-like effectors hijack the shikimate pathway of the plant to increase SA levels within the host, and decrease the biosynthesis of defense related antimicrobial compounds, thus leading to the establishment of disease.

MATERIALS AND METHODS

Plant and fungal materials

S. sclerotiorum wild-type (1980) and transformants, were maintained at room temperature on potato dextrose agar (PDA). *N. benthamiana, S. lycopersicum L. cv Moneymaker, and A. thaliana* Col-0 wild-type plants were grown and maintained under standard growth chamber conditions (Williams et al., 2011).

Pathogenicity Assays

Pathogenicity assays were conducted as previously described (Williams et al., 2011). Newly emerging leaves of tomato, *Arabidopsis* and tobacco were excised and inoculated with 5 mm PDA plugs containing actively growing *S. sclerotiorum* wild-type isolate (1980) and Δ*sscm1* mutants. Leaves were photographed at 24 and 48 hpi (hours-post-inoculation) to evaluate symptom development.

Bioinformatic analyses

Signal peptide prediction was performed using the program of SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/). Chloroplast targeting elements were identified using the Chlorop 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/). Phylogenetic tree of fungal CM domains was generated using maximum parsimony method. Tertiary structure for the chorismate mutase domain, were modeled using Phyre2 program (http://www.sbg.bio.ic.ac.uk/phyre2/).

***Sscm1* gene replacement**

The Δ*sscm1* mutant was generated using a split-marker-deletion approach (Catlett et al., 2003). To construct the *SsCM*1 gene-replacement vector, a 0.89-kb fragment upstream from *SsCM1* was amplified with primers SsCM1 AF and SsCM1 AR and cloned into the *Kpn*I and *Xho*I sites on pCX62 as pWL20-1. A 0.84-kb fragment downstream from *scm1* was amplified with primers SsCM1 BF and SCM1 BR and cloned between the *Bam*HI and *Sac*I sites on pCX62 as pWL21-1. PCR primer pairs (SCM1 AF + HY split, YG split + SCM1 BR) were used to amplify fragments from pWL20-1 and pWL21-1, respectively. PCR products were transformed into protoplasts of the wild-type strain 1980 as described (Liu et al., 2011). Hygromycin-resistant transformants were screened by quantitative PCR with primers SCM1 UA and HY split, and YG split and SCM1 DB. The Δ*sscm1* mutant was purified after several rounds hyphal tipping and further confirmed by RT-PCR with the *SsCM1* gene ORF primers (Scm1NF and Scm1NR). For complementation experiments, the full-length *Sscm1*gene with its native promoter was cloned into pFY1 as described (Bruno and Mitchell, 2004). The resulting construct (pWL22-2) was transferred into Δ*sscm1*. Vegetative growth, sclerotia formation and oxalic acid production were examined in the null mutants.

SsCM1 secretion assay

S. sclerotiorum strain SsCM1-HA was generated by transformation with plasmid pWL73 (SsCM1-HA driven by *Aspergillus nidulans* trpC promoter) into the wild-type fungal strain 1980. To analyze SsCM1-HA secretion, protein extracts of mycelia and liquid culture supernatants after precipitation with trichloroacetic acid, were used for western blot analysis with mouse-anti HA (Roche) and mouse anti-α-actin antibodies (Sigma).

To visualize SsCM1 secretion, the transformed *S. sclerotiorum* strains harboring *SsCM1* SP-cTP-GFP driven by its native promoter or Control GFP driven by trpC promoter were generated and inoculated on 4-week-old *N. benthaniama* leaves. Fluorescence was examined and photographed under the Olympus IX81 inverted fluorescence microscopy with the GFP filter sets (excitation at 460–490 nm, emission at 510 nm) as described (Williams et al., 2011; Kabbage et al., 2013).

Quantitative real-time PCR

RNA was extracted from mycelia grown in PDB culture as well as from infected Arabidopsis plants at the indicated time points with the RNeasy Plant Mini Kit (Qiagen), treated with DNase (Qiagen) and subsequently used for cDNA synthesis. Quantitative real-time PCR was conducted as described (Kabbage et al., 2013). All reactions were performed with a minimum of three biological replicates. Relative *SsCM1* expression levels were calculated in relation to the values obtained for the constitutively expressed actin gene of *S. sclerotiorum*.

SsCM1 chloroplast assays

Immunogold labelling

To determine the location of SsCM1, *N. benthamiana* leaves were inoculated with fresh *S. sclerotinia* Scm1::HA strain. The immunogold labelling assay for SsCM1-HA was performed as described in Kabbage et al. (2013).

Organelle isolation

Intact chloroplasts were isolated from Arabidopsis leaves infected by transformed *Sclerotinia* strain Scm1::HA through a Percoll gradient (Goyal et al., 1988). Four-week-old Arabidopsis leaves were inoculated with the *Sclerotinia* strain Scm1-HA. Intact Arabidopsis chloroplasts were isolated from the leaves 16 hours after inoculation. All isolation steps were performed at 4°C and, where applicable, on wet ice. Typically, 50 leaf discs from infected leaves were sampled and ground in organelle isolation buffer (400 mM sorbitol, 20 mM MES with pH 5.2, 0.5 mM $CaCl_2$), and then digested with 4% cellulase `Onozuka' R-10 (Yakult, Tokyo, Japan), 0.08% Macerozyme R-10 (Yakult) in isolation buffer for 4 hours at room temperature (sampled as "Total"). The released organelles were filtered through 200 mm nylon mesh, collected by centrifugation at 2600 *g* for 2 minutes and washed once with isolation buffer (Sampled as "Supernatant" and "Pellet"). The pellet was resuspended in 300 mM sorbitol, 20 mM Tricine, pH 8.4, 5 mM EDTA, 5 mM EGTA, 10 mM NaHCO3, 0.1% BSA and forced twice through 20 and 10 mm nylon mesh, and then immediately purified on a 50% Percoll continuous gradient and centrifuged at 250 x g. The fractions of the gradient and pellet were used for SsCM1-HA analysis tracking with the relevant antibodies (anti-cytochrome C, anti-rubisco activase and anti-HA).

To visualize SP- or SP/cTP- null SsCM1, SP- or SP/cTP- truncated SsCM1 were fused to eGFP and then transiently expressed in *Arabidopsis* protoplasts using methods as described elsewhere (Negrutiu et al., 1987). GFP fluorescence and chloroplast autofluorescence were examined and

photographed with the Olympus IX81 inverted fluorescence microscopy with GFP and RFP filter sets as described above (Williams et al., 2011).

Yeast assays

SsCM1 interacting proteins were assayed *in vitro* by using the yeast-two hybrid system from Clontech Co. (Clontech, CA). Arabidopsis chorismate mutase genes (*AtCM1*and *AtCM2*) were amplified from an Arabidopsis cDNA library and introduced into the yeast prey vector pGADT7. Fungal chorismate mutase gene *SsCM*1 was cloned into both the prey (pGADT7) and bait (pGBKT7) vectors. The prey and bait constructs were confirmed by sequencing and co-transformed into yeast strain HF7C by Alkali-Cation yeast transformation (MP Biomedicals, OH). Trp^+ and Leu^+ transformants were identified and assayed for growth on SD-Trp-Leu-His medium. LacZ reporter gene expression and analysis were provided by Clontech Co. (CA). Empty vector plasmids encoding only the GAL4-activation or -binding domain, respectively, served as negative controls.

Yeast wild-type strain Y054679 and the *aro7* mutant (kindly provided by Dr. Regine Kahmann) were used in a complementation assay as described (Djamei et al., 2011). The *aro7* mutant strain was transformed with the corresponding pYES260 derivative constructs using the alkali-cation yeast transformation kit as mentioned above and tested for growth on medium lacking phenylalanine and tryptophan as described (Djamei et al., 2011).

Enzyme Assays for Chorismate Mutase Activity

In vitro enzyme activity assay for CM activity was used to determine the ability of a given CM to convert chorismate into prephenate was performed (Westfall et al., 2014). SsCM1, Cmu1,

and *Arabidopsis* AtCM1 to AtCM3 were cloned into the pET expression system (http://www.novagen.com) and expressed in *E. coli* as described previously (Westfall et al., 2014).

Quantification of Salicylic Acid in *N. benthamiana*

To determine the effect of SsCM1 on plant SA level, SsCM1 lacking SP, Cmu1 without SP, and control eGFP were inserted into *A. tumefaciens* vector pGWB402Ω driven by CMV35S promoter and infiltrated into 4- to 5-week-old *N. benthamiana* at the end of day cycle indicated on growth chamber. As suggested by Goodspeed et al., 2012, SA accumulates at highest level at night in Arabidopsis, therefore, leaf samples were collected at night for better representation of SA quantity. Two days after infiltration, approximately 200 mg of infiltrated leaf tissue per sample and 6 replicates per treatment were collected in liquid nitrogen during the middle of the night cycle. Leaves were homogenized into fine powder by homogenizer at 1,500 rpm for 15 secs and SA from the leaf powder were extracted by homogenizing with 600 µl of 65% methanol at 1,500 rpm for 10 mins (MiniG, SPEX Sample Prep). SA from samples were then dried by speedvac and resuspended in 150 µl 65% methanol. One hundred µl of suspension per sample was analyzed by HPLC using program modified from Rozhon et al., 2005 and Bonawitz et al., 2014.

Quantification of Salicylic Acid in *E. coli*

To examine the IPL activity, tested proteins were cloned into pRham N-His SUMO vector and expressed in *E. coli* 10G cells according to the manufacturer's protocol (Lucigen). Mature SsCM1 without cTP (Δ1-66 AA, based on the ChloroP prediction) or SsCM1 without shorter cTP (Δ1-53 AA), predicted by alignment with other prokaryotic homologs from NCBI multiple alignment excluding eukaryotes (**Suppl. Fig S2**), SP-null Cmu1 (Δ1-21 AA), or PchB from *P. aeruginosa* were synthesized with codon optimized (IDT) and expressed in *E. coli* 10G. SA

production in bacterial cells were extracted as described by Mercado-Blanco et al., 2001 with slight modifications. To obtain SA from *E. coli,* 1 ml of bacterial culture were acidified to pH 2 and SA was extracted with chloroform (v/v of culture supernatant:chloroform, 1:0.5) upon vigorous shaking. Chloroform layer at lower phase was then generated by centrifugation and collected into new tube. Chloroform extraction was repeated three times and SA in the lower phase were dried by speedvac. Pellets containing SA were then resuspended with 100 µl of 65% methanol (v/v) and SA quantity was analyzed by HPLC as described in methods for plant SA quantification.

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FIGURES AND TABLES

Table 1. Frequency of Signal Peptide (SP) /Chloroplast Targeting Peptide (cTP) in SsCM1 homologs within Ascomycota.

	Eurotiomycete	Leotiomycete	Dothideomycete
Total Species No.	22	32	1
Frequency of SP	86.4%	90.6%	100.0%
Frequency of cTP	9.1%	62.5%	100.0%
Frequency of cTP (no SP)	10.5%	69.0%	100.0%

Figure 1. Domain architecture and phylogeny of select chorismate mutases (CMs).

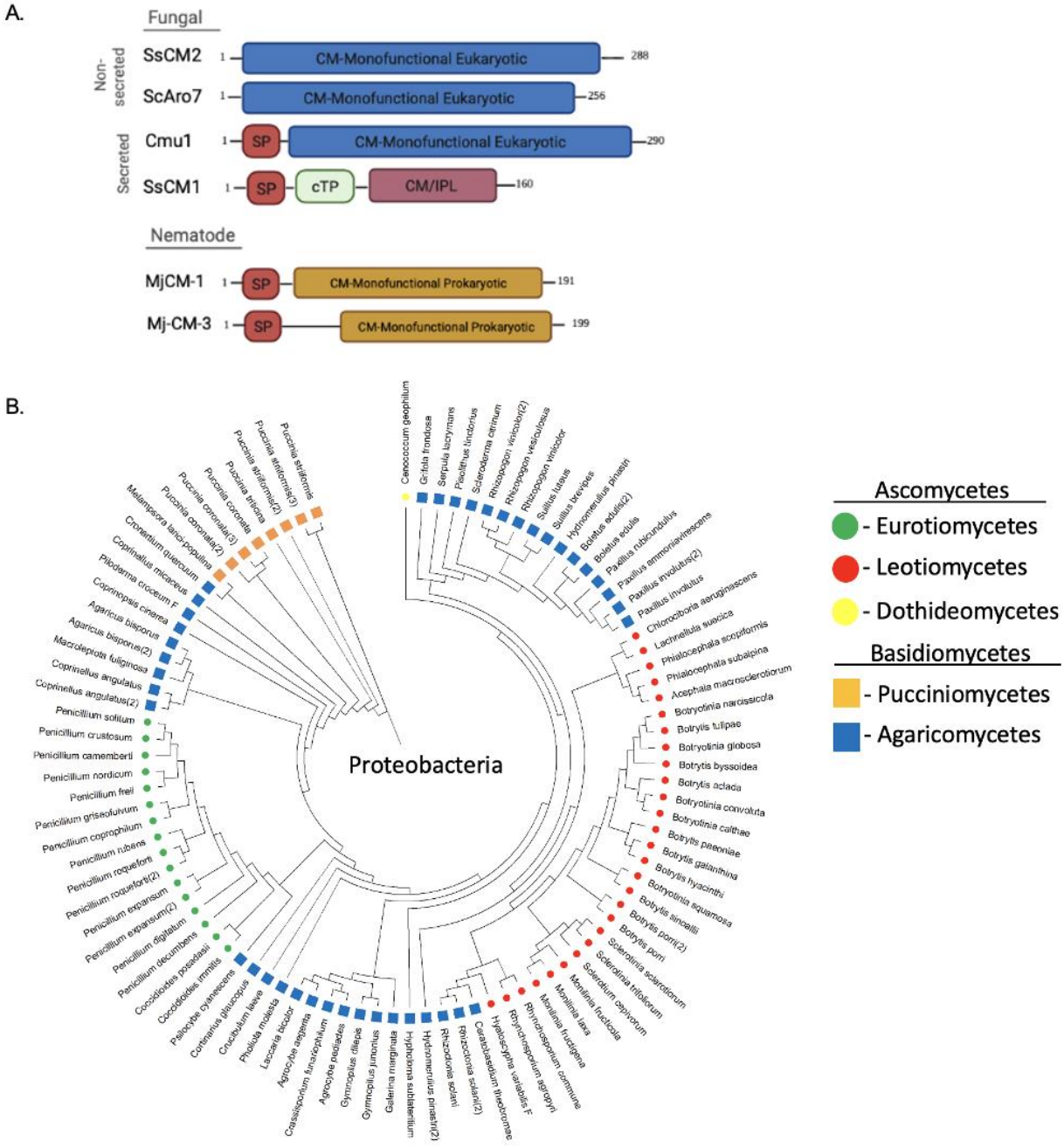

Figure 1. Domain architecture and phylogeny of select chorismate mutases (CMs). (A) Domain architecture of four fungal and two nematode-derived chorismate mutases, including the putative *Sclerotinia sclerotiorum* secreted CM (SsCM1), *Ustilago maydis* secreted CM (Cmu1), *Meloidogyne javanica* secreted CM (MjCM-1) and *M. incognita* CM (Mi-CM-3), non-secreted

SsCM2 from *S. sclerotiorum*, and non-secreted ScARO7 from *Saccharomyces cerevisiae*. SP – Signal Peptide, CM – Monofunctional eukaryotic, CM - Monofunctional prokaryotic, IPL - Isochorismate pyruvate lyase, cTP - Chloroplast targeting peptide. Numbers represent amino acid length. (B) Maximum likelihood phylogenic tree of proteins showing homology to SsCM1. Tree was rooted in Proteobacteria and bacterial sequences were collapsed.

Figure 2. Deletion of SsCM1 didn't affect fungal growth and development.

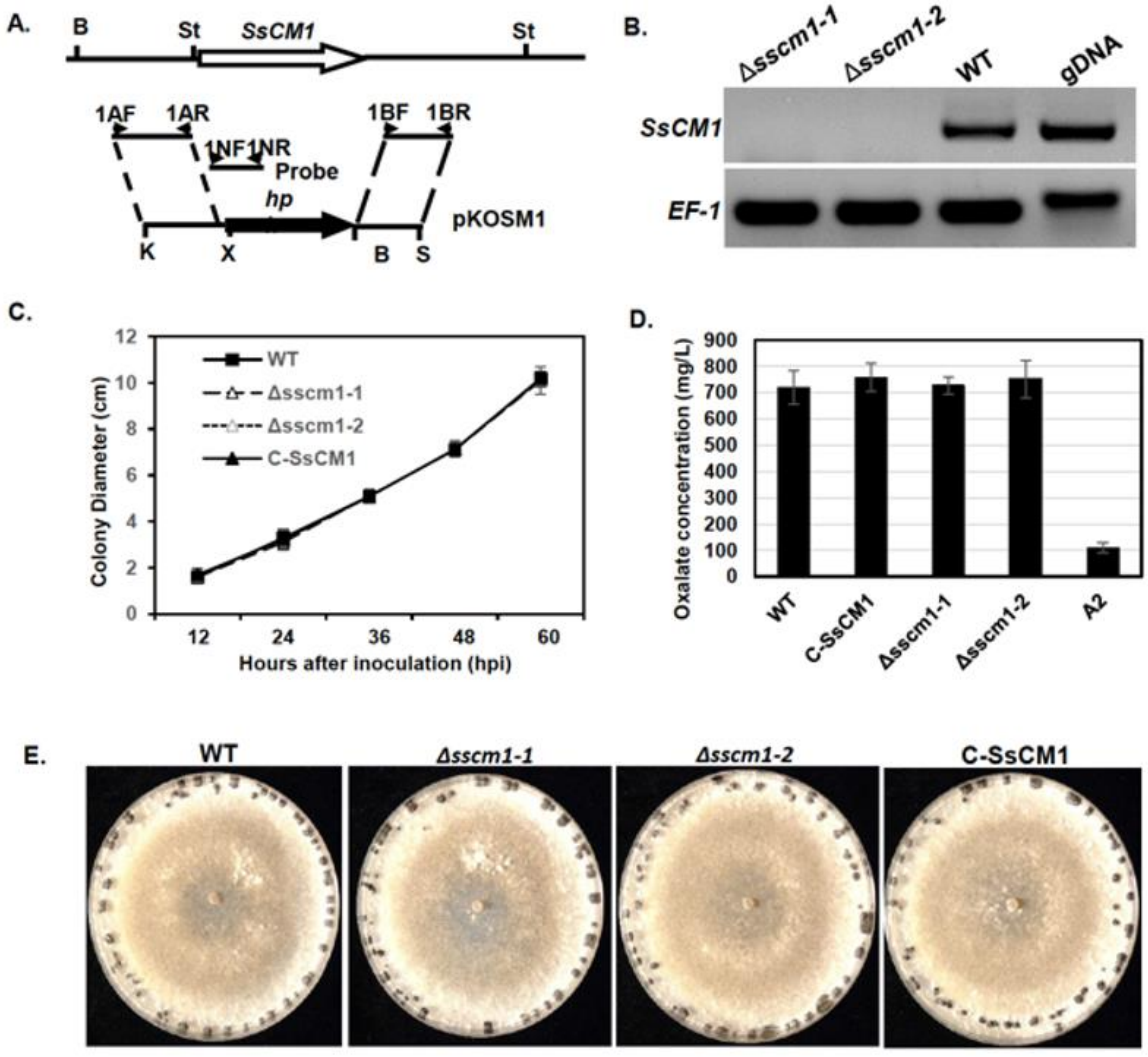

Figure 2. Deletion of SsCM1 didn't affect fungal growth and development. *SsCM1* was replaced with the hygromycin phosphotransferase (hp) through a split-marker-deletion approach (A). *SsCM*1 gene-replacement vector (pKOSM1) contains a 0.89-kb fragment upstream from *SsCM1* that was amplified with primers SsCM1 AF and SsCM1 AR in between *Kpn*I (K) and *Xho*I (X) sites on pWL20-1 and a 0.84-kb fragment downstream from *scm1* that was amplified with primers SsCM1 BF and SCM1 BR in between the *Bam*HI (B) and *Sac*Isites (S) on pWL21-1. Deletion of *SsCM1* in the *Δsscm1* mutants is confirmed by semi-quantitative reverse transcript

PCR with the *SsCM1* gene ORF primers (Scm1NF and Scm1NR) with *EF-1* served as the internal control (B). Among the tested *S. sclerotiorum* strains (*SsCM1* null mutants: *Δsscm1-1* and *Δsscm1-2; C-SsCM1:Δsscm1-1 complemental strain; S. sclerotiorum* wild-type), there were no observable differences in fungal vegetative growth (C), oxalic acid (OA) production (D) and Sclerotia formation (E). A2 is a non-pathogenic *S. sclerotiorum* OA defective mutant. All treatments represent a minimum of 3 replicates.

Figure 3. Inactivation of *SsCM1* results in reduced fungal virulence.

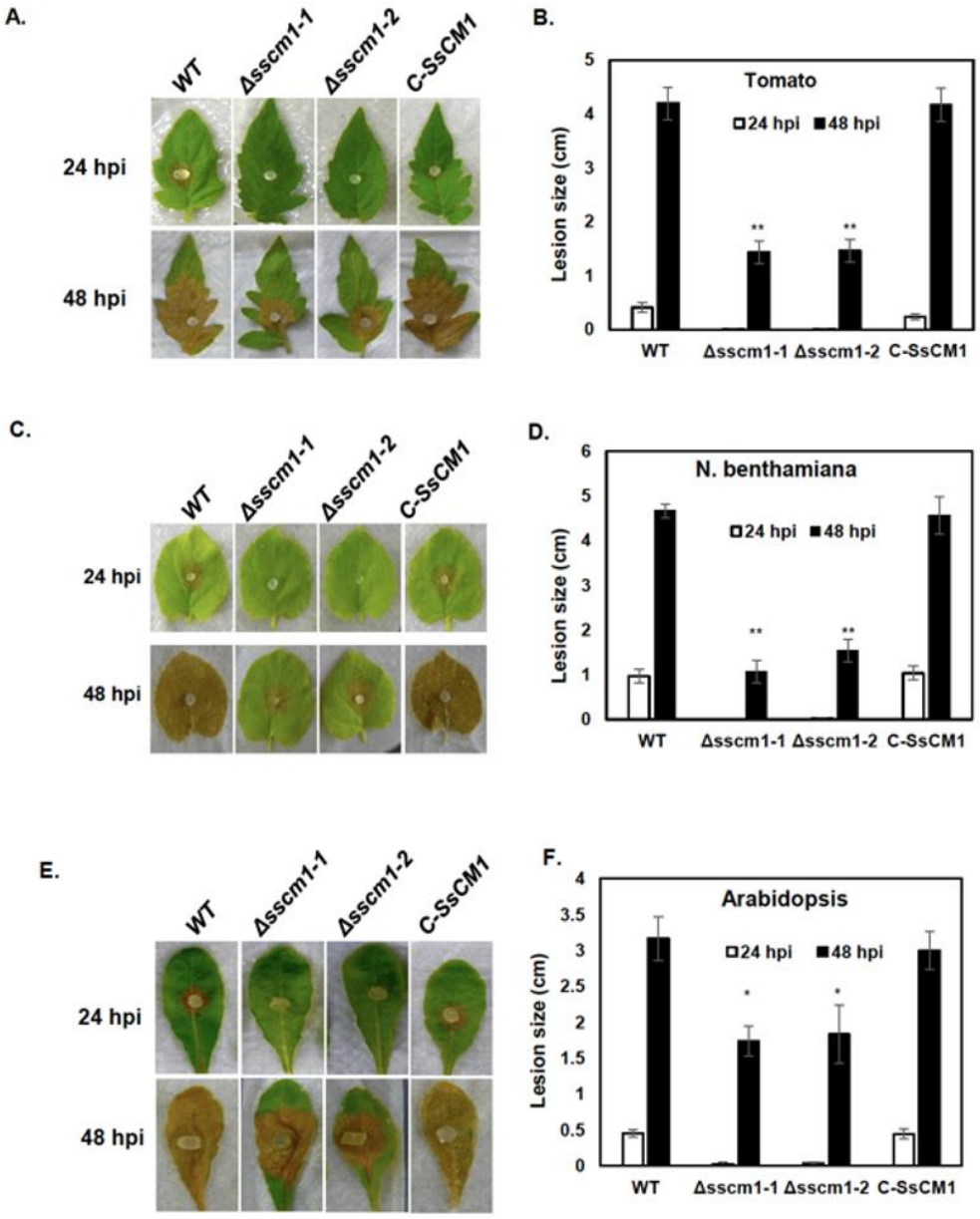

Figure 3. Inactivation of *SsCM1* results in reduced fungal virulence. Lesions on the detached leaves of tomato (A, B), tobacco (C, D) and Arabidopsis (E, F) following inoculation of the following *S. sclerotiorum* strains (*SsCM1* null mutants: *Δsscm1-1* and *Δsscm1-2; C-SsCM1:Δsscm1-1 complemental strain; S. sclerotiorum* wild-type) were photographed 24 and 48

hours post inoculation (hpi) and analyzed with a minimum of 3 replicates (n>10; *: *P* value <0.05 level; **: *P* value <0.01 level).

Figure 4. SsCM1 is secreted and localized to chloroplasts during infection.

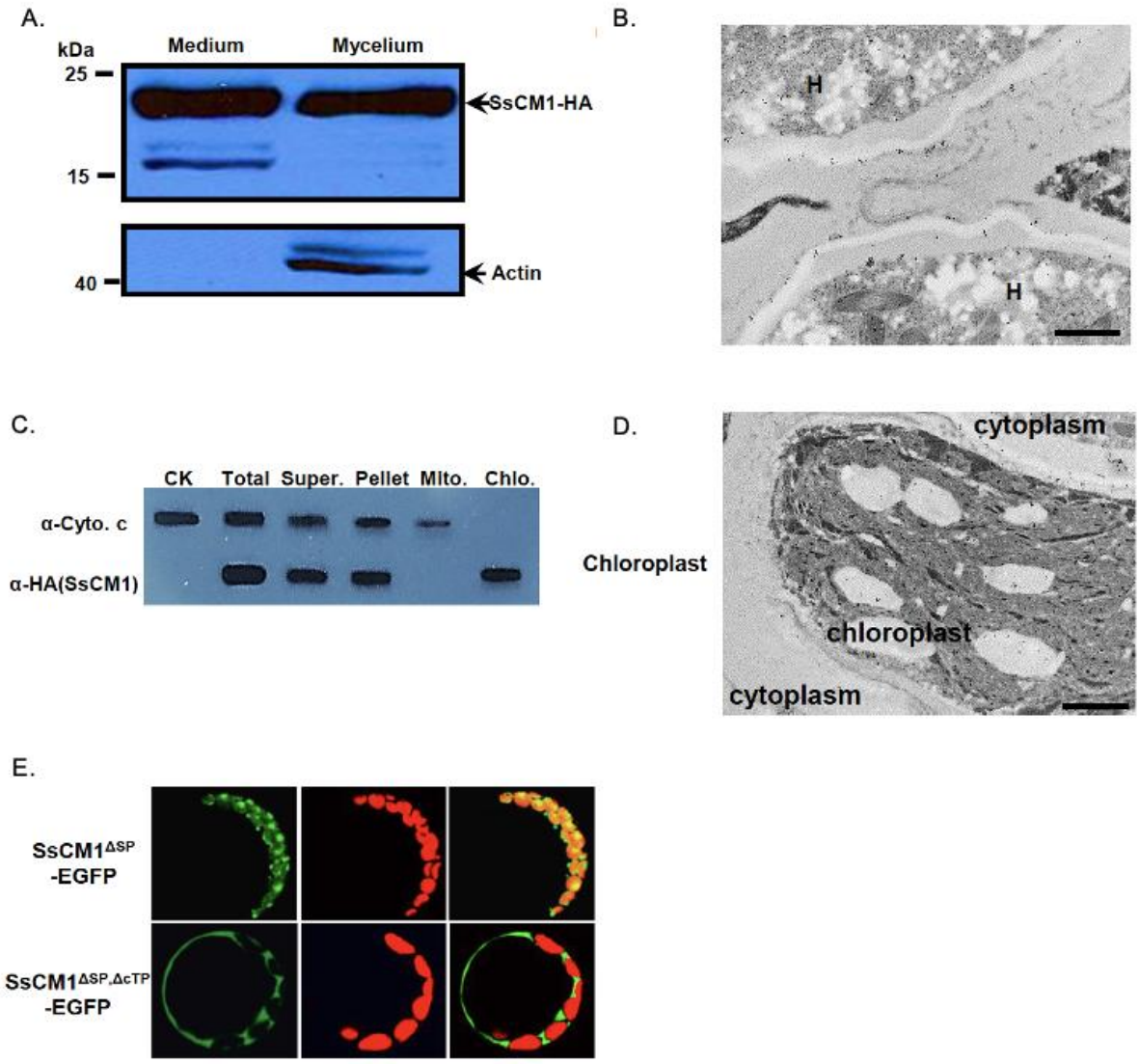

Figure 4. SsCM1 is secreted and localized to chloroplasts during infection. Crude proteins were extracted from cultural medium (for secreted proteins) and fungal mycelium (for non-secreted proteins) and were analyzed using Western blotting (A) and immunogold labeling (B). HA-tagged SsCM1 was detected not only in the medium but also in fungal hyphae; while non-secreted actin control was only present in fungal hyphae, indicating SsCM1 can be secreted from

the fungus. (C) Chloroplast and mitochondria fractions from Arabidopsis leaves infected by *Sclerotinia* expressing *HA-SsCM*1 were isolated and analyzed by western blotting. Total proteins from infected tissues which include proteins from all components from the cells; supernatant (Super.) represents most of cytoplasmic proteins; pellet includes proteins from organelles such as chloroplast and mitochondria; Mito is for most proteins from mitochondrial organelle and Chlo for proteins from intact Chloroplast. (D) Subcellular localization of HA-tagged SsCM1 during infection were also visualized by Immune-gold assay. SsCM1 signals are present both inside and outside of fungal hyphae (H); infected tissue shows HA-tagged SsCM1 presents in Chloroplast (Ch) during infection, as well as cytoplasm. (E) Fluorescence microscopy shows SsCM1 with cTP element transiently expressed in Arabidopsis protoplasts. Co-localization of fluorescence on SsCM1$^{\Delta SP1-20}$-GFP is evident with the host chloroplast (red autofluorescence); while without cTP, SsCM1 (SsCM1$^{\Delta SP1-20,\ \Delta cTP21-66}$-GFP) does not translocate to the chloroplast.

Figure 5. Overexpression of SsCM1 upregulates SA in *Nicotiana benthamiana*, possibly not through IPL activity.

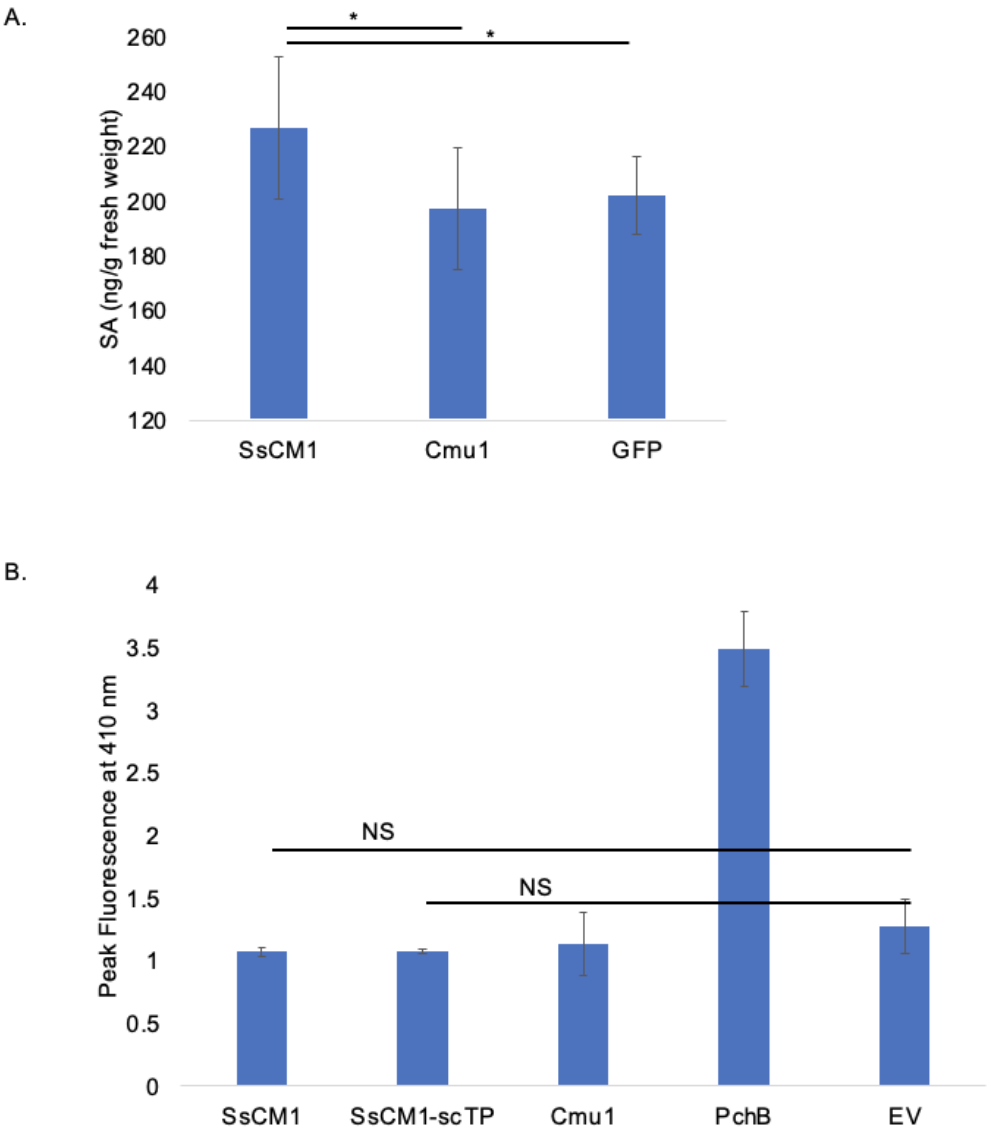

Figure 5. Overexpression of SsCM1 upregulates SA in *Nicotiana benthamiana*, possibly not through IPL activity. (A) Relative abundance of SA in *N. benthamiana* which transiently expresses SsCM1, Cmu1, or GFP through *Agrobacterium*-mediated transformation. Constructs pGWB402Ω containing SsCM1, Cmu1, or eGFP were agro-infiltrated into *N. benthamiana*. Three days after inoculation, SA was extracted from leaves and analyzed through HPLC. Quantities of

SA were determined by fluorescence peak at 410 nm wavelength. (B) Relative quantity of SA in *Escherichia coli* expressing SsCM1, SsCM1-scTP, Cmu1, PchB, and EV. *E. coli* with pET28a carrying SsCM1$^{\Delta SP/cTP1-66}$, SsCM1-scTP $^{\Delta SP/shorter\ cTP1-53}$, Cmu1 $^{\Delta SP1-21}$, PchB, or EV were cultured in LB to reach to OD_{600} of 0.5. Bacterial cultures were added with IPTG and rhamnose with final concentrations of 0.2 mM and 0.1% w/v, respectively, and shaked at 275 rpm, 24°C for overnight to induce expression of proteins. SA was extracted from 1 ml of bacterial culture and analyzed by HPLC. Relative abundance of SA was determined by fluorescence peak at 410 nm wavelength. (*: *P* value <0.05 level; NS: no significance, *P* value >0.01 level)

Figure 6. SsCM1 interacts with plant host chorismate mutases (CMs), therefore attenuating CM activity towards phenylpropanoids pathway.

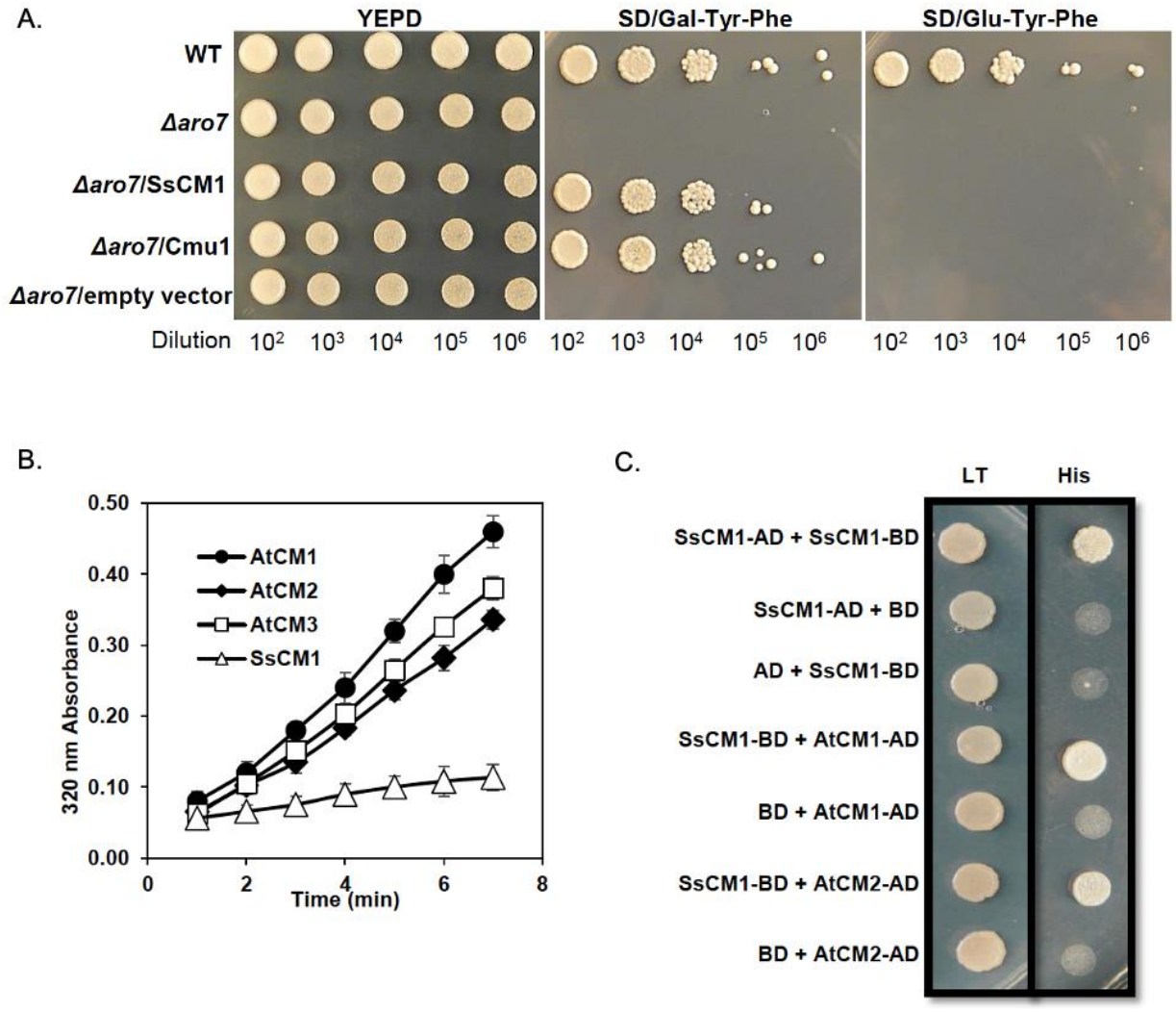

Figure 6. SsCM1 interacts with plant host chorismate mutases (CMs), therefore attenuating CM activity towards phenylpropanoids pathway. Biochemical function of SsCM1 catalytic activity were explored by using bio-function complementary assay (A). The yeast *Δaro7* mutant, *Δaro7* complemented by SsCM1 (*Δaro7*/SsCM1), Cmu1(*Δaro7*/Cmu1), or empty vector (*Δaro7*/empty vector), respectively, were cultured on the aromatic amino acid-null medium (-Tyr and -Phe) with supplementation of glucose (Glu) or galactose (Gal). Biochemical function of *in*

vitro expressed SsCM1 and Arabidopsis CMs were carried out (B). Enzymatic activity of CMs was analyzed by their capability of converting chorismate into prephenate which is then automatically converted into phenylpyruvic acid with high absorbance in 320nm wavelength. Binding assay between SsCM1 and Arabidopsis CM (AtCM1) was performed *in vitro* (C) by Yeast-2-Hybrid assay.

Figure S1. SsCM1 displays weaker chorismate mutase activity in comparison to Cmu1.

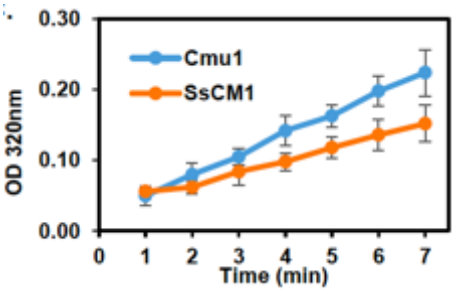

Figure S1: SsCM1 displays weaker chorismate mutase activity in comparison to Cmu1. Biochemical function of *in vitro* expressed SsCM1 and Cmu1 were tested by the catalytic activity of converting chorismate into prephenate and further phenylpyruvic acid with high absorbance in 320nm wavelength.

Figure S2: Alignment of SsCM1 protein sequence with select prokaryotic SsCM1-homologs.

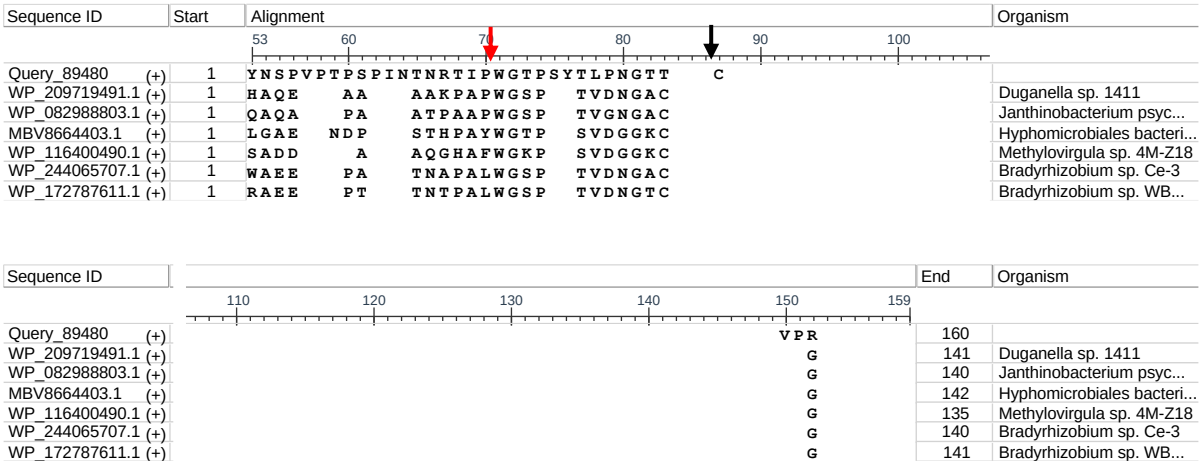

Figure S2: **Alignment of SsCM1 protein sequence with select prokaryotic SsCM1-homologs**. Amino acid sequence of SsCM1 was used to blast against prokaryotic homologs and compared using Constraint-based Multiple Alignment Tool on NCBI site. Alignment of SsCM1 with select homologs is presented. Black arrow implies the cut site for chloroplast targeting peptide (cTP) in SsCM1 according to prediction from ChloroP. Red arrow indicates modified cut site for shorter chloroplast targeting peptide (scTP) in SsCM1 based on the conservation with select prokaryotic SsCM1-homologs.

Figure S3: SDS-PAGE gel for protein expression of SsCM1, SsCM1-scTP, Cmu1, and PchB.

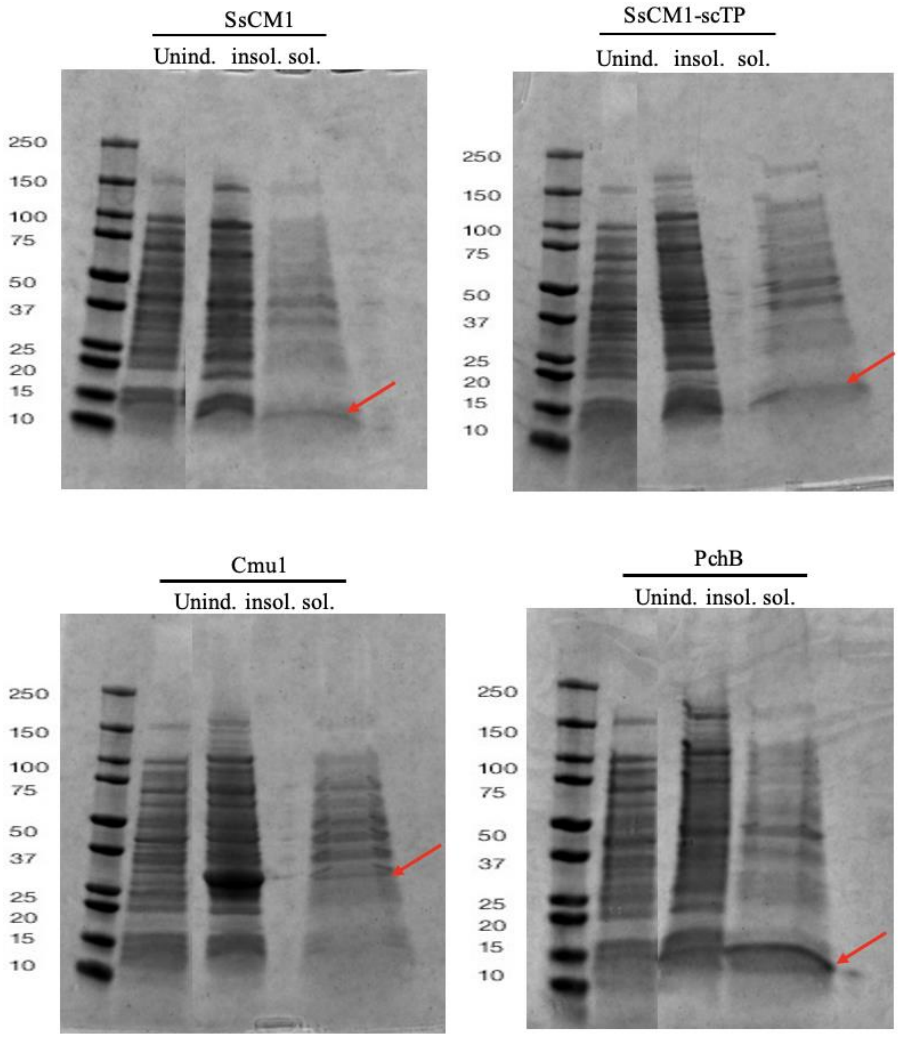

Figure S3: SDS-PAGE gel for protein expression of SsCM1, SsCM1-scTP, Cmu1, and PchB. SsCM1$^{\Delta SP/cTP1-66}$, SsCM1-scTP $^{\Delta SP/shorter\ cTP1-53}$, Cmu1 $^{\Delta SP1-21}$, and PchB were codon-optimized and cloned into *Escherichia coli* pET28a vector. *E. coli* with pET28a carrying SsCM1$^{\Delta SP/cTP1-66}$, SsCM1-scTP $^{\Delta SP/shorter\ cTP1-53}$, Cmu1 $^{\Delta SP1-21}$, PchB, or EV were cultured in LB to reach to OD_{600} of 0.5 and 1 ml of each bacterial culture was collected for uninduced protein control (unind.). Remaining bacterial culture were then added with IPTG and rhamnose with final concentrations

of 0.2 mM and 0.1% w/v, respectively, and shaked at 275 rpm, 24°C for overnight to induce expression of proteins. Insoluble (insol.) and soluble (sol.) fractions of total proteins were then separated for protein analysis on SDS-PAGE gel. Arrows indicate soluble protein with expected sizes.

Table S1. Primers used in this study.

Name	Sequence (5'-3')	Use
attB_SsCM1_F	GGGGACAAGTTTGTACAAAAAAGCAGGC Tatgaaattcaccaccatttccca	Expression in pGWB402Ω
attB2_SsCM1_R	GGGGACCACTTTGTACAAGAAAGCTGGG TCagaagaaatcgcccaaacaca	Expression in pGWB402Ω
attB_Cmu1_F	GGGGACAAGTTTGTACAAAAAAGCAGGC Tatgaagttgagcgtgtccat	Expression in pGWB402Ω
attB_Cmu1_R	GGGGACCACTTTGTACAAGAAAGCTGGG TCggtgcacttgttggcgtg	Expression in pGWB402Ω
attB1_GFP_F	GGGGACAAGTTTGTACAAAAAAGCAGGC TATGGTGAGCAAGGGCGAG	Expression in pGWB402Ω
attB2_GFP_R	GGGGACCACTTTGTACAAGAAAGCTGGG TCCTTGTACAGCTCGTCCATGC	Expression in pGWB402Ω
SsCM1_IF_F	CGCGCGGCAGCCATATGTGCGATTCACT GGATCAAGTC	Expression in pET28a
SsCM1_IF_R	GCTCGAATTCGGATCCTTAGCTGCTTATC GCCCAAACAC	Expression in pET28a
SsCM1 scTP_IF_F	CGCGCGGCAGCCATATGTGCGATAGCCT GGATCAAGTCC	Expression in pET28a
SsCM1 scTP_IF_R	GCTCGAATTCGGATCCTTAGCTCGAAATC GCCCAGACAC	Expression in pET28a

Cmu1_IF_F	CGCGCGGCAGCCATATGGCGGCCGTAAG CGGCAAG	Expression in pET28a
Cmu1_IF_R	GCTCGAATTCGGATCCTTAGGTGCACTTA TTGGCGTGGTC	Expression in pET28a
PchB_IF_F	CGCGCGGCAGCCATATGAAAACTCCCGA AGACTGCACCG	Expression in pET28a
PchB_IF_R	GCTCGAATTCGGATCCTCATGCGGCACCC CGTGTCTGG	Expression in pET28a

CHAPTER 4: Conclusions and Perspectives

S. sclerotiorum, causative agent of Sclerotinia stem rot (SSR), is a cosmopolitan necrotrophic fungal pathogen that causes huge yield loss on many economically important crops (http://www.whitemoldresearch.com). Like other necrotrophs, studies on pathogenicity/virulence factors for *S. sclerotiorum* have been largely focused on cell wall degrading enzymes (CWDE) (Amselem et al., 2011) and the major secreted metabolite oxalic acid (OA) (Williams et al., 2011; Liang et al., 2015). Compared to hundreds of effectors that have been characterized in biotrophic and hemibiotrophic fungal pathogens, the effectors of necrotrophic fungal pathogens are comparatively understudied (Louet et al., 2021). Current knowledge about plant necrotrophic fungal effectors is reviewed in **chapter 1**. Recent genome sequencing and transcriptomics analysis of *S. sclerotiorum* suggest that this pathogen not only encodes a large repertoire of proteinaceous effectors, but utilizes these effectors in a dynamic fashion across a range of pathosystems (Guyon et al., 2014; Seifbarghi et al., 2017; Westrick et al., 2019). Our recent RNA-Seq analysis of *S. sclerotiorum* during infection of soybean identified a list of effectors that are highly upregulated throughout infection time courses (Westrick et al., 2019). To date, only a few *S. sclerotiorum* effectors from this list have been examined for their specific virulence mechanisms in host plants (Zhu et al., 2013; Lyu et al., 2016; Yang et al., 2017; Tang et al., 2020; Wei et al., 2022). Given this lack of information, this project aims to explore additional effector candidates from *S. sclerotiorum* and expand the current knowledge of necrotrophic fungal effector biology. This will facilitate a better understanding of the plant biological and physiological components that are involved in specific necrotroph-induced responses. Additionally, *S. sclerotiorum* effectors characterized from this research work will also serve as targets to develop genetic tools for disease management against *S. sclerotiorum*.

So far, three candidates from the predicted effector list (Westrick et al., 2019) have been investigated for their contributions to SSR disease development. SsCM1, which encodes an *S. sclerotiorum* secreted chorismate mutase (CM), was characterized in **chapter 3**. SsCM1-like effectors represent a novel group of chloroplast-localized and prokaryotes-originated CMs that are widely distributed in fungi with diverse lifestyles. We showed SsCM1 enhances plant susceptibility to necrotrophic infection by upregulating salicylic acid (SA) in the plastids, which runs contrary to the mechanism used by CMs from biotrophic pathogens. Specifically, this SA induction is likely driven by SsCM1 binding to and reducing the efficiency of plant plastidic CMs or by directly converting isochorismate to SA through isochorismate pyruvate lyase (IPL) activity. Moving into the future, additional characterization of the mechanism used by SsCM1 will be key. This includes *in-planta* fluorescent complementation assays to confirm that SsCM1 is capable of binding to plant plastidic CMs within chloroplasts. Additionally, further *in-planta* validation would help to prove or disprove SsCM1's potential role as an IPL. As we were unable to confirm this activity through expression in the prokaryotic model organism *Escherichia coli*, further analysis will include the co-infiltration of SsCM1 with plant isochorismate synthase to validate isochorismate dependency of the observed increase in SA during SsCM1 transient expression in *Nicotiana benthamiana*. From a broader phylogenomic perspective it would additionally be valuable to determine if *ΔSsCM1* could be functionally complemented with orthologs found in the genomes of fungi with both related (*Rhizoctonia solani*) and unrelated (*Puccinia* spp.) lifestyles.

Two other effector candidates, designated as *S. sclerotiorum* putative effector 1 and 2 (SsPE1 and SsPE2) were also verified to be important for fungal virulence(**Appendix 1 and 2**). Knock-outs of SsPE1 or SsPE2 reduced the level of *in-planta* infection (**Appendix 3**). Identification of specific virulence mechanisms for SsPE1 and SsPE2 are currently ongoing.

Ultimately, information from effector studies will be applied to develop approaches for *S. sclerotiorum* control. A potentially valuable tool for genetic resistance, host-induced gene silencing (HIGS), was assessed in **chapter 2** as a potential strategy to target and reduce the production of a *S. sclerotiorum* non-proteinaceous effector, OA, during infection. Specifically, *Ssoah1*, a gene encoding an oxaloacetate acetylhydrolase enzyme that catalyzes the last step in OA biosynthesis, was used to synthesize dsRNA/siRNAs. External *Ssoah1*-dsRNA/siRNAs were shown to be successfully taken up by *S. sclerotiorum* and reduced *Ssoah1* gene expression in *S. sclerotiorum.* HIGS was assessed in soybean using a viral vector which produced *Ssoah1*-targetting double stranded RNA (dsRNA) and small interfering RNA (siRNA). This *Ssoah1*-dsRNA/siRNA actively decreased *Ssoah1* transcript abundancy and reduced *S. sclerotiorum* infection. Taken together, HIGS and exogenous applications of ds/siRNAs targeting *S. sclerotiorum* OA and other effectors are viable strategies for the control of SSR and can be deployed as discovery tools to identify additional *S. sclerotiorum* effectors and virulence factors in future studies. As this study served as a proof of concept for the HIGS-mediated control of SSR, future work will focus on the stacking of virulence targets in a way that will optimize disease control while minimizing off-target effects to the mycobiome. A major advantage of HIGS is that it allows for a targeting of specific transcripts important to pathogen virulence without the unwanted repercussions of broad-scale fungicide application. For these benefits to be realized, however, pathogen-specific virulence targets must be identified, characterized, and ultimately used to generate stable transgenic plants expressing targeted dsRNA/siRNA. Currently our lab is collaborating with the Wisconsin Crop Innovation Center (WCIC) to generate transgenic plants targeting some of these virulence factors and validation of SSR control in these lines will be the goal of future work.

In summary, this project has accomplished the assessment of HIGS as a potential control strategy against *S. sclerotiorum* infection by targeting fungal virulence factors including effectors. Additional *S. sclerotiorum* effectors have been evaluated for their contribution to disease development and are likely valuable targets for HIGS-mediated control. Particularly, one *S. sclerotiorum* effector which encodes a novel secreted CM has been functionally characterized. This newly identified *S. sclerotiorum* effector revealed a conserved virulence strategy used by necrotrophs and elucidated plant biological processes specifically involved in plant-necrotrophic interactions, therefore greatly extending our current understanding of this highly successful pathogenic fungal lifestyle.

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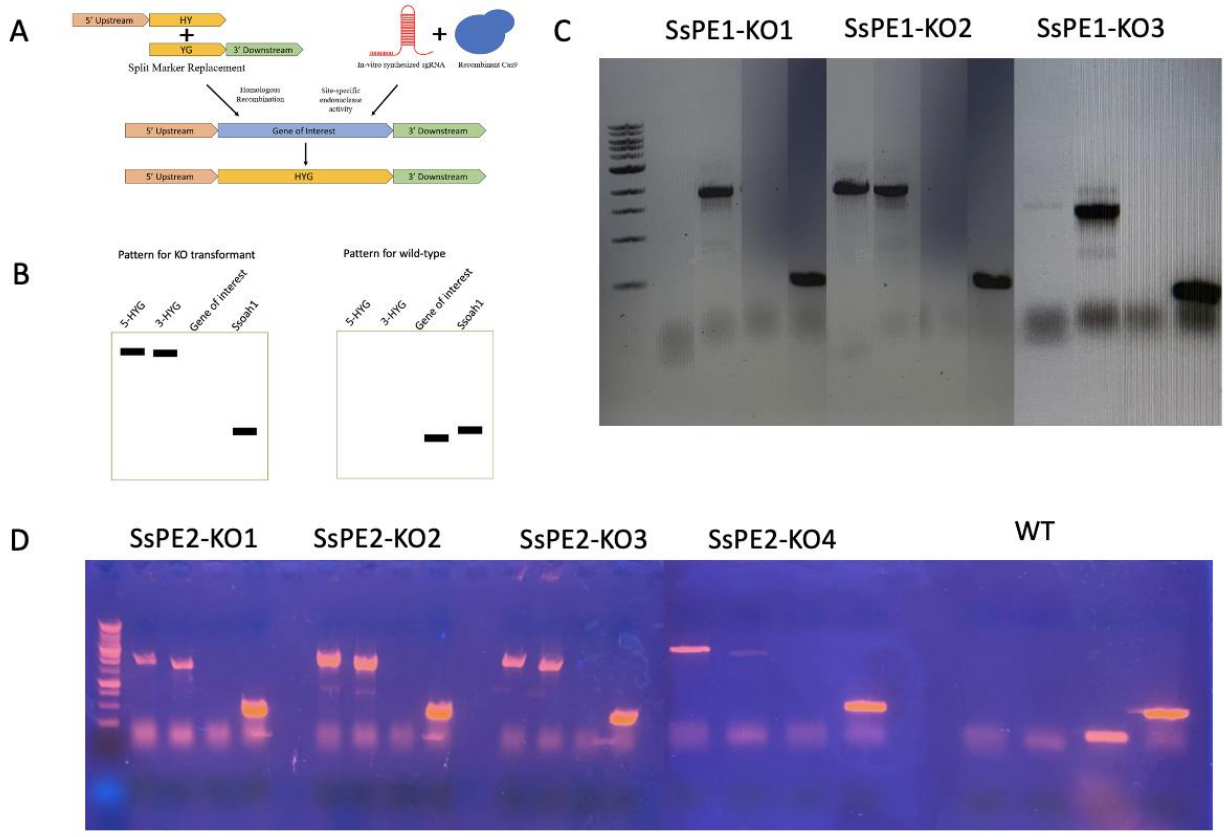
APPENDICES

Appendix 1: Deletion of SsPE1 and SsPE2 in *Sclerotinia sclerotiorum* 1980.



Appendix 1. Deletion of SsPE1 and SsPE2 in *Sclerotinia sclerotiorum* 1980. *SsCM1* was replaced with the hygromycin phosphotransferase (HYG) through approach combining split markers and CRISPR/Cas9 (A). Deletion of *SsPE1* in the *SsPE2* mutants is confirmed by reverse transcript PCR for the absence of gene of interest, the presence of 5' and 3' flanking regions of HYG (5-HYG and 3-HYG, respectively), and the expression of non-targeted *Ssoah1*. Agarose gel patterns for checking true mutants or wild-type are displayed in (B). Screening results for three SsPE1 deletion mutants and four SsPE2 deletion mutants were presented in (C) and (D). Gene ID for SsPE1: Sscle07g055350. Gene ID for SsPE2: Sscle05g045060.

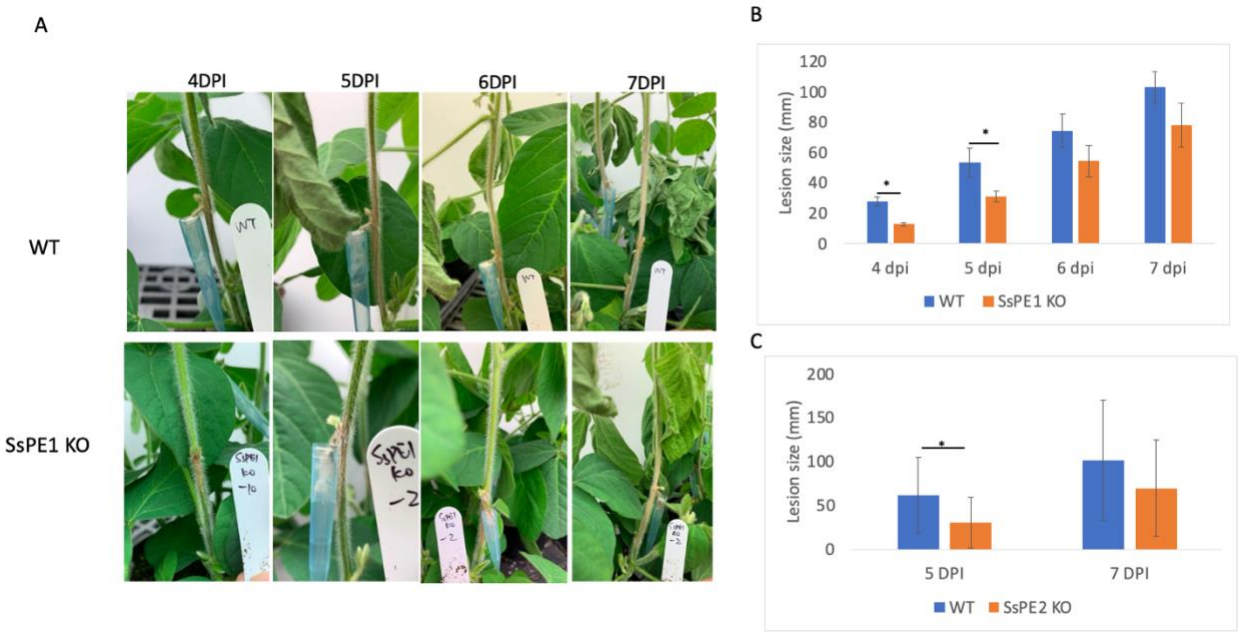
Appendix 2: Table for primers used to generate knock-outs of SsPE1 and SsPE2.

Name	Sequence	Use
SsPE1 5-F	CAAGCGTGGTAACGGGAAAA	Forward primer for amplifying 5' Flanking region of SsPE1
SsPE1 5-R	TCTAAACAAGTGTACCTGTG AATATATGCGTGGTGTGCG	Reverse primer for amplifying 5' Flanking region of SsPE1
SsPE1 3-F	AATCCAATGCGTCTAGAGGG AAGTACTGGCAGGAGGTTGA	Forward primer for amplifying 3' Flanking region of SsPE1
SsPE1 3-R	AGCTTGGTACGACGACACA	Reverse primer for amplifying 3' Flanking region of SsPE1
SsPE1 sgRNA1	CCTCtaatacgactcactataGGAAAA GGTCACATACACCACgtttaagag ctatgc	sgRNA1 for CRISPR targeting SsPE1
SsPE1 sgRNA2	CCTCtaatacgactcactataGGTCAC TTTGGATAGAAGAGAgtttaagag ctatgc	sgRNA2 for CRISPR targeting SsPE1
SsPE1 KO-F	CTTCAGATGTGAGGTCACCT	SsPE1 KO Confirmation F primer and hyg insertion confirmation, paired with hyg Split Marker R for 5' insertion of hyg cassette.
SsPE1 KO-R	TCCATGTCACAGGTTACCCA	SsPE1 KO Confirmation R primer and hyg insertion confirmation,

		paired with hyg Split Marker R for 5’ insertion of hyg cassette.
SsPE1 Detection F	TGCTCAAGCACCGACAACTA	detect any unsuccessful deletion of SsPE2
SsPE1 Detection R	TGCGGTAGTCAATCCACAAG	detect any unsuccessful deletion of SsPE2
SsPE2 5-F	GATTGAGGGAGTTGACTCAC T	Forward primer for amplifying 5’ Flanking region of SsPE2
SsPE2 5-R	TCTAAACAAGTGTACCTGTG TGAATGTGACTGGAGAGCT	Reverse primer for amplifying 5’ Flanking region of SsPE2
SsPE2 3-F	AATCCAATGCGTCTAGAGGG TCGCACAGTTATGCACCAA	Forward primer for amplifying 3’ Flanking region of SsPE2
SsPE2 3-R	TACACCTGGGTATCTTCGCT	Reverse primer for amplifying 3’ Flanking region of SsPE2
SsPE2 sgRNA1	CCTCtaatacgactcactataGGCGCT GGCGATGAAGAATAgtttaagagc tatgc	sgRNA1 for CRISPR targeting SsPE2
SsPE2 sgRNA2	CCTCtaatacgactcactataGGAGTA CGGTCAAGAGACTCAgtttaagag ctatgc	sgRNA2 for CRISPR targeting SsPE2
SsPE2 KO-F	CATGCAAGCTTCAATTCAAG C	SsPE2 KO Confirmation F primer and hyg insertion confirmation,

		paired with hyg Split Marker R for 5’ insertion of hyg cassette.
SsPE2 KO-R	GAGACGAGAATGCAATTCCT	SsPE2 KO Confirmation R primer and hyg insertion confirmation, paired with hyg Split Marker R for 5’ insertion of hyg cassette.
SsPE2 Detection F	TGAGATTGGAAACCTCGATTG	detect any unsuccessful deletion of SsPE2
SsPE2 Detection R	GCATGGAGTACGGTCAAGAGA	detect any unsuccessful deletion of SsPE2

Appendix 3: Pathogen aggressiveness was reduced in SsPE1 knock-out and SsPE2 knock-out mutants infected soybean.



Appendix 3. Pathogen aggressiveness was reduced in SsPE1 knock-out and SsPE2 knock-out mutants infected soybean. Soybean plants Williams 82 were challenged with *S. sclerotiorum* strain 1980, using a cut petiole technique. Lesions were measured 4-7 days post inoculation (DPI). Data are the average lesion size of fifteen to seventeen plants. (A) Lesion development was delayed, and lesions were smaller in plants that are infected by SsPE1 knock-out mutant (SsPE1 KO), while plants that are infected by wild type (WT) often showed girdling lesions starting from 4 DPI. (B) Lesion size was reduced in SsPE1 KO infected plants after four DPI, with a significant reduction in lesion size at 4 DPI and 5 DPI ($P<0.05$). (C) Lesion size was significantly reduced in SsPE2 KO infected plant at 5 DPI ($P<0.05$).