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REVIEW

The complexity of the *Sclerotinia sclerotiorum* pathosystem in soybean: virulence factors, resistance mechanisms, and their exploitation to control Sclerotinia stem rot

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Abstract

Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum*, is a globally important, yield limiting disease of soybean. Progress has been made in our understanding of this pathosystem at the plant level, such as the key role of oxalic acid in disease development and the importance of cell wall-degrading enzymes and other secreted proteins. Unfortunately, advances have largely focused on the fungal side of this interaction and only provide glimpses into the plant mechanisms governing resistance to this pathogen. With the absence of commercially available resistant soybeans, chemical and cultural solutions are being used by farmers to manage SSR with limited success. Additional research is needed to identify *S. sclerotiorum* resistance mechanisms that can be exploited to improve genetic resistance in soybean and decrease reliance on spray regimes. Technologies such as transgenics and RNAi could be exploited to improve the level of resistance to *S. sclerotiorum* in soybean. This review offers insight into the hurdles of managing SSR at the plant level and potential solutions that might be adopted in the future.

Keywords Sclerotinia sclerotiorum · Soybean · Resistance · Oxalic acid · Virulence factors · Control

Abbreviations

BiFC	Bimolecular fluorescence complementation
CAZymes	carbohydrate active enzymes
Chs	chitin synthase
CWDE	cell wall-degrading enzymes
DSI	disease severity index
dsRNA	double-stranded RNA
HIGS	host-induced gene silencing
HR	hypersensitive response
MAS	marker assisted selection
OxDC	oxalate decarboxylase
OxO	oxalate oxidase
OA	oxalic acid
sRNA	small RNA
SSR	Sclerotinia stem rot
PG	endopolygalacturonases
OAH	oxaloacetate acetylhydrolase
PGIP	polygalacturonase-inhibiting protein
QTL	quantitative trait loci
RIL	recombinant inbred lines
RNAi	RNA interference
ROS	reactive oxygen species
siRNA	small interfering RNA
SIGS	spray-induced gene silencing
VIGS	virus-induced gene silencing

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Sclerotinia sclerotiorum lifestyle characterization

Sclerotinia sclerotiorum (lib.) deBary has traditionally been considered a prototypical necrotrophic pathogen. Necrotrophic pathogens are characterized by nutrient acquisition from cells killed by a host of cell wall-degrading enzymes and toxins produced by the pathogen. Necrotrophs are further characterized by their wide host range and lack of complete resistance in their plant hosts. Partial, quantitative resistance of soybean to Sclerotinia stem rot (SSR) has been observed in field trials of commercial varieties (Conley et al. 2017) and through QTL mapping (Bastien et al. 2014; Vuong et al. 2008;

Zhao et al. 2015). However, reliance on this type of quantitative resistance can result in limited control. This is in contrast to biotrophic pathogens which depend on living plant tissue for nutrient acquisition and propagation. Biotrophic fungi release effectors, which are small, secreted proteins that contribute to virulence by manipulating host physiology and suppressing plant basal defenses (Rafiqi et al. 2012). Resistance to biotrophic pathogens is associated with a strong hypersensitive (HR) response which can result in programmed cell death through the process of effector-triggered immunity (Raffaele and Kamoun 2012) after effectors ("avirulence proteins"; Bourras et al. 2016) are recognized (directly or indirectly) by immune receptors ("resistance proteins") (Williams et al. 2016). It has been assumed that the presence of cell wall-degrading enzymes may render effectors less crucial in necrotrophic pathogens, and thus secreted proteins have been an understudied area of *S. sclerotiorum* research.

Recent studies indicate that establishment of SSR is more intricate than brute degradation of host tissues. Due to the complicated nature of *S. sclerotiorum* and host interactions, Kabbage et al. (2015) emphasized the importance of a more holistic view of this pathosystem that considers a hemibiotrophic lifestyle of *S. sclerotiorum*. Contrary to the inelastic classification of *S. sclerotiorum* as strictly nectrotrophic, Kabbage et al. (2015) describe two phases of infection based on microscopic imaging. In the early stages, the fungus grows intracellularly without killing host cells, during which the pathogen is presumed to secrete pathogenicity factors, including oxalic acid (OA), that modulate the host response. Following the brief establishment stage, necrotrophy is initiated, and the fungus procures nutrients from dead host tissue. Transient biotrophic growth persists at the leading edge of lesions while cell death is initiated at the trailing end. This model argues that a more relevant measure of host-pathogen interactions is the damage caused to the host *versus* the pathogen lifestyle and the process leading to damage. This relationship is demonstrated in "damage-response" curves where the outcome of the interaction is the primary consideration.

Important *S. sclerotiorum* virulence factors

Oxalic acid functions to suppress host defenses and induce cell death

In support of the idea of an early biotrophic stage, virulence factors related to the suppression of host defenses have been characterized and are known to be important to *S. sclerotiorum* infection (Fig. 1). The most studied of these virulence factors, in *S. sclerotiorum,* is oxalic acid (OA) and the conjugate base, oxalate. Oxalate is a di-carboxylic acid and alters an array of plant physiological processes that facilitate fungal colonization in the host plant. Oxalic acid is not directly toxic but may have a more refined role as a signaling

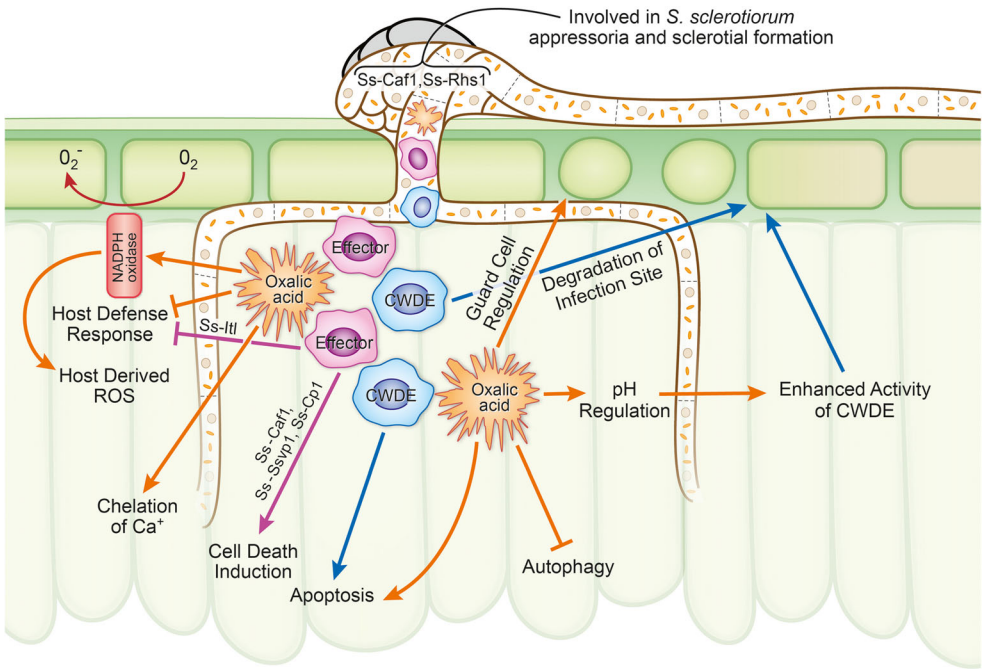

Fig. 1 *S. sclerotiorum* virulence factors serve various regulatory functions. Oxalic acid (OA: orange lines), broadly, dampens host defense responses and suppresses autophagy at the leading edge of infection while enhancing reactive oxygen species (ROS), apoptosis, and the action of cell wall-degrading enzymes (CWDE) at the trailing edge of the lesion. Effector-like secreted proteins (purple lines) similarly function to evade host defense responses and induce cell death. Cell wall-degrading-enzymes (blue lines) are essential for degradation of infection surfaces, and endopolygalacturonases (PGs) are involved in the induction of programmed cell death *via* cytosolic Ca^{2+} signaling

molecule that hijacks the host cell and provides a more conducive environment for fungal growth (Kim et al. 2008; Williams et al. 2011; Kabbage et al. 2013).

Oxalate deficient *S. sclerotiorum* mutants were shown to be poorly pathogenic (Cessna et al. 2000; Dickman and Mitra 1992; Godoy et al. 1990; Kim et al. 2008; Liang et al. 2015a; Williams et al. 2011), and these mutations provoked morphological changes in the fungus in several studies (Rollins 2003; Rollins and Dickman 2001; Liang et al. 2015a). Studies with the OA mutants showed that in the early stages of infection, OA dampens the oxidative burst of the host plant by creating a reductive state, thus suppressing host defenses (Williams et al. 2011). These oxidative bursts are caused by the release of reactive oxygen species (ROS). Reactive oxygen species occur endogenously in cells as byproducts of the reduction of molecular oxygen (O_2), but plants produce higher ROS levels under stressful conditions as a protective strategy (Kotchoni and Gachomo 2006; Mittler et al. 2011; Petrov and Van Breusegem 2012; Noctor et al. 2014; Sewelam et al. 2016; Xia et al. 2015; Wojtaszek 1997). Indeed, oxalate-deficient mutants of *S. sclerotiorum* appear to be actively recognized by the host plant, leading to classical defense responses that include an ROS burst and callose deposition (Williams et al. 2011). Processes involving recognition of pathogens by the host and evasion of such defenses by the pathogen have been largely described in response to biotrophs. Thus, the initial pathogenic phase of this well-established necrotroph, surprisingly, displays features akin to those observed during biotrophic interactions.

In contrast to this early phase, *S. sclerotiorum* was shown to elicit programmed cell death during disease development and concordantly increase ROS levels in the plant at the later stages of the infection process (Kim et al. 2008; Williams et al. 2011). ROS induction is associated with plant defense mechanisms involving HR to biotrophic pathogens. However, this response was shown to be exploited by pathogens with a predominantly necrotrophic lifestyles (Liu et al. 2012; Curtis and Wolpert 2002) including *S. sclerotiorum* (Kim et al. 2008). At later stages, when necrotic tissues are necessary for nutrient acquisition by *S. sclerotiorum*, OA flips the switch on ROS regulation and enhances its production to cause apoptosis-like programmed cell death in the host, as indicated by DNA laddering and TUNEL reactive cells (Kim et al. 2008). Recent work showed that this increase in ROS levels by *S. sclerotiorum* is achieved by hijacking host NADPH oxidases in soybean (Ranjan et al. 2018).

Additional proposed roles for OA have been reported including the acidification of the surrounding environment (Xu et al. 2015). This acidification enhances the activity of cell wall degrading enzymes and enzymes involved in signaling, such as the MAP-kinases associated with sclerotial development (Bateman and Beer 1965; Chen et al. 2004; Rollins and Dickman 2001). OA was also proposed to function as chelating agent, particularly to chelate calcium as evidenced by the presence of calcium oxalate crystals in the infection court (Uloth et al. 2015). Calcium is known to be an important component of cellular signaling and structure. *In toto*, this simple dicarboxylic acid is a key molecule with multifaceted roles in the pathogenic success of *S. sclerotiorum*. Thus, OA constitutes a prime target to achieve resistance to this pathogen, hence the importance of studying the genes and pathways involved in its biogenesis.

Genes related to OA production are known to be important for pathogenicity

Functional genetic studies have identified various genes related to OA synthesis and accumulation in *S. sclerotiorum*. For example, *Ss-Oah1*, an oxaloacetate acetylhydrolase (OAH)-encoding gene has been demonstrated to be essential for the accumulation of OA; *S. sclerotiorum* deletion *Ss-oah1* mutants are unable to accumulate OA in culture or during plant infection (Liang et al. 2015a; Xu et al. 2015). *Ss-Oah1* catalyzes the conversion of oxaloacetate to OA during the final step in OA synthesis, (Dutton and Evans 1996; Munir et al. 2001) and accumulation of *Ss-Oah1* transcripts is dependent on the transcription factor, Ss-Pac1. *Ss-Pac1* was previously observed to be necessary for neutral pH-induced OA accumulation (Rollins 2003), and four Pac C binding sites were identified in the Ss-oah1 promotor region (Liang et al. 2015a). Gene deletion mutants also lacked compound appressoria and were deficient in sclerotia development, however, subsequent T-DNA and targeted gene replacement *Ss-oah1* mutants exhibited normal growth and sclerotial development in a pH lower than 5.5 compared to wildtype *S. sclerotiorum* (Xu et al. 2015), indicating the pH-dependency of *Ss-Oah1*. Upon challenging plant hosts, mutants did cause small lesions on tomato, *Arabidopsis*, and soybean leaves, but the hyphae gradually lost viability (Liang et al. 2015a). The development of even small lesions suggests that other pathogenicity factors, besides OA, contribute to initial infection and primary lesion development. Oxalate decarboxylase genes *Ss-Odc1* and *Ss-Odc2* have also been characterized (Liang et al. 2015b), and mutants of *Ss-Odc2* were, similarly, hindered in their ability to form appressoria and lesions affecting virulence on soybean leaves. Wounding restored lesion development, indicating that the contribution of *Ss-Odc2* to virulence was, likewise, incomplete.

Secreted proteins function to dampen the host immune response

Despite the importance of OA as a major virulence factor in *S. sclerotiorum*, recent studies have singled out other virulence components. While necrotrophic pathogens are thought

to have fewer effectors due to an ephemeral interaction with their host plants compared to biotrophic pathogens, exploratory research has identified several secretory proteins with effector-like activity. In agreement with the early biotrophic trope of *S. sclerotiorum*'s lifestyle, the potential effectors identified may assist *S. sclerotiorum* in evading host detection and condition the plant for the impending necrotrophic stage.

As observed in other pathosystems, *S. sclerotiorum* secretory proteins may dampen the host immune response by the suppression of jasmonic/ethylene signaling pathway. Ss-Itl is one such effector candidate (Zhu et al. 2013). The RNAi silencing of *Ss-Itl* in *S. sclerotiorum* led to a strong defense response in *Arabidopsis* as evidenced by expression of marker genes involved in the JA/ET and SA signaling pathway, and the *Ss-Itl*-silenced version of *S. sclerotiorum* was unable to successfully invade *Arabidopsis*. The protein is also critical for normative physiology of *S. sclerotiorum*, with silenced strains having aberrant, detrimental hyphal and sclerotial structures. Another *S. sclerotiorum* secreted protein, Ss-Rhs1, was also found to be critical for virulence. The silencing of *Ss-Rhs1* led to aberrant sclerotia and hindered the formation of compound appressoria on *A. thaliana.* Virulence of the silenced strain was reduced on *A. thaliana* and *Brassica napus* despite the continued production of OA, amylases, cellulases, proteases, and pectinases (Yu et al. 2017). Yet, another effector-like protein, Ss-Caf1, was identified by Xiao et al. (2014), and was shown to be important for virulence. The identified gene, *Ss-Caf1,* encodes a secretory protein candidate with a Ca^{2+} binding EF hand motif likely involved in compound appressorium formation and sclerotial development. Lesions on rapeseed failed to form when challenged with the mutant, despite greater quantities of OA being produced, likely due to impeded tissue penetration as hyphae lacked modification into the melanin-rich infection cushions known as compound appressoria. This protein also induces cell death within the host as observed following transient expression in *Nicotiana benthamiana* (Xiao et al. 2014). Similarly, the protein Ss-Ssvp1 was shown to induce plant cell death (Lyu et al. 2016). SsSsvp1 appears to induce cell death through the disruption of the mitochondrial respiration chain. Indeed, Bimolecular fluorescence complementation (BiFC) assays demonstrated an interaction between Ss-Ssvp1 and QCR8 (a subunit of the cytochrome b-c_1 complex) that disrupted localization of QCR8 in the mitochondria. *Ss-Cp1,* a certato-platanin protein, was recently identified as yet another inducer of cell death when expressed in *N. benthamiana*. This secreted protein was found to interact with At-PR1 in the apoplast *via* yeast two-hybridization, Glutathione S-Transferase, Co-immunoprecipitation, and BiFC assays (Yang et al. 2018). Due to their role in virulence and modification of host plants, targeting secreted, effector-like proteins from *S. sclerotiorum* or elucidating and altering interacting host proteins may be an effective SSR control strategy.

Though functional studies describing secreted proteins from *S. sclerotiorum* are limited, genomics studies identified a large repertoire of potential secreted proteins that can serve as effectors (Amselem et al. 2011). Using bioinformatics approaches, Guyon et al. (2014), identified 78 effector candidates based on protein domains and motifs found in known fungal effectors, signatures of positive diversifying selection, and recent gene duplication events. Derbyshire et al. (2017) identified 70 effector candidates, only nine of which overlapped with Guyon et al., using a more complete genome derived from the optical map of the *S. sclerotiorum* genome combined with PacBio and Illumina sequencing. Twenty-two sequences contained predicted functional domains, and four have previous associations with effector-like activity in other fungi. Furthermore, RNAseq data by Derbyshire et al. (2018) identified 374 abundant sRNAs targeting immunity components in 10 host plants, demonstrating that S. sclerotiorum has another arsenal of virulence factors, besides effectors, that can be deployed to avoid detection or immune responses. While these results are intriguing, the importance and deployment of these effectors and sRNAs for invasion and host defense suppression by *S. sclerotiorum* will have to be elucidated through functional studies.

The brute force of cell-wall-degrading enzymes is vital for necrotrophic infection stages

Cell-wall-degrading-enzymes (CWDEs) were a principle focus of early studies in *S. sclerotiorum* pathogenicity. As a necrotrophic pathogen that requires plant cell death for growth and infection, *S. sclerotiorum* possesses an arsenal of enzymes to transverse host barriers, including cell walls. The *S. sclerotiorum* genome contains 106 carbohydrate active enzymes and affiliated proteins (CAZymes) which assist in the degradation of cell wall substrates including cellulose (20), hemicellulose (40), hemicellulose and pectin side chains (13), and pectin (33) (Amselem et al. 2011). Depolymerization of cellulose, hemicellulose, and pectin and subsequent degradation of plant structural components facilitates pathogenesis and access to mono and oligo-saccharides. Levels of pectinolytic and cellulolytic enzymes are correlated with disease severity and pathogenicity (Lumsden 1969; Prade et al. 1999; Willats et al. 2001). Using polysaccarhide depolymerases and glucosidases, the fungus converts cellulosic, pectinolytic, and hemicellulosic substrates into simple sugars for assimilation (Riou et al. 1991). Glycoside hydrolase activities accompany polysaccharidase enzymes to release monosaccharides from polymers of the cell wall.

Several hydrolases are produced which differ in the isoelectric point and molecular weight, thus increasing the efficiency of hydrolytic applications. Furthermore, these enzymes sometimes function in a nonspecific, multifunctional manner, targeting multiple proteins, and induction can often occur *via* multiple substrates. Cellulolytic and pectinolytic enzymes, for example, are induced by both cellulolytic or pectinolytic substrates, perhaps indicating a common regulatory system. In *S. sclerotiorum*, 69 enzymes involved in cellulose depolymerization and 24 hemicellulolytic enzymes have been identified through comparative genomics (Kubicek et al. 2014; Zhao et al. 2013). The deployment of such a vast number of enzymes facilitates infection in a wide range of polysaccharide cell wall compositions, thus facilitating its promiscuous ability to infect numerous hosts.

In turn, plants do combat enzymes to an extent, as indicated by the production of polygalacturonase-inhibiting proteins (PGIPs) which target the activity of the pectin-degrading enzymes, endopolygalacturonases (PGs) (De Lorenzo and Ferrari 2002). Endopolygalacturonase production by *S. sclerotiorum* induces cytosolic Ca^{2+} signaling and cell death which can be suppressed with administration of PGIP (Zuppini et al. 2005). These PGs are important pathogenicity determinants and *S. sclerotiorum* isolate screening has revealed a positive correlation between pectinase activity, using pectin agar medium, and the virulence of 25 isolates (Asoufi et al. 2007). Sclerotinia has been found to upregulate 11 genes related to pectin degradation during infection of sunflower. However, genes involved in cellulose and hemicellulose degradation are scarcer than those encoded by nearly all plant cell wall degrading pathogens (Amselem et al. 2011). This observation may be related to *S. sclerotiorum's* propensity to infect *via* flowers, which are abundant in pectin, and, indeed Huzar-Novakowiski and Dorrance (2018) observed differential host-plant resistances when soybean lines were inoculated at the flower with ascospores *versus* at the petiole with mycelia. The activity of PGs and OA is synergistic, with OA acidifying the cellular environment (Cessna et al. 2000; Dutton and Evans 1996; Zhou and Boland 1999), thus facilitating PG activity and weakening pectic polymers (Kurian and Stelzig 1979). Cell wall-degrading enzymes have an important functional role in the necrotrophic nutrient acquisition ability of SSR and host counter-response to these CWDEs is a prospective area of plant defense research. Additionally, the comparatively high activity of pectinic *versus* cellulosic and hemicellulosic enzyme activity explains why *S. sclerotiorum* is an effective pathogen of dicots with relatively higher quantities of pectinic polysaccarhides compared to monocots which have primary cell walls characterized by a richness of hemicellulosic polysaccharides.

The genetics of quantitative resistance to SSR

Various QTL conferring resistance to SSR in soybean have been identified

In the absence of elicitors of strong resistance to the pathogen, polygenic alleles with minor effects are widely believed to contribute to resistance to *S. sclerotiorum*. Partially resistant soybean genotypes have been selected and identified (Bastien et al. 2014; Boland and Hall 1987; Grau et al. 1982; Han et al. 2008; Huynh et al. 2010; Iquira et al. 2015; Kim and Diers 2000; Li et al. 2010; McCaghey et al. 2017; Sebastian et al. 2010; Zhao et al. 2015). Overall, 103 quantitative trait loci (QTL) that contributed to resistance have been recorded in Soybase on 18 out of 20 chromosomes (Soybase.org 2010), however the contribution to klenducity, or disease escape mechanisms, *versus* physiological resistance has often not yet been elucidated. Three QTL were identified by Kim and Diers (2000) and 28 QTL were identified by Arahana et al. (2001) which individually explain 4–10% of the phenotypic variation between 153 and five, respectively, recombinant inbred lines compared to SSR-susceptible parent, 'Williams 82.' Additionally, Vuong et al. (2008) mapped four QTL for Sclerotinia stem rot resistance that each explained 5.5 to 12.1% of the phenotypic variance in Sclerotinia stem rot development, and Guo et al. (2008) identified seven QTLs which explained 6.0–15.7% of resistance phenotype differences in their populations. Identification of these loci provide an opportunity to use marker assisted selection (MAS) as a potential tool for the screening of lines resistant to SSR. However, such an approach presents practical challenges that must be overcome to deploy SSR resistance.

Polygenic resistance to SSR presents breeding challenges

While polygenic resistance (quantitative resistance) is thought be more durable than qualitative resistance; breeding using quantitative resistance is complicated. This includes the "drag" of deleterious and undesirable traits within and near QTL regions, existence of numerous QTL with minimal sole contribution to SSR resistance, and epistatic interactions that pose a challenge to heritability (Moellers et al. 2017). Furthermore, the genetics of physiological resistance to *S. sclerotiorum* are not well understood. Current 'field tolerant' soybean cultivars may be tolerant due to avoidance phenotypes such as flowering time and plant height or entangled environmental and genetic interactions. For example, Kim and Diers (2000) used Novartis S19–90 as a source of resistance in breeding lines and mapped three QTL that accounted for 8–10% of disease severity index (DSI) variability. However, two

were associated with disease escape mechanisms of greater height, increased lodging, and later flowering date. These escape mechanisms make screening for physiological disease resistance in a field setting difficult. Furthermore, flowering time or canopy closure may differentially align with apothecial development in varied environments, thus impacting disease resistance across environments. In disease nurseries, screening is complicated by aggregated distributions of inoculum and differing favorable microenvironments for infection within a field which may result in differential disease pressure. The same study observed differential disease resistance and heritability between research environments with broad-sense heritability of DSI ranging from 0.30–0.71 across research sites within Michigan (Kim and Diers 2000). These heritabilities are lower than those found by Miklas and Grafton (1992), ranging from 0.50–0.77. Therefore, while resistance to SSR can be selected for in a field setting, there remains a persistent need to identify consistent, heritable resistance across environments.

Methods have been developed to screen for physiological resistance to SSR

To circumvent resistance conferred by escape mechanisms, breeders have mapped QTL and screened lines using inoculation methods that avoid selecting exclusively klendusity-associated traits (Arahana et al. 2001; Guo et al. 2008; Vuong et al. 2008). However, only few, partially resistant lines were identified. These inoculation methods include severing the stem of soybean and placing a mycelial plug on the cut stem (Vuong et al. 2004, 2008), inoculation of a cut petiole (Del Rio et al. 2001), or ascospore inoculation of flowers (Cline and Jacobsen 1983; Huzar-Novakowiski and Dorrance 2018; Pérès 1995; Rousseau et al. 2004). Floral bud inoculations with cotton pads dipped in mycelial suspensions have also been used with reproducible inoculations (Iquira et al. 2015). The results of these inoculation techniques for resistance differentiation may vary depending on the inoculation technique (Rousseau et al. 2004; Huzar-Novakowski and Dorrance 2018) and the isolate used (Willbur et al. 2017). Huzar-Novakowiski and Dorrance (2018) suggest that multiple screening methods are needed to successfully capture varied resistance mechanisms in selections. Similarly, Willbur et al. (2017) evaluated a panel of *S. sclerotiorum* isolates from the United States and observed differential aggressiveness on soybean cultivars, suggesting that multiple isolates are needed to screen for broad resistance. McCaghey et al. (2017), therefore, utilized both SSR field nurseries and greenhouse evaluations with several *S. sclerotiorum* isolates to identify recombinant inbred lines (RIL) with consistent, moderate to high levels of resistance. Inoculation methods are useful for capturing physiological resistance, but screening methods should be comprehensive to capture the quantitative nature of SSR resistance and differences in *S. sclerotiorum* pathogenicity.

Methods of evaluating lines in the field may include the infestation of fields with sclerotia collected from alternate hosts (Kim et al. 1999) or from naturally infested fields rotated with susceptible hosts such as sunflower (McCaghey et al. 2017). Infestation of soil and planting alternate hosts homogenizes inoculum and encourages more uniform infections in field trials. Lines may be evaluated using a disease severity index or DSI (Grau et al. 1982; Kim and Diers 2000) which differentiates between lateral branch and mainstem infections.

$$DSI = \left(\frac{\sum rating \quad of \quad each \quad plant}{3 \times number \quad of \quad plants \quad rated} \right) 100$$

Modern soybean breeding has emphasized lateral branching, and mainstem infections are associated with the disruption of the mainstem vascular system, which results in severe yield loss. A system which uses both artificial inoculations and field evaluations (McCaghey et al. 2017) allows one to select for both physiological resistance and field tolerance in addition to favorable agronomic properties.

Biotechnology to control Sclerotinia stem rot

Genetic engineering can reduce SSR but is not commercially deployed

While traditional breeding for SSR resistance has identified lines ranging from field tolerance to high levels of quantitative resistance, traditional breeding is time intensive and has its challenges when dealing with alleles with additive effects as described above. Thus, the identification of specific genes and pathways that can be targeted *via* genetic engineering will be helpful to introgress SSR resistance into commercial soybean germplasm. Efforts to use transgenic approaches targeting SSR have not been widely explored. Enhanced resistance in soybean targeting OA for degradation has been observed (Cunha et al. 2010; Donaldson et al. 2001). Donaldson et al. (2001) first developed a transgenic soybean line which expressed an oxalate oxidase (OxO). This physically resilient wheat germin is conserved in monocots and is proposed to oxidize oxalic acid to carbon dioxide and hydrogen peroxide (Dumas et al. 1993; Lane et al. 1993). Oxalate oxidase serves multiple purposes (Donaldson et al. 2001). First, it can hinder the action of oxalate on cell walls. Second, it can remove oxalate, which suppresses host oxidative bursts (Cessna et al. 2000), from the cellular environment. Third, the production of hydrogen peroxide is known to both trigger programmed cell death involved in the HR response, and it mediates the cross-linking of cell wall glycoproteins (Levine et al. 1994). The transformation event occurred without

obvious yield penalties and resistance in a field setting was equivalent to the most resistance inbred line tested by Cober et al. (2003). Cuhna et al. (2010) also targeted oxalate but with a transformation event using the decarboxylase gene (OxDC) which resulted in delayed lesion development. Oxalate decarboxylase converts oxalate into carbon dioxide and formate with no hydrogen peroxide production (Kesarwani et al. 2000), and transgenic lines had reduced lesion size by up to 96%. The supposed avoidance of ROS production may prevent programmed cell death related to fungal growth and colonization (Kim et al. 2008; Ranjan et al. 2018), though no comparative studies examine differences in resistance or the induction of programmed cell death in these lines. While lesion development is successfully impaired, these transgenic lines have yet to be exploited commercially.

The use of RNAseq to assess global gene expression during *S. sclerotiorum* infection of soybean is a promising tool to develop additional genetic engineering technologies for enhanced resistance. Expression data from soybean and other crop plants will provide valuable information on soybean susceptibility factors for gene editing or post transcriptional gene silencing and gene candidates for cis or transgenic plant development. RNAseq methods were used to evaluate the interaction of *S. sclerotiorum* with crop hosts such as, canola (Joshi et al. 2016; Girard et al. 2017; Seifbarghi et al. 2017), pea (Chang et al. 2018), and common bean (Oliveira et al. 2015).

RNA-interference strategies show promising results

More recently, RNA interference (RNAi) strategies have been experimentally developed as a defense strategy against *S. sclerotiorum* and other fungal pathogens. Through various studies in fungal pathogens, it is known that large and small double-stranded RNA (dsRNA) molecules can be transported within and between species for effective gene silencing (Wang et al. 2016). The phenomenon, which occurs endogenously in eukaryotic organisms for gene regulation and the control of viruses, has been exploited *via* host-induced gene silencing (HIGS) to target pathogenicity factors of biotrophic and mycotoxigenic fungal pathogens. These include, but are not limited to *Verticillium dahliae* (Song and Thomma 2018), *Puccinia striiformis* (Qi et al. 2017), *Fusarium spp.* (Chen et al. 2016; Cheng et al. 2015; Ghag et al. 2014; Hu et al. 2015; Koch et al. 2013), and *Blumeria graminis* (Nowara et al. 2010). Mycotoxin production has also been targeted for *Aspergillus flavus* in corn (though with off-target effects on kernals; Masanga et al. 2015) and peanut, with near 100% aflatoxin reduction in B_1 and B_2 chemotypes (Arias et al. 2015).

RNAi has also shown success for controlling necrotrophic pathogens. RNAi was shown to be an effective strategy for controlling *Botrytis cinerea* (Wang et al. 2016) through the targeting of both Dicer proteins, Bc-Dcl1 and Bc-Dcl2. Dicer proteins process dsRNA into small interfering RNA (siRNA) which are used by *B. cinerea* to interfere with host immunity genes. Similarly, *dcl1* and *dcl2* double disruption mutants of *S.sclerotiorum* exhibited pathogen debilitation and enhanced mycovirus susceptibility, indicating a functioning and important silencing machinery that can be targeted or utilized for silencing with foreign dsRNA (Mochama et al. 2018). These studies demonstrate that targeting single genes *versus* multiple, functionally redundant genes might improve RNAi silencing efficacy. Additionally, RNA silencing machinery genes such as argonaute proteins (which forms a RISC complex to guide sRNAs to targets) or RNA-dependent RNA polymerases can be potential targets. Andrade et al. (2016) also used RNAi to reduce virulence of *S. sclerotiorum* through the targeting of the structural gene, chitin synthase (*Chs*). RNAi can also be deployed to alter pathogen targets or plant genes otherwise exploited by pathogens. Recently, the silencing of NADPH oxidases involved in ROS production needed for *S. sclerotiorum* infection was undertaken by Ranjan et al. (2018). The introduction of dsRNA using a viral vector through the process of virus-induced gene silencing (VIGS), in this system, enhanced soybean resistance to *S. sclerotiorum*. RNAi technologies using host-induced silencing provide a promising and precisely-targeted method to enhance host resistance.

Furthermore, new technologies, such as spray-induced gene silencing (SIGS) provides the opportunity to apply dsRNA constructs in a spray form without the regulatory hurdles of bringing genetically engineered technologies to market. Sheet-like clay nanoparticles have been found to extend the integrity of dsRNA on plant surfaces past the five to seven days observed with naked dsRNA, and dsRNA can persist in the presence of clay nanoparticle for up to 30 days (Mitter et al. 2017). These exogenous dsRNA applications can be used in conjunction with RNAseq data for functional studies to understand the importance of various genes to *S. sclerotiorum* pathogenicity (McLoughlin et al. 2018), although virulence factors may differ in importance depending on the host, as is observed in the case of *Ss-Oah1* which is more important for disease development on soybean than pea and faba bean (Xu et al. 2015). One can also speculate that a mycovirus-induced gene silencing vector can be used to silence fungal genes. Gene silencing and editing technologies such as RNAi provide opportunities for the specific, targeted control of SSR and would abate the use of chemical control.

Conclusions

As previously discussed, genetics are one of the most powerful tools to reduce the severity and incidence SSR in soybean. While progress has been made in the identification of resistant cultivars (Bastien et al. 2014; Boland and Hall 1987; Grau

et al. 1982; Han et al. 2008; Huynh et al. 2010; Iquira et al. 2015; Kim and Diers 2000; Li et al. 2010; McCaghey et al. 2017; Sebastian et al. 2010; Zhao et al. 2015), a need persists to identify cultivars that sustain heritable resistance across environments (Kim and Diers 2000) and with multiple isolates of *S. sclerotiorum* (Willbur et al. 2017). Our understanding of the *S. sclerotiorum* pathosystem has been enhanced by better understanding the role of oxalic acid in immune suppression as well as the identification of specific CWDEs and secreted proteins implicated in pathogenic success. Transgenic and RNAi approaches provide a unique opportunity for the precise targeting of both susceptibility factors on the host side and virulence factors on the pathogen side. However, in the absence of commercially deployed biotechnological approaches in SSR management, soybean farmers across the globe will be left with incomplete management options.

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