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Sclerotinia sclerotiorum: An Evaluation of Virulence Theories

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oxalic acid, oxaloacetate acetylhydrolase, pH regulation, pH responsiveness, molecular Koch's postulates

Abstract

Oxalic acid production in *Sclerotinia sclerotiorum* has long been associated with virulence. Research involving UV-induced, genetically undefined mutants that concomitantly lost oxalate accumulation, sclerotial formation, and pathogenicity supported the conclusion that oxalate is an essential pathogenicity determinant of *S. sclerotiorum*. However, recent investigations showed that genetically defined mutants that lost oxalic acid production but accumulated fumaric acid could cause disease on many plants and substantiated the conclusion that acidic pH, not oxalic acid per se, is the necessary condition for disease development. Critical evaluation of available evidence showed that the UV-induced mutants harbored previously unrecognized confounding genetic defects in saprophytic growth and pH responsiveness, warranting reevaluation of the conclusions about virulence based on the UV-induced mutants. Furthermore, analyses of the evidence suggested a

hypothesis for the existence of an unrecognized regulator responsive to acidic pH. Identifying the unknown pH regulator would offer a new avenue for investigating pH sensing/regulation in *S. sclerotiorum* and novel targets for intervention in disease control strategies.

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INTRODUCTION

Sclerotinia sclerotiorum has a remarkable ability to adapt to diverse environmental conditions (8, 52, 62, 108), particularly to ambient pH (2, 13, 21, 23, 73, 96, 107, 115). It is exceptionally able to not only sense and respond to but also modulate ambient pH. Under neutral/alkaline pH conditions, the fungus grows slowly and secretes organic acids, especially oxalic acid, that acidify the environment (73). It can also respond to acidic pH by increasing its growth rate (96), initiating production of melanized survival structures called sclerotia (21, 115), and secreting an array of cell wall–degrading enzymes (CWDEs) and other pathogenicity factors that macerate host tissue and release nutrients (22, 88, 94, 105). Hydrolytic enzymes are sequentially expressed and secreted during ambient pH acidification and disease development such that the lytic enzymes are produced when the ambient pH is optimal for their activities (22, 44, 64, 78, 104, 116). If the ambient pH is too low for optimal growth or for degradation of the available substrates, the fungus may generate ammonia and/or produce oxalate decarboxylase that can degrade oxalate, thereby raising the ambient pH (13, 81, 93, 126). This ability to correctly sense, actively modulate, and dynamically respond to ambient pH allows *S. sclerotiorum* to properly deploy its vast arsenal of disease weaponry, enabling it to survive and thrive under diverse environmental conditions, thereby becoming one of the most successful plant pathogens. It has a wide host range of more than 400 plant species, including almost all dicotyledonous and some monocotyledonous plants, and causes significant economic losses globally every year (14, 15, 108). Because of the enormous negative economic impact of the diseases it causes in many crops, a large body of literature has been published on this pathogen in population biology (5, 7, 18, 69, 72), disease management (1, 117, 121, 134), and pathogenicity/virulence (28, 53, 61, 62, 80, 140) as well as about its association with mycoviruses (58, 138). This review is focused on recent developments in understanding pertinent virulence genes and virulence theories.

The genome sequences of *S. sclerotiorum* have been available for more than a decade (4). Analyses of the genome sequences showed a massive pathogenesis arsenal possessed by this necrotrophic pathogen, including CWDEs, phytotoxins, and other secondary metabolites (4). The availability of the genome sequences also facilitated comparative transcriptomic analysis (91, 99, 104, 109, 116, 150), allowed bioinformatics analyses in predicting possible secreted pathogenicity factors (47, 51, 91) and surveying eukaryotic protein kinases potentially involved in development and pathogenesis (52), and permitted applications of targeted candidate gene approaches in studying biological processes, morphological differentiation, and pathogenicity/virulence mechanisms (13, 80, 84, 139, 142, 149). The *S. sclerotiorum* genome was recently updated with a more complete sequence and more accurate gene annotation (27). This recent analysis provided an updated prediction of potential effectors that will stimulate investigations into the host-pathogen interactions, particularly in the age of effector biology (54). This is happening at a breathtaking pace. For example, Derbyshire et al. (27) predicted an effector based on sequence properties (a cerato-platanin domain) and conservation in other phytopathogens and speculated on its functions. This effector has been empirically validated using a gene-deletion approach by Yang et al. (142). Yang et al. (142) showed that the *S. sclerotiorum* cerato-platanin protein (SsCP1) specifically interacts with

the host pathogenesis-related protein PR1 and plays important roles in virulence. The roles of SsCP1 in pathogenicity were independently confirmed using RNAi technology (100).

Disruption or knockout of specific genes using molecular genetic approaches came late to *Sclerotinia* relative to other phytopathogenic fungi, as there was no mention of *Sclerotinia* pathogenicity genes in a 2001 review (57), primarily because of difficulty in purifying the disrupted allele to a homokaryotic condition in a fungus lacking a conidial state (113). The first account of a specific gene knockout in *S. sclerotiorum* was published by Rollins in 2003 (113) and used repeated hyphal-tip transfer under selection. Later, additional techniques for transformation and purification of mutant alleles became available (10, 84, 133, 142). Others have employed RNAi technology to bypass the requirement of the homokaryotic state of the disrupted allele (21, 39, 41, 66, 79, 90, 91, 100, 101, 110, 137, 144, 146, 149) or to study genes whose deletion could be lethal (49). Recently, the CRISPR-Cas9 technology has been applied to *S. sclerotiorum* (74). The genes of *S. sclerotiorum* that have been specifically studied are listed in **Table 1**. Although many genes can be involved in

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Table 1 List of genes specifically investigated and their potential roles in the life cycle and virulence of *Sclerotinia sclerotiorum*

Gene symbol	Gene	Gene name	Main function and/or putative mechanisms	Method[a]	References
Cell wall–degrading enzymes					
PG2	S13661	Endopolygalacturonase	NA	P	130
PG3	B60155	Endopolygalacturonase	NA	P	130
sspg3	AY312510 (SS1G_10698)	Endopolygalacturonase	Virulence	C, Q	78
pg7	AJ539087 (SS1G_10698)	Endopolygalacturonase	Virulence	C, Q	22
pg5	Y13669 (SS1G_04177)	Endopolygalacturonase	Virulence	C, Q	64
sspg5	AY496277 (SS1G_04177)	Endopolygalacturonase	Virulence	C, Q	78
PGa	CAF05669 (SS1G_04177)	Endopolygalacturonase	Virulence	C, Q	42
pg6	AJ539086 (SS1G_11057)	Endopolygalacturonase	Virulence	C, Q	64
sspg6	AF501308 (SS1G_11057)	Endopolygalacturonase	Virulence	C, Q	78
pg1	L12023 (SS1G_10167)	Endopolygalacturonase	NA	C	111
pg2	L29040 (SS1G_10167)	Endopolygalacturonase	NA	C	43
pg3	L29041 (SS1G_10167)	Endopolygalacturonase	NA	C	43
sspg1d	AF501307 (SS1G_10167)	Endopolygalacturonase	Virulence	C, Q	78
PGb	CAF05670 (SS1G_10167)	Endopolygalacturonase	Virulence	C, Q	42
ssxpg1	AY312511 (SS1G_04207)	Exopolygalacturonase	NA	C, Q	78

(*Continued*)

Table 1 *(Continued)*

Gene symbol	Gene	Gene name	Main function and/or putative mechanisms	Method[a]	References
ssxpg2	AY312512 (SS1G_02553)	Exopolygalacturonase	NA	C, H	78
Ss-Sl2	SSIG_05917	Cell wall protein	Sclerotial development, cellular integrity	R	144
Endo2	XP_001597632 (SS1G_01828)	Endo-β-1,4-glucanase	NA	C, P	20
SsCut	XM_001590986.1 (SS1G_07661)	Cutinase	Induces hypersensitive response	C, H	148
SsXyl1	SS1G_07749	Endo-β-1,4-xylanase	Sclerotial development, virulence	D	145
Signal cascade components					
SNF1	AJ238009 (SS1G_10426)	Sucrose nonfermenting 1	Growth, virulence	C, Q	125
pka1	AY545583 (SS1G_03171)	Protein kinase A (PKA)	PKA activity	C, D	59
Smk1	AY351633 (SS1G_11866)	ERK (extracellular signal-regulated kinase)-type mitogen-activated protein kinases (MAPKs)	Growth and sclerotial development via a pH-dependent signaling pathway	C, R	21
cna1	DQ182488 (SS1G_01788)	Catalytic subunit calcineurin-encoding gene	Sclerotial development, virulence	R	49
Sac1	SS1G_07715.1	Adenylate cyclase	Sclerotial development, virulence, cAMP-signaling pathway	D	60
pph1	SS1G_08489.1	Type 2A Ser/Thr phosphatase catalytic subunit (PP2Ac)	Growth, sclerotial development	R	39
rgb1	SS1G_07871)	Type 2A Ser/Thr phosphatase B subunit	Sclerotial development, virulence, infection cushions	R	39
SsNep1	SS1G_03080.1	Necrosis- and ethylene-inducing peptides	Induce necrosis and cell death	C, H	9
SsNep2	SS1G_11912.1	Necrosis- and ethylene-inducing peptides	Induce necrosis and cell death and calcium and cAMP signaling	C, H	9
Ss-Ggt1	SS1G_14127.3	γ-Glutamyl transpeptidase	Sclerotial and appressorium formation	D	75
Shk1	SS1G_12694	Histidine kinases	Growth, sclerotial development, stress tolerance	D	36
SSITL	SS1G_14133	Integrin-like protein	Growth, sclerotial development, virulence, germination, suppression of host defense	R	149
SMK3	SS1G_05445; incomplete in the genome version 1	Slt2 ortholog (MAPK associated with the cell wall integrity pathway)	Sclerotial development, cuticle penetration	D	8

(Continued)

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Table 1 (*Continued*)

Gene symbol	Gene	Gene name	Main function and/or putative mechanisms	Method[a]	References
Proteases and hydrolase					
aspS	AF271387 (SS1G_03629)	Aspartyl protease	Virulence	C, Q	106
acp1	AF221843 (SS1G_07836)	Acid protease	Virulence	C, Q	105
Ssaxp	SS1G_02462.1	Arabinofuranosidase/ β-xylosidase	Virulence	D	141
Ss-pth2	SS1G_13339.3	Peroxysomal carnitine acetyl transferase	Sclerotial development, virulence	D	84
Ss-odc1	SS1G_08814	Oxalate decarboxylases	NA	D	81
Ss-odc2	SS1G_10796	Oxalate decarboxylases	Appressorium formation, virulence	D	81
Ss-oah1	SS1G_08218.3	Oxaloactetate acetylhydrolase	Oxalate production, sclerotial and appressorium formation, infection	D	80
Ssoah	SS1G_08218	Oxaloactetate acetylhydrolase	Oxalate production, virulence	D, T	140
Scd1	SS1G_13314	Scytalone dehydratase	Sclerotial development, growth	D	82
Thr1	SS1G_13315	Trihydroxynaphthalene reductase	Sclerotial development, growth	D	82
Transcription factor					
pac1	AY005467 (SS1G_07355)	pH-Responsive transcription factor	Growth, sclerotial development, virulence	D	113
SsMADS	SS1G_05588	MADS-box proteins	Growth, virulence	R	110
Ss-FoxE2	SS1G_05834	Forkhead-box transcription factors	Apothecial development	D	132
SsFKH1	SS1G_07360	Atypical forkhead (FKH)-box-containing protein	Growth, sclerotial formation, virulence	R	41
Genes involved in fungal nutrition and responding to environment					
CRE1	AJ000976 (SS1G_09934)	Putative glucose repressor	Carbon catabolite repression	C, D	123, 124
cry1	SS1G_05163	Cryptochrome family CRY-DASH (members of this branch exhibited no or trace levels of DNA repair activity) ortholog	Sclerotial development, response to UV light	D	127
sop1	SS1G_01614	Microbial opsin homolog gene	Growth, sclerotial development, virulence	D	89
Fungal development					
ssp1	SS1G_14065.1	Development-specific protein	Sclerotial development	D, Q	76, 77
ssp2	SS1G_12133.1	Development-specific protein	Sclerotial development	Q	76, 77
MAT1-1-1	SS1G_04004	Mating-type gene	Apothecial development	D	35
MAT1-2-1	SS1G_04006	Mating-type gene	Apothecial development	D	35
MAT1-1-5	SS1G_04003	Mating-type gene	Apothecial development	D	35
MAT1-2-4	SS1G_04005	Mating-type gene	Carpogenic germination, disc morphology	D	35

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Table 1 (*Continued*)

Gene symbol	Gene	Gene name	Main function and/or putative mechanisms	Method[a]	References
Suppress reactive oxygen species (ROS)					
Ssnox1	SS1G_05661	NADPH oxidase	Sclerotial development, virulence, ROS regulation	R	66
Ssnox2	SS1G_11172	NADPH oxidase	Sclerotial development, ROS regulation	R	66
Sssod1	SS1G_00699	Cu/Zn superoxide dismutase	Growth, sclerotial development, virulence, stress tolerance	D	128
SsSOD1	SS1G_00699	Cu/Zn superoxide dismutase	Virulence, stress tolerance	D, T	139
Scat1	SS1G_02784	Type A catalase gene	Growth, sclerotial development, virulence	D	143
Secreted proteins					
Ssv263	SS1G_00263.1	Hypothetical secreted protein	Virulence	D	83
SsCVNH	SS1G_02904	Secreted protein	Virulence, sclerotial development	R	91
Ss-Caf1	SS1G_02486	Secreted protein with a putative Ca^{2+}-binding EF-hand motif	Appressorium formation, sclerotial development, induction of host cell death	T, R	137
Ss-Bi1	SS1G_05839	Bax inhibitor-1 protein	Development, virulence, putative antiapoptosis	R	147
SsPemG1	SS1G_07345	Elicitor-homologous protein	Elicitor, negative virulence factor	R	101
SsNACα	SS1G_05284	Nascent polypeptide-associated complex α-subunit	Sclerotial development, virulence	R	79
SsSSVP1	SS1G_02068	Small secreted virulence-related protein	Virulence, induction of plant cell death	R	90
SsCP1	SS1G_10096	Cerato-platanin protein	Virulence, induction of plant cell death, interaction with PR1	D	142
SsSm1	SS1G_10096	Cerato-platanin protein	Virulence, growth, sclerotial formation, induction of plant cell death	R	100
Ss-Rhs1	SS1G_07404	Rearrangement hot spot repeat-containing protein	Virulence, sclerotial formation, appressorium formation	R	147

[a]Techniques used in studying the genes: C, cloning; D, disruption or targeted deletion; H, heterologous expression; P, protein purification; Q, quantitative RT-PCR; R, RNA interference; T, T-DNA insertion.

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pathogenicity, only a few are true pathogenicity genes according to the definition of Van de Wouw & Howlett (122), as many of the pathogenicity genes are required for completion of the life cycle. Some of the genes are involved in gene regulation and signal transduction (9, 60, 149), some are involved in morphology and development (49, 76, 91), some elicit resistance response from host plants and/or negatively regulate virulence (101, 148), and some are secreted and interact with and manipulate host plants, acting as effectors (79, 83, 90, 101, 137, 142, 146, 147). So far, only a few studies actually identified a host receptor of the *S. sclerotiorum* effectors (90, 142). Although the effector-like gene *SsCm1* was identified in *S. sclerotiorum* (61, 62) as having strong structural and functional similarity to the *Ustilago maydis* effector *Cmu1* that encodes a secreted chorismate mutase (32), a formal description and documentation for *SsCm1* are not found.

Of all the pathogenicity/virulence factors of *S. sclerotiorum*, oxalic acid has received the most attention. It has been extensively studied in the past quarter-century using ultraviolet (UV) light–induced oxalate-deficient mutants (hereafter, UV mutants) as a model. A virulence theory that oxalate is a pathogenicity determinant has been developed over the years (28, 62, 135). In recent years, however, research using genetically defined mutation approaches has yielded evidence that showed mutants of *S. sclerotiorum* defective in oxalate production are still capable of causing disease (140), thus contradicting the notion that oxalate is the determinant of pathogenicity. In this review, we attempt to reconcile the contradictory results for the role of oxalate in virulence of *S. sclerotiorum* by critically evaluating the evidence available in the literature and providing a synthesis. In so doing, we show that the UV-induced, genetically undefined mutants exhibit phenotypes characteristic of defects in growth and in response to acidic pH, which could explain the lack of pathogenicity and other observed phenotypes of the UV mutants.

Based on different experimental approaches used in uncovering the mechanisms involving oxalic acid in virulence, we divide these approaches into three eras: pre-UV mutant era, UV mutant era, and post-UV mutant era. Experimental approaches used in the pre-UV mutant era are mostly observational, using naturally occurring isolates of *S. sclerotiorum*. The experimental approaches used in the UV mutant era, as the name implies, are mostly based on the UV-induced genetically undefined mutants generated in 1990 (45). The experimental approaches used in the post-UV mutant era involved more site-specific mutational approaches expected to produce genetically defined mutants and will expand to use next-generation -omics approaches.

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THE PRE-UV MUTANT ERA

Early studies have consistently observed a correlation of oxalic acid accumulation, pH reduction, and production of CWDEs and virulence of *S. sclerotiorum*, as discussed in several reviews (37, 53, 108). For example, Maxwell & Lumsden (96) reported production of oxalic acid in culture and in infected bean hypocotyls, which accompanied an initial decrease in pH in disease development, and subsequent detection of polygalacturonase activities. Marciano et al. (94) showed the synergistic interactions among oxalic acid, CWDEs, and pH reduction in pathogenesis of *S. sclerotiorum* on sunflower. Magro et al. (92) showed oxalic acid in the inoculum preparation as an important factor for disease development and found a correlation among disease severity, accumulation of oxalic acid, decrease in pH, and inhibition of host polyphenol oxidase. All evidence showed involvement of oxalic acid in disease. Oxalic acid lowers environmental pH, providing a pH level optimal for the lytic activities of CWDEs, and chelating calcium in host cell walls, making cell wall pectin more accessible to enzyme degradation (37, 53).

THE UV MUTANT ERA

The concept that oxalic acid is involved in virulence was solidified in a landmark publication using UV mutants of *S. sclerotiorum* (45). These UV mutants lost oxalic acid production and pathogenicity. They also lost the ability to form sclerotia, which means these UV mutants could not complete their life cycle (15). These UV mutants have since been used in a series of extensive studies investigating the roles of oxalate in the virulence of *S. sclerotiorum*. Oxalic acid is a multifunctional molecule. In addition to the functions mentioned above, oxalate was reported to have others, such as suppressing host oxidative burst (19), deregulating host guard cells (46), and eliciting host programmed cell death (PCD) to facilitate nutrient acquisition by the pathogen (67). Oxalic acid can suppress host defense by manipulating (initially reducing and subsequently increasing) the redox environment of the host cell (30, 135). In these experiments, a lack of a response from the host plant to inoculation with the UV mutants was attributed to lack of oxalate in the UV mutants.

Conversely, a disease response to inoculation with the wild-type strain was attributed to oxalate production in the wild type. This approach assumes that the differences between the UV mutants and the wild type lie in the lack of oxalate production in the former (19, 31, 67, 135).

The current state of this virulence theory based on the UV mutants is that *S. sclerotiorum* needs oxalate to transition from a biotrophic to a necrotrophic state to initiate disease (61, 62, 135). Specifically, *S. sclerotiorum* via oxalate initially suppresses host oxidative burst for infection to take place, then increases oxidative burst to induce host PCD, for disease development (62). Because this virulence theory emphasizes the importance and necessity of oxalate, for the convenience of discussion it is hereafter referred to as the oxalate-dependent theory.

There are a number of studies that show reduced virulence without affecting oxalate production, seemingly contradicting this oxalate-dependent theory. For example, Harel et al. (49) found that calcineurin is required for sclerotial development and pathogenicity, independent of oxalic acid. Xiao et al. (137) found that disruption of *Ss-Caf1*, a secreted protein, resulted in loss of pathogenicity and normal sclerotial formation but more oxalic acid accumulation than the wild-type strain (137). Yang et al. (142) found that deletion of the effector cerato-platanin protein Sscp1 significantly reduced virulence without affecting oxalic acid production (142). The oxalate-dependent theory explains this type of phenomenon of reduced virulence without negatively affecting oxalate production (39, 49, 60, 100, 137, 139, 142) as "the presence of oxalate cannot always compensate for other pathogenicity defects" (62, p. 56). In other words, according to the oxalate-dependent theory, presence of oxalate is a necessary but not always a sufficient condition determining pathogenic success for *S. sclerotiorum*.

Gaps in the Oxalate-Dependent Theory

Although the studies using the UV mutants mentioned above represent the most comprehensive investigations on the adverse effects of oxalate on plants in connection with the development of *Sclerotinia* diseases, there are still some significant gaps in this theory. Its central premise is that the differences between the UV mutants and the wild-type strain are mainly, if not solely, a lack of oxalate production in the UV mutants (19, 31, 67); thus, the UV mutants can be used as a proxy of oxalate deficiency. It is still a mystery as to why the UV mutants cannot develop sclerotia (28, 45, 49, 50, 76), which is a necessary life stage of the life cycle of *S. sclerotiorum* (15, 108). The explanation that sclerotial development requires oxalic acid (28, 29, 67) is obviously not satisfactory because (*a*) oxalic acid could not restore sclerotial development in the UV mutants (45, 115), (*b*) the UV mutants are actually capable of producing oxalic acid (80, 135), (*c*) sclerotial formation in *S. sclerotiorum* requires low pH but not oxalate (115), and (*d*) genetically defined oxalate-minus mutants can form sclerotia (80, 140). Could the reason(s) behind the lack of sclerotial development also account for, at least in part, the lack of pathogenicity in the UV mutants? This is a legitimate question because studies have shown that a number of genes required for normal sclerotial development are also involved in pathogenicity, independent of oxalic acid (39, 49, 60). Because of the confounding factors in the UV mutants, the question still remains whether oxalic acid is required by *S. sclerotiorum* to cause disease. To answer this question, we need to complete molecular Koch's postulates. Similar to Koch's postulates applied in identifying microbial pathogens, molecular Koch's postulates, proposed by Falkow (40), are used in identifying and proving a molecular agent responsible for disease. The molecular Koch's postulates stipulate that, along with other requirements, (*a*) specific deletion or inactivation of the suspected agent causes measurable reduction of virulence and (*b*) reversion of the inactivation or allelic replacement restores virulence (40). The second criterion is necessary to ensure that there are no unintended mutations introduced during the first inactivation step. Because the mutation in the UV mutants is unknown, fulfilling the molecular Koch's postulates could not be attempted with the UV mutants.

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THE POST-UV MUTANT ERA

Improved abilities to generate genetically defined mutants through recombinant DNA techniques facilitated the production of oxalate-minus mutants that could be used to fulfill the molecular Koch's postulates. Toward this goal, two independent studies published in 2015 gave completely different results with respect to virulence (80, 140). In the first study, Liang et al. (80) generated targeted deletion mutants of the oxaloacetate acetylhydrolase (*oah*) gene responsible for oxalate production in *S. sclerotiorum* (Liang's *oah* mutants). The Liang's *oah* mutants completely abolished oxalate production, proving that the *oah* gene is the sole route for oxalic acid production in *S. sclerotiorum*. Liang's *oah* mutants produced aberrant sclerotia (loose, unmelanized stroma-like aggregates) (80) and could not form compound appressoria (infection cushions), and thus could not penetrate the host cuticle layer and therefore could not initiate infection. Even after wounding, Liang's *oah* mutants still caused very limited lesions and lesion expansion. In fulfilling the second criterion of the molecular Koch's postulates, introducing the wild-type *oah* allele to Liang's *oah* mutant restored only oxalic acid production but could not restore normal sclerotial development or compound appressorium formation and therefore could not initiate infection. The complemented strain caused disease only after wounding (80). Thus, the second criterion of the molecular Koch's postulates was not fulfilled. Because Liang's *oah* mutants could not initiate infection, Liang et al. (80) suggested continued use of the UV mutants to study the role of oxalate in virulence. It was concluded that the results based on Liang's *oah* mutants further support the oxalate-dependent theory (62, 80).

In the second study, Xu et al. (140) first generated a T-DNA insertion mutant that had the *oah* gene disrupted and then created targeted *oah*-deletion mutants. Both the T-DNA insertion *oah* mutants and *oah*-deletion mutants showed similar phenotypes and are together referred to as Xu's *oah* mutants. Similar to Liang's *oah* mutants, Xu's *oah* mutants completely lost oxalic acid production and could not change the color of pH-indicating plates (**Figure 1*a***) (80). Instead of producing oxalic acid, Xu's *oah* mutants accumulated fumaric acid in culture (140). The fumaric acid accumulation in Xu's *oah* mutants remained pH responsive; more fumaric acid accumulated at pH 7 than at pH 5 or 3 (L. Xu, G.Q. Li, D.H. Jiang, W. Chen, unpublished data), and this is the same trend/kinetics as oxalic acid accumulation found in the wild type (68, 113, 115). Xu's *oah* mutants were able to lower ambient pH but to a lesser extent than the wild-type strain (**Figure 1*c***) because fumaric acid is a weaker acid than oxalic acid (140). Fumaric acid is one of the organic acids produced and commonly secreted by *S. sclerotiorum* (73). Xu's *oah* mutants produced normal functional sclerotia (capable of carpogenic germination) (**Figure 1*a***) (140), demonstrating that oxalate is not required for normal sclerotial formation. Xu's *oah* mutants also produced compound appressoria similar to those formed by the wild type (**Figure 1*b***)

Most importantly, Xu's *oah* mutants retained pathogenicity on a wide range of plants in detached leaf assays as well as on intact plants (**Figure 2**) (140). The virulence of Xu's *oah* mutants varied depending on host plants. On many host plants, such as faba bean, gourd, pea, potato, tomato, and sunflower, the lesions caused by Xu's *oah* mutants were similar in size to those caused by the wild-type strain, (**Figures 2**) (140). However, Xu's *oah* mutants could not cause any appreciable lesions on soybean leaves (**Figure 2**) (140). Similarly, in *Botrytis cinerea*, another oxalic acid–producing pathogen, *oah*-deletion mutants could not produce oxalic acid but caused lesion expansion on most, although not all, host plants (118, 136). On four tested legume plants, Xu et al. (140) found that lesion expansion by the *oah* mutants was correlated with the host pH and buffering capacity. High tissue pH and buffering capacity inhibited lesion expansion of Xu's *oah* mutants (140). Artificially lowering the pH of common bean leaves with citric acid increased lesion expansion caused by the *oah* mutants, whereas supplying oxalate without buffer to lower the pH did not (140). To fulfill the

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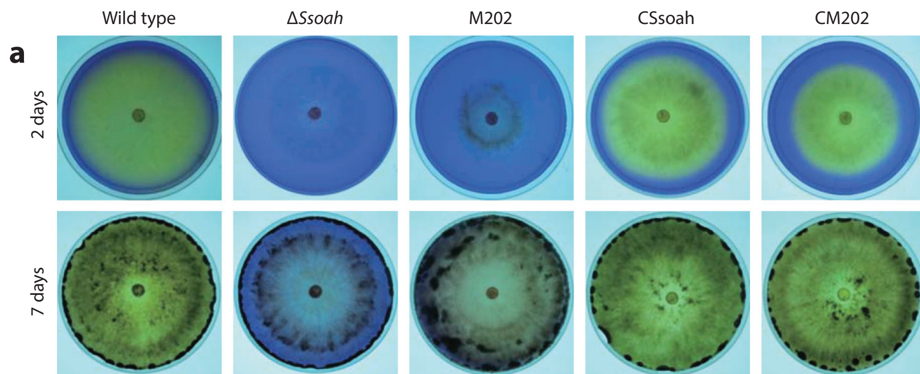

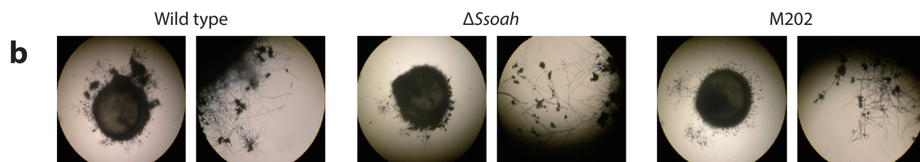

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Figure 1 (*Figure appears on preceding page*)

Colony morphology, appressorial formation and radial growth of *Sclerotinia sclerotiorum* wild-type strain *oah*-deletion mutant Δ*Ssoah*, T-DNA insertion *oah* mutant M202, and wild-type *oah* allele-complemented strains of the mutants CSsoah and CM202. (*a*) Growth on pH-indicating media (PDA amended with bromophenol blue) two days (*top*) and seven days (*bottom*) after inoculation. Yellow indicates acidification by secreted oxalic acid. (*b*) Formation of compound appressoria two days after placing agar plugs with actively growing mycelia on microscopic cover slides. (*c*) Radial growth and accompanying pH changes in unbuffered PDA, and PDA buffered at various pH conditions with citric acid–sodium phosphate buffer. Panels *a* and *c* are adapted from Reference 140. Abbreviations: hpi, hours post inoculation; PDA, potato dextrose agar.

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second criterion of the molecular Koch's postulates, complementation by introducing the wild-type *oah* allele restored oxalic acid production and virulence on all tested plants, including soybean plants (140). Because the oxalate-dependent theory cannot explain the fact that the oxalate-minus mutants can still cause considerable disease at least on some plants (**Figure 2**), Xu et al. (140) proposed that it is the acidic pH, not oxalic acid per se, that sets the optimal environment for virulence expression (140). This virulence theory emphasizes the importance of acidic pH and, for the convenience of discussion, is hereafter referred to as the pH-dependent theory.

The pH-dependent theory does not mean pH itself is the disease agent but rather recognizes the importance of pH-regulated gene expression that in turn determines virulence (2, 3, 107). Thus, the pH-dependent theory does not exclude the roles of oxalic acid because oxalic acid, probably the strongest organic acid, can efficiently induce low pH. It is oxalic acid that enables *S. sclerotiorum* to have such a broad host range (**Figure 2**) (14, 140). The pH-dependent theory differs from the oxalate-dependent theory in that in the absence of oxalate, *S. sclerotiorum* is capable of initiating infection and causing disease at least on some plants provided that the ambient pH is low enough. The pH-dependent theory also permits evocation of pathogen-induced PCD even in the absence of oxalate because PCD is not necessarily a host response specific to oxalate (56). Pathogenicity factors other than oxalate produced by *S. sclerotiorum* are capable of inducing host PCD (11, 12, 151), and low pH itself can also induce PCD (56). In the pH-dependent theory, the primary importance of oxalic acid is its strong ability to lower the ambient pH rather than providing oxalate. Such a distinction is important for understanding the basic mechanisms of virulence of *S. sclerotiorum*, and exonerating oxalate as the primary pathogenicity determinant will stimulate research to identify true pathogenicity determinants and design management strategies to control *Sclerotinia* diseases.

The pH-dependent theory can also explain the fact that transgenic plants expressing exogenous oxalate-removing enzymes, such as oxalate decarboxylase (OXDC) or oxalate oxidase (OXO), resulted in enhanced resistance against *S. sclerotiorum* (24, 25, 33, 34, 131). Because oxalate is intrinsically related to low pH, removing oxalate can help prevent its associated pH reduction. Indeed, Dong et al. (34) showed that OXO-transgenic plants maintained higher pH than non-transgenic control plants after application of oxalic acid. Additionally, other mechanisms besides oxalate removal are also involved in the enhanced disease resistance in the transgenic plants (33, 55, 65).

A SYNTHESIS

The striking differences in disease phenotypes among the *oah* mutants and the UV mutants prompted a search for explanations to reconcile the differences and, in the process, gain a better understanding of the pathogenicity/virulence mechanisms of *S. sclerotiorum*. It is useful to compare the features of the three sets of mutants that were used in the experimental approaches: Xu's *oah* mutants, Liang's *oah* mutants, and Godoy's UV mutants. One obstacle in making the comparison is that the actual genotype (mutation) of the UV mutants is unknown, and physiological

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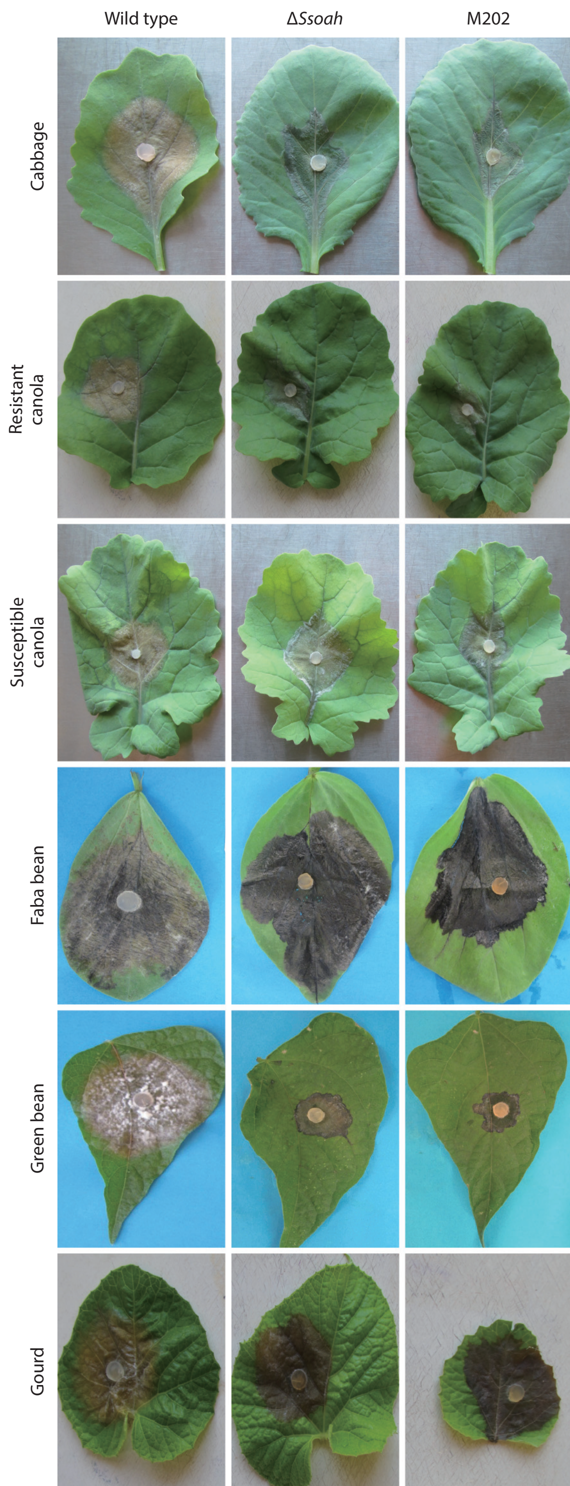

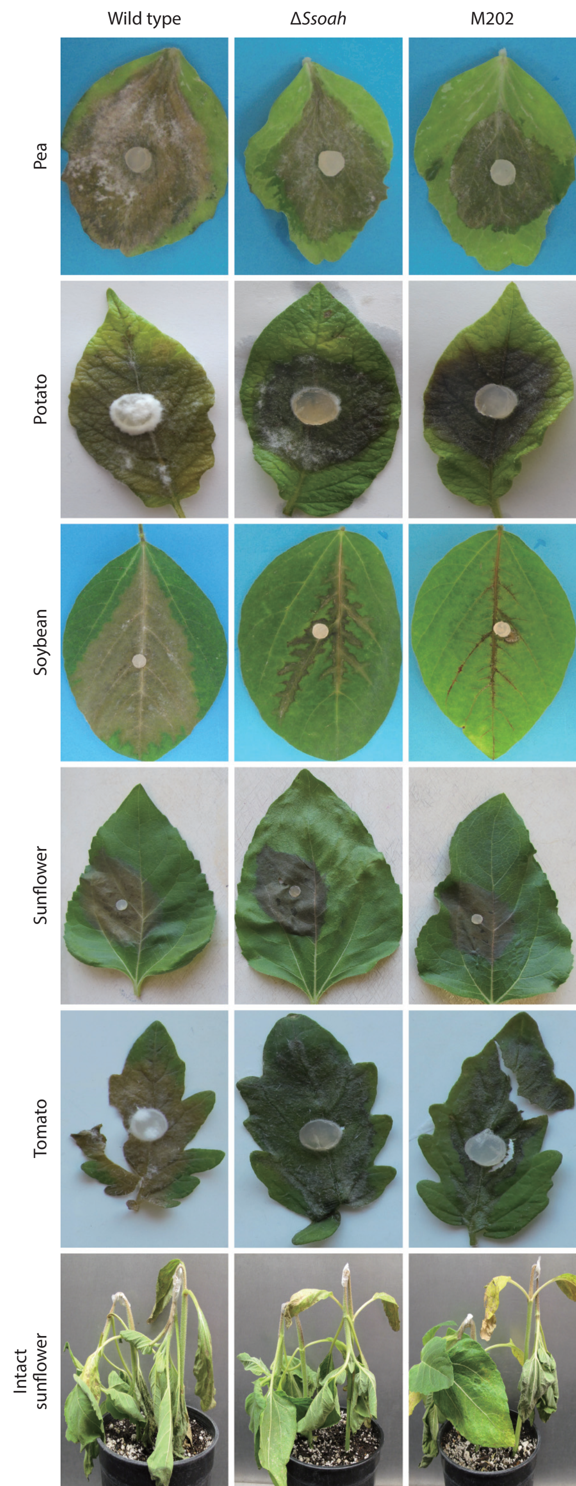

(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Pathogenicity assays of *Sclerotinia sclerotiorum* wild-type strain, *oah*-deletion mutant Δ*Ssoah*, and T-DNA insertion *oah* mutant M202 on detached leaves from two- to three-week-old plants of cabbage (variety Stonehead hybrid), susceptible canola (DKL 30–42), resistant canola (45556), faba bean (Broad Windsor), green bean (Great North Tara), gourd (Birdhouse), pea (Guido), potato (Russet Norkotah), soybean (Skylla), sunflower (Giganteus), and tomato (Moneymaker) as well as on intact sunflower (Giganteus) plants. All photos, including the intact sunflower plant assay, were taken two days post inoculation (dpi) (except the photos from Reference 140 which were three dpi). All experiments were performed at least twice. The photos of faba bean, green bean, pea, and soybean are from Xu et al. (140).

information about the UV mutants is scattered. In comparing the known phenotypes of the UV mutants with those of the *oah* mutants for which the genotype is known, we discovered that although the UV mutants are frequently called and used as oxalate-deficient mutants, their phenotypes are inconsistent with those of genetically defined *oah* mutants (**Table 2**). We discuss each of the traits that differentiate the three sets of the mutants.

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Genetically Defined Mutation

Xu's *oah* mutants and Liang's *oah* mutants (80, 140) are all genetically defined; the mutations were at the *oah* gene encoding for oxaloacetate acetylhydrolase responsible for the final biosynthesis step in the formation of oxalic acid from the hydrolysis of oxaloacetate (95, 98). However, the UV-induced mutation is still unknown, even though the UV mutants have been extensively used since 1990 as a model of oxalate deficiency (19, 31, 45, 61, 67, 135).

Production of Oxalic Acid

Both Liang's and Xu's *oah* mutants completely lost oxalic acid production (**Figure 1*a***) (80, 140). However, the UV mutants are capable of producing oxalic acid (supplementary figures 1 and 2 of Reference 135) and could produce up to 6 mg/ml of oxalic acid, approximately 50% of that

Table 2 Comparison of the features of Xu's *oah* mutants, Liang's *oah* mutants, and Godoy's UV mutants used in studying roles of oxalic acid in virulence of *Sclerotinia sclerotiorum*

Trait	Xu's *oah* mutants[a]	Liang's *oah* mutants[b]	Godoy's UV mutants[c]
Genetically defined mutation	Yes	Yes	No
Production of oxalic acid	No	No	Yes[d]
Development of sclerotia	Yes	Yes (but abnormal)	No[e]
Development of appressoria	Yes[f]	No[g]	Yes[g]
Pathogenicity/virulence	Yes	No	No
Genetic complementation	Yes	No	Not attempted
Growth rate (colony diameter)[h]	>4.0 cm/day	>4.0 cm/day	<2.0 cm/day
pH-Responsive growth	Yes	Yes	No[i]

[a]Both T-DNA insertion and *oah*-deletion mutants generated by Xu et al. (140).
[b]The *oah*-deletion mutants generated by Liang et al. (80).
[c]The mutants A1, A2, and A3 generated using UV radiation by Godoy et al. (45) and used in the study by Liang et al. (80).
[d]Supplemental figure S1 in Williams et al. (135); supplemental figure S2 in Liang et al. (80).
[e]Godoy et al. (45); Dickman (28); Harel et al. (49, 50); Li & Rollins (76).
[f]See **Figure 1*b***.
[g]Liang et al. (80).
[h]Growth rate (colony diameter) at pH 3.5 or 3.6 when no medium acidification is required for optimal growth, from **Figures 1** and **3** (80, 140).
[i]**Figure 3**; supplemental figure S2 in Liang et al. (80).

produced by the wild-type strain under the optimal pH 7 (80). The UV mutant A2 and the wild-type strain have "identical *Ss-oah1* nucleotide sequences" (80, p. 566). Thus, the mutation in the UV mutants must be located at a regulatory locus or another locus that modulates or pleotropically limits oxalic acid production. For example, oxalate accumulation in *S. sclerotiorum* was negatively impacted by disruption of a seemingly unrelated peroxysomal carnitine actyl transferase gene (*Ss-pth2*) (84). Oxalic acid production in *S. sclerotiorum* is a trait responsive to neutral/alkaline pH (115, 126). Under neutral/alkaline conditions, *S. sclerotiorum* secretes oxalic acid and a number of other organic acids (glycolic acid, fumaric acid, malic acid, and succinic acid) (73, 97, 126), thereby acidifying the environment. At low pH conditions or once the environmental pH decreases, acid secretion is reduced or ceases (115, 126), correlating with *oah* gene expression in relation to ambient pH (80). The fact that the UV mutants can produce higher levels of oxalate under neutral pH than at lower pH conditions (80, 135) shows that oxalate production in the UV mutants is still responsive to neutral/alkaline pH.

Although oxalic acid invariably accumulated in the late stages of cultures of *S. sclerotiorum* and in diseased plant tissues, the profiles of secreted organic acids by *S. sclerotiorum* in the early stages of cultures and plant infection have not been thoroughly investigated. There is growing evidence that oxalic acid is not involved or not required in the early stages of disease development (13, 25, 116). During colonization of sunflower cotyledons, the early stage pH reduction was associated with increases in citric acid and succinic acid concentrations, but not oxalic acid concentration until after more than 40% of the tissue was colonized (13). Davidson et al. (25) compared the *Sclerotinia* infection process between wild-type soybean and its OxO overexpression transgenic plants and found no evidence that oxalic acid is necessary for initial infection in the primary lesion formation, although it is required later for lesion expansion because of its ability to lower ambient pH and chelate calcium (25). Soybean, known for high levels of oxalate accumulation in the plant (70), happens to have the highest tissue pH and buffering capacity among four tested legume plants (140). Pathogenicity assays using genetically defined oxalate-minus mutants also showed that oxalic acid is required for lesion expansion on some host plants but not for the establishment of initial primary infection (**Figure 2**).

Development of Sclerotia

The sclerotium is central to the life cycle and disease cycle of *S. sclerotiorum* (15, 108). It is the survival structure and also integral in both vegetative and sexual reproduction. Because of its importance, the mechanisms of sclerotial formation have been extensively studied at morphogenesis and at molecular levels (49, 50, 73, 76). The sclerotial development process can be divided into various developmental stages (76, 120) and involves several interconnected signal transduction pathways (such as cAMP-dependent and pH-dependent signaling pathways and RAS/MAP kinase–dependent signal transduction pathways) (21, 38, 114). Sclerotial formation in *S. sclerotiorum* is a pH-responsive trait (responsive to acidic pH), although it also responds to mechanical disruption and desiccation (or exposure to air) (50). It is low pH, not oxalate, that triggers sclerotial initiation along with expression of sclerotial development-associated genes, such as *Ss-ssp1* (76) and *Smk1* (21). Neutral pH inhibits sclerotial development (115). Xu's *oah* mutants can develop fully functional sclerotia in the absence of oxalic acid (140). Liang's *oah* mutants can also develop sclerotia, although sclerotial formation is aberrant, suggesting that the *oah* mutants can at least respond to pH and start the sclerotial initiation process. However, the UV mutants are defective in sclerotial formation and did not respond to acidic ambient pH (115), and the development-specific protein gene *Ss-ssp1*, the sclerotium-related MAPK *Smk1*, and protein kinase PKA were not expressed or remained at the background levels (21, 50, 76).

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Development of Compound Appressoria

Formation of compound appressoria (or infection cushions) is required by *S. sclerotiorum* for infecting plants unless infection is directly through stomata, but direct stomatal infection is rare (88). *S. sclerotiorum* forms compound appressoria in response to physical environmental cues such as contact with the cuticle layer of host tissue or hydrophobic surfaces such as Petri dishes, microscopic cover slides, and parafilm (88). Xu's *oah* mutants formed compound appressoria similar to those formed by the wild type (**Figure 1*b***), suggesting oxalate is not required for compound appressorial formation. Godoy's UV mutants were also capable of forming compound appressoria (80). However, Liang's *oah* mutants were defective in forming compound appressoria and did not form compound appressoria on Petri dishes, cover slides, or parafilm (80). Thus, the Liang's *oah* mutants could not initiate infection (80). Mutants with disrupted or silenced genes that affect appressorial formation such as *Smk3* (8), *Ss-Caf1* (137) and *Ss-Rhs1* (147) are also nonpathogenic.

Pathogenicity/Virulence

Virulence of *S. sclerotiorum* is a complex and dynamic process involving external factors of the host and the environment and is a trait responsive to acidic pH. It has been long observed that oxalic acid accumulation–accompanied reduction in pH is associated with disease development (48, 87, 96). Acidic pH also induces expression of a number of CWDEs and at the same time provides the most optimal condition for their activities (22, 86, 115). Xu's *oah* mutants can initiate infection on all plants, but their virulence (degree of lesion expansion) is dependent on host pH and buffering capacity because of their reduced ability to acidify the surroundings of the infection site (**Figure 1*c*** and **Figure 2**) (140). The Liang's *oah* mutants could not penetrate the host cuticle layer (due to lack of appressorial formation) and even after wound inoculation could not cause disease. The UV mutants are nonpathogenic on all tested plants (23, 28, 31, 45, 80). Although the UV mutants were able to produce a number of CWDEs in culture (45), the hydrolytic activities of the CWDEs are inevitably curtailed due to lack of optimal ambient pH in the UV mutant infection sites, as pointed out more than ten years ago by Bolton et al. (15).

Genetic Complementation

Genetic complementation here refers to a process intended to prove the presumptive function of an agent (gene) by its ability to restore the missing function in a deficient strain and is the second criterion of the molecular Koch's postulates (40). Because the nature of the mutation in the UV mutants is not known (a genetically intractable system), genetic complementation could not be attempted with the UV mutants. One of the original UV mutants (A-3) reverted to the wild-type phenotype and became pathogenic (45). That revertant has been frequently cited (19, 21, 28, 31, 45), seemingly implying fulfillment of the second criterion of the molecular Koch's postulates. However, that reversion is not the same as fulfilling the molecular Koch's postulates because the actual mutation in the mutant A-3 was not determined and thus cannot be assumed to be the same as in the other UV mutants because that mutant (A-3) was so unstable and reverted after mere three transfers (45), whereas the other UV mutants have been stable for more than 25 years. If the reversion is not a controlled process, we believe that to fulfill the molecular Koch's postulates the mutation should be at least an identified genetic event. That is not the case with the revertant (A-3) of the UV mutants. In the Liang's *oah* mutants, although introducing the wild-type *oah* allele complemented oxalic acid production, it did not restore the defects in sclerotial development and appressorial (infection cushion) formation (80). Liang et al. (80) offered two alternative explanations: the precise and dynamic regulation of oxalic acid metabolism for these

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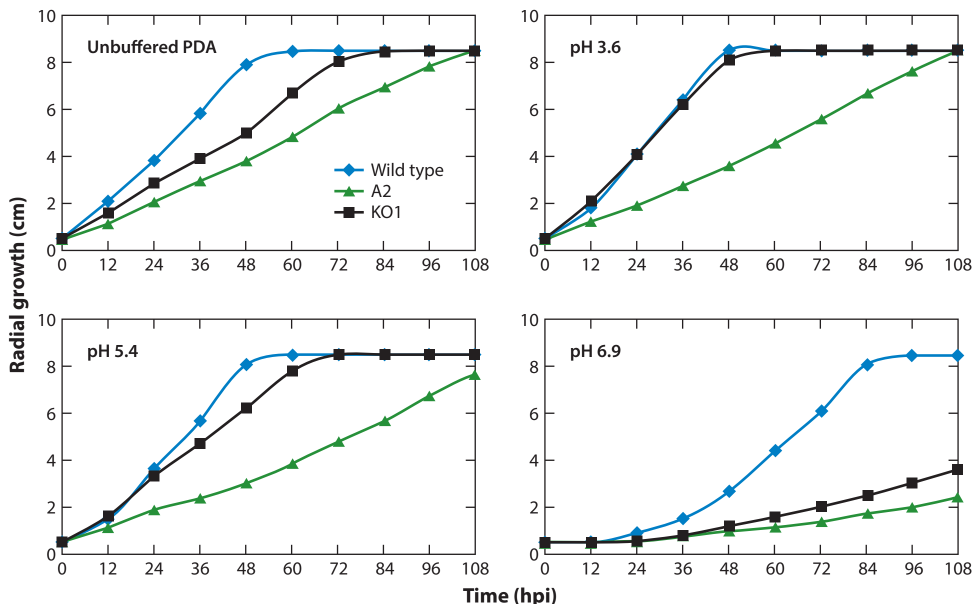

Figure 3

Radial growth comparison of the UV mutant (A2) with Liang's *oah* mutant (KO1) and wild-type strains of *Sclerotinia sclerotiorum* on unbuffered PDA and PDA buffered with citric acid–sodium phosphate buffer at pH 3.6, 5.4, and 6.9. The other two UV mutants A1 and A3 grew at the same rate as mutant A2 (*not shown*) according to Liang et al. (80). Values are means of three replicates, and standard deviations for all data points did not exceed 0.3 (*not shown*) according to Liang et al. (80). Adapted from supplemental figure S2 in Liang et al. (80) with permission from the publisher. Abbreviation: hpi, hours post inoculation.

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developmental events in *S. sclerotiorum* could not be accomplished by ectopic complementation or the epigenetic processes that attenuate sclerotial development, and infection cushion formation in the absence of oxalic acid accumulation could not be reversed by genetic complementation (80). Another likely and more intuitive explanation is that unintended mutations were introduced during the first step of deletion of the *oah* gene, especially in this case where no control transformation with the empty vector was presented. This explanation is supported by a recent study in which the *oah* gene was disrupted with CRISPR-Cas9 technology (74). The CRISPR-Cas9-mediated mutants lost production of oxalic acid but formed compound appressoria and infected healthy host tissue (74). Nevertheless, the fact that introducing the wild-type *oah* allele could not fully restore wild-type phenotypes creates doubts in attributing all the mutant phenotypes to the loss of oxalic acid production alone. In the Xu's *oah* mutants, introducing ectopically the wild-type *oah* allele restored oxalate production (**Figure 1**) and full virulence on all tested host plants, including soybean plants (140).

Growth Rate

The UV mutants are severely impaired in saprophytic growth, growing at only 50% of the rate of the wild-type strain (**Figure 3**) (80). Such reduced growth rates, as well as the differences in growth rates between the UV mutants and the wild type reported by Liang et al. (80) in 2015 and shown in **Figure 3**, are consistent with the original description of the UV mutants in 1990 (45). In the original description, the colony radii (∼3.66 cm) of four-day-old cultures of the UV mutants

(excluding the revertant A-3) were significantly smaller than those of three-day-old cultures (4.25 cm) of the wild type (the wild type reached the edge of plates by day three) (45). Because the growth rate of *S. sclerotiorum* is affected by ambient pH, it is often confounded by its ability to acidify media due to secretion of oxalic acid. At low pH conditions (pH 4 or below) when medium acidification is not required for optimal growth, growth rate should reflect the intrinsic ability of growth. All the *oah* mutants, as well as the mutants of *pac1* (an alkaline pH-responsive transcription factor, discussed below), showed growth rates similar to those of the wild-type strains at low pH (**Figure 1*c*** and **Figure 3**) (68, 80, 113, 140). However, the UV mutants grew at only 50% of the growth rate of the wild type at pH 3.6 (**Figure 3**) (80). The slow growth rate of the UV mutants could have affected their ability to colonize host tissue, as it would allow more time for the host plant to mount a defense response (122). Any general growth defect could explain the lack of pathogenicity (29). Indeed, their low virulence compared to that of Liang's *oah* mutants after wound inoculation was attributed to the slow growth rate of the UV mutants (80).

pH-Responsive Growth

pH-Responsive growth refers to an increase in growth rate in response to decreasing ambient pH. What is most telling is that the UV mutants not only had impaired growth rate but also lacked a growth response to acidic pH. Growth rate is a complex trait. The effect of ambient pH on growth rate could be through changing nutrient availability by affecting efficiencies of permeases and transporters (63) and/or through changing general metabolic rate by affecting intracellular pH (16). However, when a single mutation could eliminate the response of such a complex trait, as seen in **Figure 3**, it is likely through a pH-regulated genetic control (6). *S. sclerotiorum* is inhibited by neutral pH, but its growth rate increases as ambient pH decreases (113, 140). This typical growth response to acidic pH is more dramatic with the *oah* mutants than with the wild-type strains (**Figure 1*c*** and **Figure 3**) (80, 140). When the ambient pH is at 5 or above, the *oah* mutants grow slower than the wild types (**Figure 1*c*** and **Figure 3**) (80, 140). The wild-type strains and the *oah* mutants (both Liang's and Xu's *oah* mutants) respond to decreasing ambient pH by increasing growth rate. When the ambient pH is <4 where medium acidification is not required for optimal growth, the *oah* mutants grew at the same rate as the wild types (**Figure 1*c*** and **Figure 3**) (80, 140). However, the UV mutants did not show such a growth response to acidic pH. They grew essentially at the same rate under three pH conditions, pH 3.6, 5.4 and unbuffered PDA (initial pH about 5.5), taking four and a half days to reach the edge of the plates (**Figure 3**) (80). This essentially unchanged growth rate at three pH conditions indicates that the UV mutants could not perceive or respond to the decrease of the ambient pH from 5.4 to 3.6. A plausible explanation of this phenomenon is that the UV-induced mutation affected the UV mutants' ability to sense or respond to acidic ambient pH. The hypothesis that the UV mutants lost the ability to sense/respond to acidic ambient pH could help explain the mystery of why the UV mutants could not form sclerotia even under favorable conditions, or why their growth rate slowed and they lost pathogenicity/virulence, as sclerotial formation, growth rate and pathogenicity are responsive to acidic pH.

S. sclerotiorum possesses the pH regulation transcription regulator Pac1 (115), a structural and functional homolog of the conserved alkaline pH-responsive transcription factor PacC, present in a number of filamentous fungi (2, 17, 85, 107) and extensively studied in the model fungus *Aspergillus nidulans* (16, 102, 119). In *A. nidulans*, pH regulation is through the pH signal transduction pathway PacC/RIM1 and involves seven genes with PacC as the final target of the pathway. After activation through proteolytic cleavages under alkaline conditions, the active form of PacC positively regulates expression of alkalinity-expressed genes and at the same time represses expression of acidity-expressed genes (16, 17, 102, 103, 119). PacC of the alkaline responsive signal

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transduction pathway is not activated under acidic pH conditions (17, 102, 103, 129). In *S. sclerotiorum*, the PacC homolog Pac1 has been identified (115) and its functions investigated through the creation of both loss-of-function and gain-of-function mutants (68, 113, 115). Sclerotinia *pac1* was not expressed at low pH, but its expression increased in parallel with increasing ambient pH (115). Any interference (either loss-of-function or gain-of-function mutations of *pac1*) with the pH regulation system resulted in severely impaired virulence (68, 113), demonstrating the importance of properly pH-regulated gene expression for virulence of this necrotrophic pathogen, consistent with the theory of pH regulation of fungal virulence of phytopathogens advanced by Prusky and associates (2, 3, 107).

In addition to the impaired virulence, phenotypic characterizations of the *S. sclerotiorum pac1* mutants also showed that oxalic acid production, a neutral/alkaline pH-responsive trait, is under control of Pac1 because its gain-of-function mutants produced more oxalic acid even at acidic pH, an alkalinity-mimicking phenotype (68). Coincidently, at least three *PacC* binding sites were found in the promoter region of the *oah* gene (80; L. Xu & W. Chen, unpublished data). However, pH-responsive growth, a pH-regulated trait responsive to acidic pH, is not under the control of Pac1 because both loss-of-function and gain-of-function mutants of *pac1* showed the same wild-type phenotypes and grew at the same rate as the wild-type strain at acidic pH (68, 113). When the phenotypes of pH-regulation mutants do not fit the PacC/RIM1 model, independent regulators are invoked to explain the situation (26, 71). It is likely that *S. sclerotiorum* has one or more pH-regulating factors independent of *pac1* that positively regulate the response to acidic pH.

Several lines of evidence support the hypothesis for the existence of a pH-regulating factor responsive to acidic pH and independent of *pac1* in *S. sclerotiorum*: (*a*) *pac1* (or its homolog *pacC* in other fungi) is responsive to neutral/alkaline pH and is not functional or not expressed at acidic pH in *S. sclerotiorum* (115) or in other fungi (2, 3, 17, 102, 103); (*b*) the trait of growth response to acidic pH is not regulated by *pac1* because either loss-of-function or gain-of-function mutants of *pac1* do not affect this trait (68, 113), but an unknown mutation in the UV mutants did (**Figure 3**); (*c*) the expression of the sclerotium-associated MAPK *smk1* is pH regulated and responsive to acidic pH, but its pH regulation is independent of Pac1 because the *pac1* expression pattern, varying with ambient pH, was not affected in either functional or suppressed *smk1* genetic backgrounds, and *smk1* transcript levels in relation to ambient pH were not affected by *pac1* deletion (21); and (*d*) the expression of the glucose repressor *cre1* of *S. sclerotiorum* and its homolog *creA* in *A. nidulans*, which regulate expression of certain CWDEs (105, 112), is controlled by both carbon source and ambient pH, but pH prevails over carbon source (105), and their pH regulation is independent of *pacC* because this pH regulation is operational in *A. nidulans* mutants where the PacC signal transduction pathway was disrupted (125). The last study also raised the possibility of the existence of a second pH-regulating system independent of PacC in *A. nidulans* (103). Also consistent with these observations, the well-conserved PacC binding sites were not found in the promoter regions of the pH-regulated genes *cre1* (125) or *smk1* (L. Xu & W. Chen, unpublished results) of *S. sclerotiorum*.

It is not a simple coincidence that all the missing traits in the UV mutants (sclerotial formation, pathogenicity/virulence, normal growth rate, and pH-responsive growth) are traits responsive to acidic pH (Note: Oxalic acid production is a trait responsive to neutral/alkaline pH and is not missing in the UV mutants) (**Table 2**). If each of the missing traits were due to a separate mutation, it would be extremely rare to have all the separate mutations affecting pH responsiveness happening simultaneously in a single ascospore, not to mention three times in three ascospores for the three UV mutants. A more parsimonious explanation would be a single mutation affecting all the acidic pH–responsive traits. It is tempting to suggest that a still unidentified factor that positively modulates response to acidic pH is impaired in the UV mutants, and a hypothesis for a

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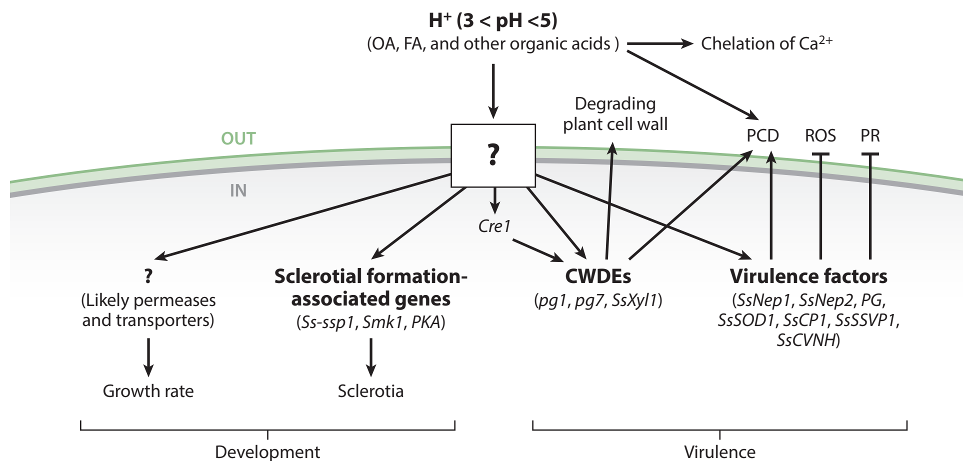

Figure 4

A diagram illustrating the hypothesis of a pH regulator responsive to acidic pH for virulence and development (growth rate and sclerotial development) in *Sclerotinia sclerotiorum*. Many of the developmental genes also affect virulence, and many of the secreted virulence factors also affect development, especially sclerotial formation. Abbreviations: CWDEs, cell wall–degrading enzymes; FA, fumaric acid; OA, oxalic acid; PCD, programmed cell death; PR, pathogenesis-related proteins; ROS, reactive oxygen species.

positive regulator responsive to acidic pH is illustrated with **Figure 4**. The pH-dependent theory emphasizes the importance of acidic pH–induced gene expression. For the acidic pH to induce gene expression, its presence must be perceived by the fungus and the signal must be transduced intracellularly to activate downstream genes. A mutation preventing this perception could block transduction of the signal and cause the mutant to lose acidic pH–responsive traits such as sclerotial formation, pathogenicity/virulence, normal growth rate, and pH-responsive growth. Although the UV mutants have been frequently called oxalate-deficient mutants (19, 67, 135), and sometimes non-sclerotium-producing mutants (50) or sclerotia-minus mutants (76) in different studies, these designations fail to convey the complete picture of the defects possessed by the UV mutants. The UV mutants are more appropriately called acidic pH–nonresponsive mutants because acidic pH nonresponsiveness can explain all the observed phenotypes of the UV mutants.

CONCLUSION AND PROSPECT

In an effort to reconcile the differences in disease phenotype among the three sets of mutants used in investigating the roles of oxalic acid in virulence of *S. sclerotiorum*, we found confounding factors in both Liang's *oah* mutants and the Godoy's UV mutants. Liang's *oah* mutants are confounded by the fact that introducing a wild-type *oah* allele could not restore appressorial formation and normal sclerotial development (80), suggesting Liang's *oah* mutants harbored other genetic defects besides mutation at the *oah* gene. Godoy's UV mutants are confounded by the fact that the mutation affected a number of traits besides oxalate accumulation (**Table 2**). It is clear that the UV mutants are not an appropriate proxy for oxalate deficiency. When the UV-induced mutation reduced the growth rate and changed the growth response to ambient pH from the wild type (**Figure 3**), it is conceivable that it also significantly changed the expression of pH-regulated genes in the UV mutants because pH is a global regulator of gene expression (2, 3, 103). Consequently, the lack of pathogenicity of the UV mutants cannot be attributed to oxalate deficiency alone. Other pathogenicity factors or effectors secreted by the wild type but missing in the UV mutants due to

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lack of response to acidic pH may also be able to induce disease-associated phenotypes such as PCD. For example, a polygalacturonase produced by *S. sclerotiorum* can induce PCD (12, 151). The UV mutants lost pathogenicity on all tested plants most likely because they lost the ability to sense and respond to ambient pH. In addition, the combination of phenotypes (**Table 2**) possessed by the UV mutants is unique and resembles that of no other known mutants, including those of *oah* (80, 140) and *pac1* (68, 113) mutants, suggesting the existence of an unknown regulator responsive to acidic pH. We hypothesize that identifying and characterizing the unknown pH regulator could provide a new avenue for studying pH sensing and ambient pH regulation in *S. sclerotiorum* and could identify novel targets for intervention in developing strategies for managing *Sclerotinia* diseases.

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SUMMARY POINTS

1. Oxalic acid is invariably accumulated in cultures of *S. sclerotiorum* and in *Sclerotinia*-diseased tissues and accompanied by pH reduction, and has been thought to be a disease agent. This concept was further supported by a landmark publication in 1990 using UV-induced mutants that concomitantly lost pathogenicity, oxalate accumulation, and sclerotial formation.
2. For more than a quarter-century, these genetically undefined UV mutants have been used as a proxy of oxalate deficiency in investigating the adverse effects of oxalate on plants in relation to virulence of *S. sclerotiorum*. An oxalate-dependent theory has been developed, which states that oxalate is an essential pathogenicity determinant required for *Sclerotinia* to cause disease. Specifically, according to the oxalate-dependent theory, *S. sclerotiorum* requires oxalate to transition from a biotrophic to a necrotrophic phase because it needs oxalate to manipulate (first suppress then increase) host oxidative burst for establishing infection and inducing host PCD for disease expansion.
3. However, two recent studies using targeted gene-deletion approaches generated *S. sclerotiorum* mutants defective in the *oah* gene but with completely different results with regard to disease phenotype. One study showed that the *oah* mutants lost oxalic acid production and are defective in the formation of normal sclerotia and compound appressoria, and therefore could not initiate infection. The study concluded that the results support the oxalate-dependent theory. But reintroducing a wild-type *oah* allele could not restore all the wild-type phenotypes, such as formation of normal sclerotia and compound appressoria, making it difficult to attribute all the mutant phenotypes to the lack of oxalic acid alone.
4. Another study showed that the *oah* mutants lost oxalic acid production but accumulated fumaric acid in culture and were still able to form normal sclerotia and compound appressoria, initiate disease, and cause disease lesions similar to those caused by the wild-type strain on many, although not all, host plants. Acidifying host tissue with citric acid restored lesion expansion by the *oah* mutants, but supplying oxalate without lowering pH did not. Because the oxalate-dependent theory could not explain this observation, this study proposed that it is acidic pH, not oxalic acid per se, that is necessary for *Sclerotinia* to cause disease.
5. Comparisons of the three sets of mutants showed that the UV mutants possess a unique combination of mutant phenotypes characteristic of genetic defects in acidic pH responsiveness, such as loss of sclerotial formation, pathogenicity/virulence, normal growth rate, and pH-responsive growth. These defects confounded oxalate deficiency, thus

warranting reevaluation of the conclusions about virulence drawn based on the UV mutants. Analyses of the evidence further suggested that the UV-induced mutation likely impaired pH sensing and pH regulation in the UV mutants, suggesting the existence of an unknown regulator that positively regulates response to acidic pH. Identifying and characterizing that unknown pH regulator will provide a new avenue for studying pH-sensing/regulation mechanisms in *S. sclerotiorum* and offer a novel target for intervention for controlling *Sclerotinia* diseases.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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